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# Efficacy of recombinant Bacillus Calmette-Guérin containing dltA in *in vivo* three-dimensional bio-printed bladder cancer-on-a-chip and *ex vivo* orthotopic mouse model

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**Purpose:** We investigated the efficacy and optimal dosage of recombinant Bacillus Calmette-Guérin-dltA (rBCG-dltA) in a high-throughput 3D bio-printed bladder cancer-on-a-chip (BCOC) and orthotopic bladder cancer mouse model.

**Materials and Methods:** We fabricated high-throughput BCOC with microfluidic systems, enabling efficient drug screening. The efficacy of rBCG-dltA was evaluated using BCOC by the cell viability assay, monocyte migration assay, and measuring cytokine levels. The anti-tumor effect was compared using the orthotopic bladder cancer mouse model.

**Results:** The cell proliferation rates of T24 and 253J bladder cancer cell lines (mean±standard error) were measured at three days after treatment. In T24 cell line, there was significantly decreased T24 cells compared to control at rBCG 1 multiplicity of infection (MOI) and 10 MOI (30 MOI: 63.1±6.4, 10 MOI: 47.4±5.2, 1 MOI: 50.5±7.5, control: 100.0±14.5, p<0.05). In 253J cell line, a statistically significant decrease in 253J cell count compared to control and mock BCG 30 MOI (30 MOI: 11.2±1.3, 10 MOI: 22.5±2.3, 1 MOI: 39.4±4.7, Mock: 54.9±10.8, control: 100.0±5.6, p<0.05). The migration rates of THP-1 cells showed increased patterns after rBCG-dltA treatment in BCOC. The concentration of tumor necrosis factor-α and interleukin-6 after rBCG-dltA 30 MOI treatment was higher than control in T24 and 253J cell line.

**Conclusions:** In conclusion, rBCG-dltA has the potential to have better anti-tumor activity and immunomodulatory effects than BCG. Furthermore, high-throughput BCOCs have potential to reflect the bladder cancer microenvironment.

**Keywords:** Bioprinting; *Mycobacterium bovis*; Tumor microenvironment

# INTRODUCTION

Bladder cancer is categorized as the non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer. Intravesical therapy with Bacillus Calmette-Guérin (BCG) is a proven strategy for the treatment of NMIBC [1,2].

However, approximately 30%–40% patients do not respond to BCG treatment [3,4]. BCG-unresponsive bladder cancer (BUBC) is defined as high-grade T1 and/or carcinoma *in situ* (CIS) of the bladder or prostate urethra within 6 months of the last BCG dose [5]. In patients for whom the intravesical BCG treatment fails, especially high-risk patients, treatment of choice is currently radical cystectomy [6]. Considering the lack of supplies of BCG and several complications of radical cystectomy, there is a continuous demand for new therapeutic options for BUBC cases [7].

Intravesical therapeutic agents such as gemcitabine, docetaxel, epirubicin, and valrubicin have been investigated as salvage therapies for BUBC. Among these, only valrubicin has been approved by the Food and Drug Administration (FDA) as a treatment for BUBC [8-10]. Pembrolizumab also obtained FDA approval for selected patients with BUBC, high-risk patients with Tis tumors who are ineligible for or have elected not to undergo cystectomy [11]. However, valrubicin's 6-month complete response rate (CRR), which was defined as absence of T lesion at any time point, was 17.9%, and the 24-month CRR was only 4.0%. Three-month CRR of pembrolizumab used in patients who failed previous BCG was 38.8%, and no subsequent long-term results were reported, requiring the development of more effective treatments.

BCG, an attenuated strain of *Mycobacterium bovis*, is the best treatment option currently. The prospect of genetically modifying BCG to generate recombinant BCG (rBCG) strains that induce more specific immune responses would be a promising approach to improve the efficacy. In our previous study, BCG insensitivity may be due to reduced internalization of BCG to bladder cancer cells and that antimicrobial peptides (AMPs) play an important role in this kind of resistance [12]. We developed a rBCG strain expressing dltA (rBCG-dltA), derived from bacteria proteins and resistant to AMP, and we reported its effective avoidance of AMP induced by BCG which had potential to be used in the treatment of bladder cancer with improved immunotherapeutic effect [13]. We further investigated the immunotherapeutic effects of rBCG-dltA in an orthotopic bladder cancer mouse model [14].

A tumor microenvironment (TME) system was required to test the effect and optimal dosage of rBCG. Bladder cancer-on-a-chip (BCOC) reflects cell-to-cell interactions and the TME *in vivo* [15]. Our high-throughput BCOC embedded with four BCOCs with a microfluidic system was manufactured through 3D bioprinting technology that could be used as drug screening platforms [16].

In this study, we conducted experiments to establish the effectiveness and determine the optimal dosage of rBCG-dltA in BCOC. In addition, we attempted to evaluate the high-throughput BCOC as an efficient tool for bladder cancer research by comparing it with the orthotopic bladder cancer mouse model.

## MATERIALS AND METHODS

Our research methodology has similarities in many ways with previous studies experimented and reported by our research team. The original source of method descriptions are as follows [16,17].

### 1. Cell culture and reagents

T24 and 253J cells (human bladder cancer cell line) and MRC-5 (human lung fibroblast cell line) were purchased from Korean Cell Line Bank. MBT2-luc cells (mouse bladder cancer cell line) were kindly provided by Dr. Sang-Jin Lee (National Cancer Center, Korea). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. *M. bovis* BCG was obtained as a commercial lyophilized preparation (Onco-Tice, Merck Sharp and Dohme). Gel4Cell, a GelMA prepolymer solution, was used (Innoregen).

### 2. Electroporation of BCG

We followed the plasmid DNA construction of dltA as previously described [13]. DNAs encoding DltA (GenBank: D86240.2) were synthesized and cloned (Macrogen). Then, dltA expression vectors were constructed in the pMV306 vector using EcoRI and HpaI restriction enzymes. For electroporation, BCG cells were washed twice with 10% glycerol by centrifugation at 3,000 ×g for 10 minutes at 4℃. Next, 2-mm gap cuvettes (BTX) were pre-chilled on ice for approximately 30 minutes. Mock BCG was electroporated with empty plasmid DNA. After electroporation, BCG was resuspended in phosphate-buffered saline (PBS) and cultured at 37℃ for overnight.

### 3. High-throughput BCOC with microfluidic system
#### 1) Fabrication of high-throughput BCOC model

Complementing the previous study [16], we designed a single-layered bladder cancer chip using GelMA prepolymer solution. Then we fabricated a microfluidic-based bladder cancer screening system that had four embedded BCOC (Fig. 1). The whole system was operated using a syringe pump (Fusion 200, Chemyx Inc.) with four syringes connected to each BCOC by Tygon® tubing (Fig. 1B). To fabricate the high-throughput BCOCs, the computer numerical control
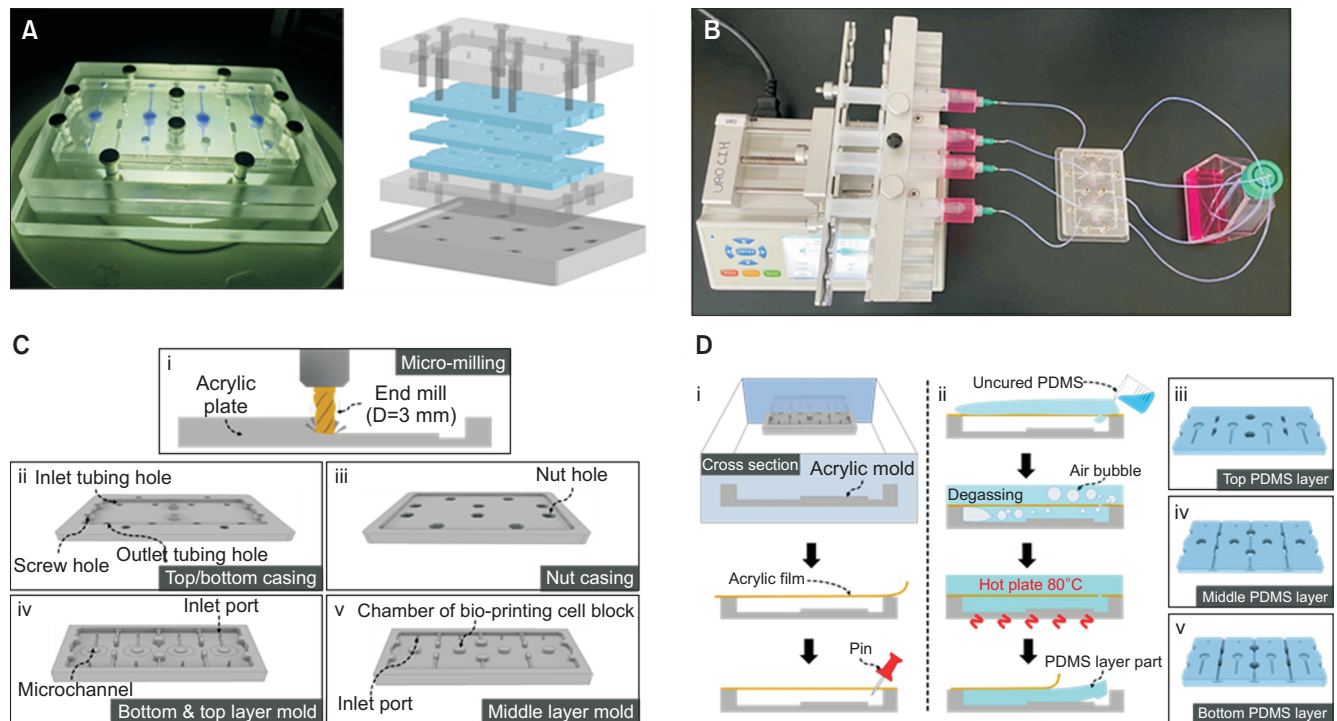
**Fig. 1.** High-throughput bladder cancer-on-a-chip (BCOC) model with microfluidic system. (A) BCOC system. Four systems can be operated with one syringe pump. (B) Actual product of the BCOC system. The system consists of a top/bottom/nut case and polydimethylsiloxane (PDMS) layer. (C) Acrylic cases and layer molds of PDMS. (D) PDMS layer manufacturing processes.

milling method and soft lithography-based polydimethylsi-loxane casting method were used.

### 2) Seeding bio-printed cell block and culture in the BCOC models

To mimic an actual bladder cancer environment, different biomaterials should be loaded onto each layer before operating the microfluidic system. On the bottom layers, monocytes were loaded and bio-printed cell blocks were inserted into the middle layer chambers, and BCGs or rBCG-dltAs were loaded on the top channels. All layers were separated by polycarbonate membranes and tightened with acrylic casing. After incubating for two hours for stabilization, the BCOCs were connected to the syringe pump by tygon tubing and operated at a flow rate of 20 µL/min.

### 3) Live/dead staining assay

The cell survival rate in the 3D cell constructs was assessed at one and three days after fabrication. A fluorescent live/dead staining solution (Thermo Fisher) was used. Each 3D cell construct and the 2D cultured cells were washed in Dulbecco's PBS (DPBS) three times before staining. The DPBS mixture with Calcein-AM (2 µM) and EthD-1 (4 µM) was filtered through a 0.22-µm syringe filter (Sigma-Aldrich). Cell morphologies were observed under a fluorescence micro-

scope (Leica DMi8, Leica). Three independent samples were analyzed.

### 4) Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo) was used to analyze cell proliferation in 3D cell constructs on one and three days. The 3D cell constructs were washed three times with DPBS. Next, 1-mL DPBS and 0.1-mL CCK-8 solution were added to each 60-mm cell culture dish and incubated in the dark for 8 hours with 5% $CO_2$ at 37℃. After incubation, 0.2 mL of medium was transferred to a 96-well plate. The absorbance of each well was measured at 450 nm using a microplate reader (SpectraMax i3x, Molecular Devices). Three independent samples were tested in each group.

### 5) THP-1 cell migration assay

THP-1 monocytes were differentiated into macrophages by incubation for 24 hours in Roswell Park Memorial Institute 1640 medium with 25 nM phorbol 12-myristate 13-acetate (Sigma). Differentiated THP-1 cells were seeded in the bottom layer of the organ-on-a-chip at a density of $2 \times 10^4$ cells/50 µL in a chip. Positive THP-1 staining was visualized using an Olympus CKX41 inverted microscope (Olympus). Three equal-sized fields were randomly selected for THP-1 cell counting, and the average was calculated.

### 6) Measurement of cytokines

After six hours of mock and rBCG-dltA treatment, the growth media was collected in the syringe for each fluidic culture solution of BCOC and centrifuged at 3,000 rpm for 10 min at 4℃. Samples were immediately analyzed, aliquoted, and stored at -80℃ prior to analysis.

The levels of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 in BCOC-cultured supernatants were measured using sandwich enzyme-linked immunosorbent assay reagent kits (DY210-05 and DY206-05), which were purchased from R&D Systems. The absorbance of the wells was measured with a microplate reader (SpectraMax i3x), and the microplate reader device was set to 450 nm.

## 4. Orthotopic bladder cancer mouse model

### 1) Experimental animals

Six-week-old female C3H mice were provided by Orient Bio Co. The animals were acclimated for one week as an adaptation period under routine laboratory conditions before the start of the experiments. All animals were housed in cages containing five animals and maintained on a daily 12-hour light/dark cycle. The mice were fed a standard balanced diet and water *ad libitum*.

### 2) Syngeneic orthotopic mouse bladder cancer model

For intravesical implantation of MBT2-Luc, female C3H mice at six weeks of age were anesthetized with isoflurane. Chemical lesions of the bladder urothelium were carried out by injecting 100 µL of poly-L-lysine (PLL) 0.1% (Sigma-Aldrich) into the bladder of each animal through a 24-gauge catheter (BD). After instilling 100 µL of 0.1 mg/mL PLL for 20 minutes, the bladders were washed with PBS and subsequently instilled with MBT2-Luc cells ($2.0 \times 10^6$) suspended in 50 µL PBS via a catheter. The cells were retained in the bladder for two hours by tying off the orifice to the urethra. The presence of tumors in the bladder was confirmed using bioluminescence imaging (BLI). After 1 week, 150 mg/kg of D-luciferin was administered intraperitoneally, and bioluminescence was detected using a VISQUE InVivo Smart-LF (Vieworks).

### 3) *In vivo* anti-cancer efficacy of rBCG-dltA

Mock BCG or rBCG-dltA were prepared in 50 µL of PBS and instilled into the bladder lumen via urinary catheterization. Each treatment was retained in the bladder for two hours by tying off the orifice of the urethra. Quantitative signal intensities were calculated and presented as regions of interest. Tumor regression after treatment with mock BCG or rBCG-dltA in the orthotopic mouse model was measured using BLI. The mice were randomized into control, mock BCG, or rBCG-dltA groups. Each mouse (5 mice/group) was intravesically administered PBS, BCG, or rBCG-dltA through a catheter twice a week. The plan was to acquire 7 images by injection and image twice a week for a total of 21 days, and 5 mice died early for 9–10 days, and finally 4 images and data were acquired from each group and terminated earlier due to inter-group balance problems. Intravesical delivery was carried out with an indwelling time of 1–2 hours. Serial BLI and body weight were measured to monitor bladder cancer progression twice a week for 2 weeks using a VISQUE Smart-LF IVIS Lumina XRMS (Vieworks). To compare the potential toxicity of rBCG with original BCG, the date of death after the BCG injection was compared, and the weight was measured every week to indirectly compare toxicity.

## 5. Ethics approval

The procedures used and the care of animals were approved by the Institutional Animal Care and Use Committee in Chung-Ang University (approval no. 2018-00050, date: May 12, 2018, Seoul, Korea) and carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Also, this study is reported in accordance with ARRIVE guidelines.

## 6. Statistical analysis

SPSS software package (version 25.0, IBM Corp.) was used for all statistical analyses. Student's t-test was used to compare the means in two groups, and statistical significance was set at $p < 0.05$.

## RESULTS

### 1. *In vitro* immunotherapeutic effects of rBCG-dltA at BCOC

We evaluated the cell viability and proliferation rate, chemotaxis of monocytic THP-1 cells, and the concentration of cytokines simultaneously after treatment with rBCG-dltA at 1, 10, and 30 multiplicity of infection (MOI) to establish the immunotherapeutic effects of rBCG-dltA in BCOCs.

In the CCK-8 assay, the cell proliferation rates of T24 and 253J cells (mean±standard error) were measured at three days after treatment (Fig. 2). In T24 cell line, there was significantly decreased T24 cells compared to control at rBCG 1 MOI and 10 MOI (30 MOI: 63.1±6.4, 10 MOI: 47.4±5.2, 1 MOI: 50.5±7.5, control: 100.0±14.5, $p < 0.05$). In 253J cell line, the effect of rBCG was more pronounced, especially at rBCG 10
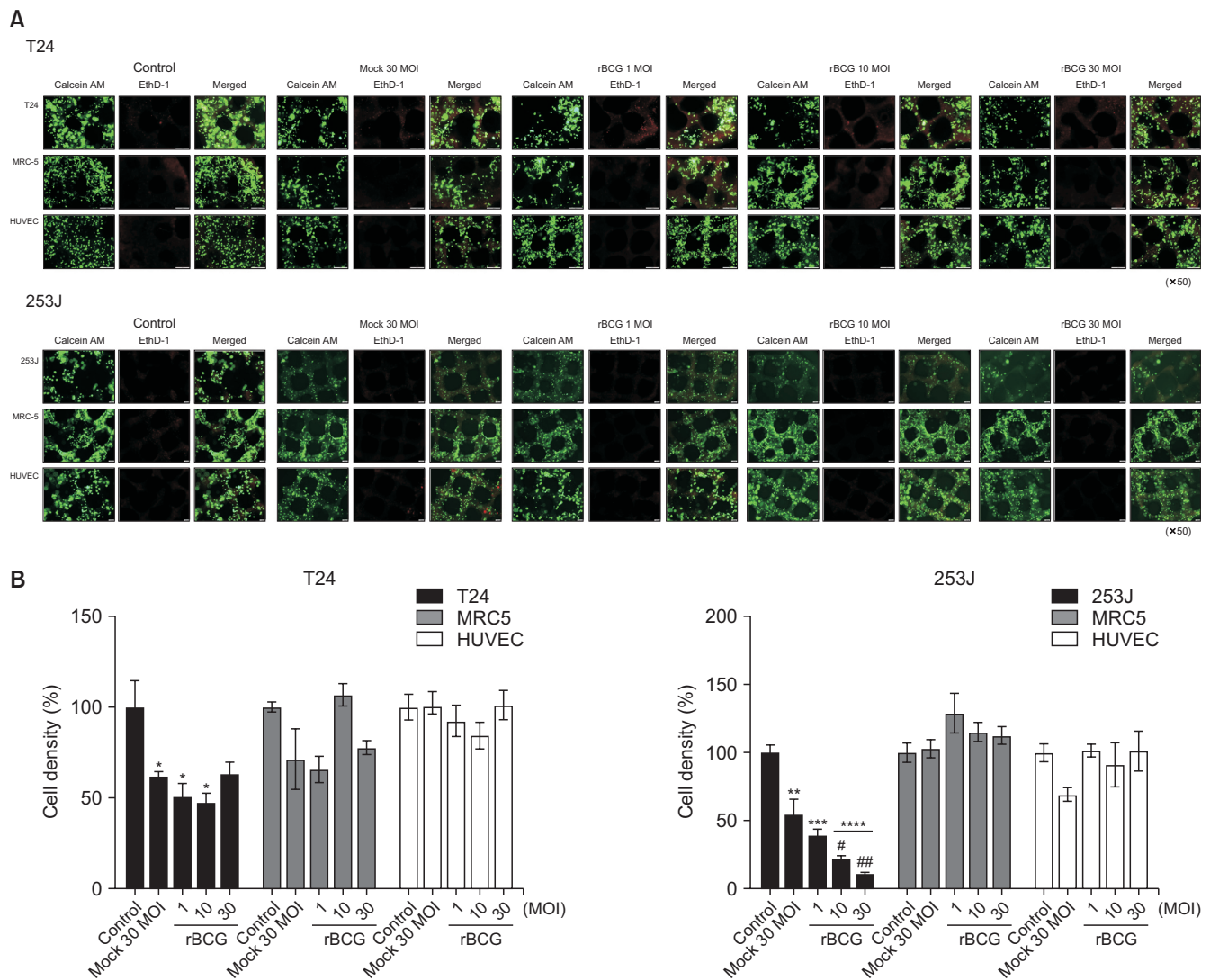
**Fig. 2.** Cell viability after recombinant Bacillus Calmette-Guérin (rBCG)-dltA treatment. (A) Live/dead staining assay of control, mock BCG, and recombinant BCG-dltA, with cell morphologies observed after 72 hours of the treatment in bladder cancer cell lines (T24 and 253J). (B) Percentage of cell viability of each treatment. Data are presented as the mean±standard error of the mean (n=3–5, per group). Control, phosphate buffered saline; Mock, non-rBCG with empty plasmid; MOI, multiplicity of infection; HUVEC, human umbilical vein endothelial cell. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs. control, #$p < 0.05$ and ##$p < 0.01$ vs. mock BCG 30 MOI.

MOI and 30 MOI, a statistically significant decrease in 253J cell count compared to control and mock BCG 30 MOI (30 MOI: 11.2±1.3, 10 MOI: 22.5±2.3, 1 MOI: 39.4±4.7, Mock: 54.9±10.8, control: 100.0±5.6, $p < 0.05$). But cell proliferation rates of MRC-5 and HUVEC, fibroblast and endothelial cells, did not show any difference between control, mock BCG 30 MOI treatment, and rBCG-dltA treatments in both cell lines (Fig. 2B).

The migration rates of THP-1 cells showed increased patterns according to rBCG-dltA treatment in BCOC (Fig. 3). The fold change in THP-1 cell migration (mean±SE, compared to control) increased dose-dependently according to 1, 10, and 30 MOI of rBCG-dltA in both T24 cell line (1 MOI: 1.53±0.07, 10 MOI: 1.82±0.10, 30 MOI: 1.99±0.06, Mock: 1.51±0.13, control: 1.00±0.03, $p < 0.001$) and 253J cell line (1 MOI: 2.72±0.54,

10 MOI: 3.50±0.65, 30 MOI: 4.47±0.20, Mock: 1.00±0.17, control: 1.00±0.20, $p < 0.05$) than control.

The concentration of TNF-α (mean±SE, pg/mL) after rBCG-dltA 30 MOI treatment was higher than control ($p < 0.0001$) and mock BCG 30 MOI ($p < 0.05$) in T24 (32.11±0.04) and 253J (38.41±1.39) cell line. Also, the concentration of IL-6 (mean±SE, pg/mL) after rBCG-dltA 30 MOI was also higher than control in T24 (13.32±1.26) and 253J (44.00±9.08) cell line ($p < 0.01$, Fig. 4).

## 2. *In vivo* antitumor efficacy of rBCG-dltA in an orthotopic bladder cancer mouse model

To evaluate the anti-tumor activity of rBCG-dltA *in vivo*, we established the orthotopic mouse bladder can-
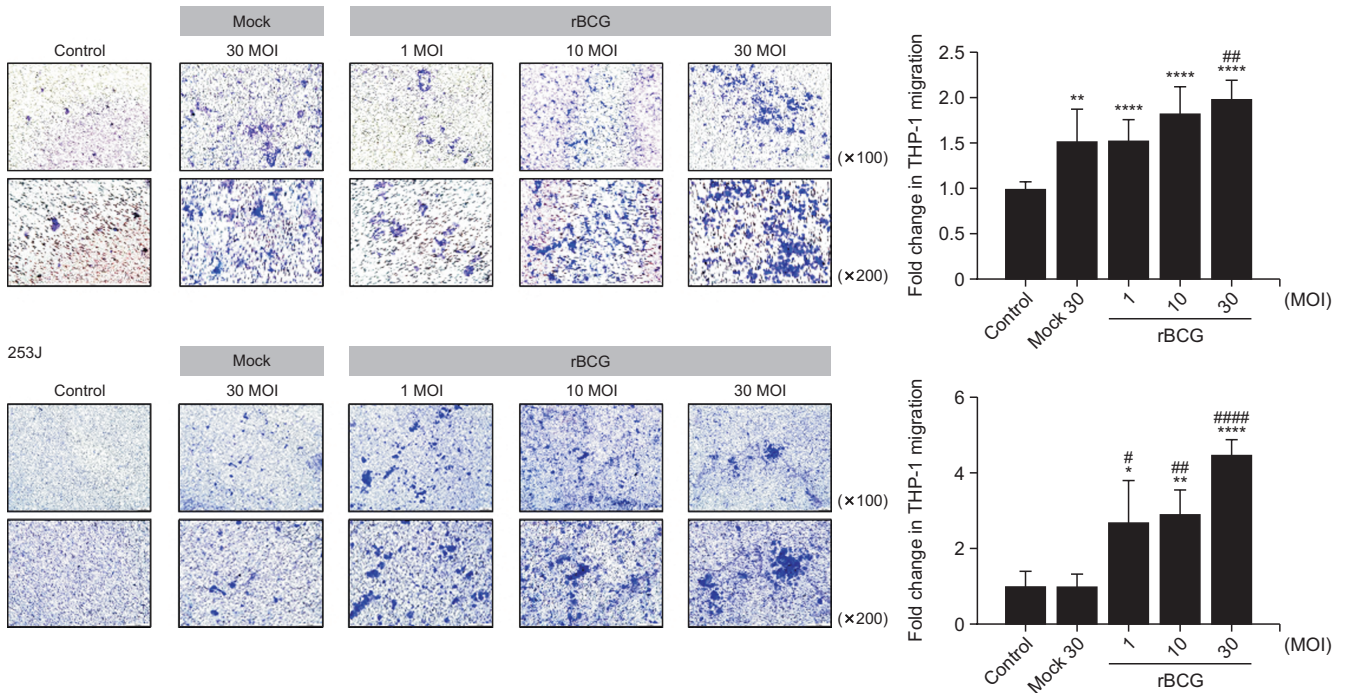
**Fig. 3.** Migration assay of recombinant Bacillus Calmette-Guérin (rBCG)-dltA. Migration of differentiated THP-1 cells by phorbol 12-myristate 13-acetate after BCG treatment in bladder cancer-on-a-chip with bladder cancer cell lines (T24 and 253J). Data are presented as the mean±standard error of the mean (n=3–5, per group). Control, phosphate-buffered saline; Mock, non-rBCG with empty plasmid; MOI, multiplicity of infection. *p<0.05, **p<0.01, and ****p<0.0001 vs. control, #p<0.05, ##p<0.01, and ####p<0.0001 vs. mock BCG 30 MOI.
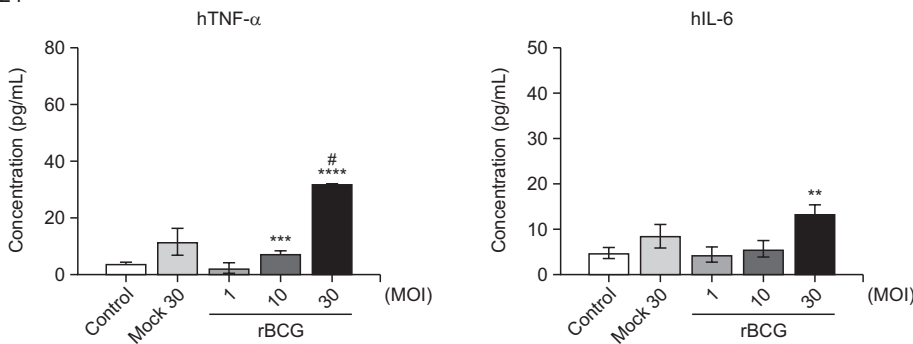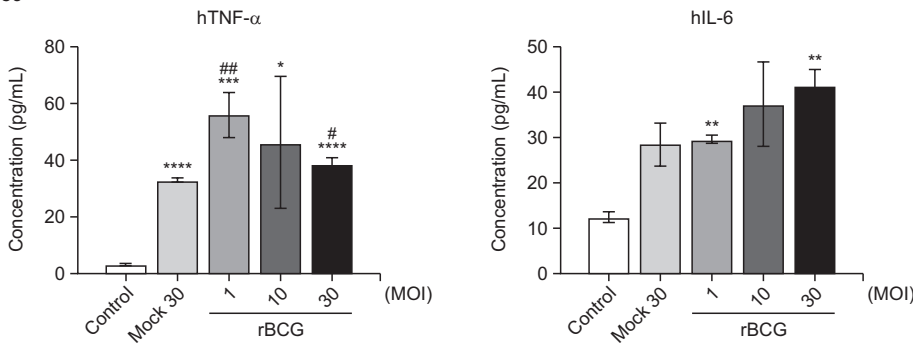


**Fig. 4.** Concentration of cytokine levels after recombinant Bacillus Calmette-Guérin (rBCG)-dltA treatment. Concentrations of cytokines (human tumor necrosis factor [TNF]-α and human interleukin [IL]-6) six hours after BCG treatment in bladder cancer-on-a-chip with bladder cancer cell lines (T24 and 253J). Data are presented as the mean±standard error of the mean (n=3–5, per group). Control, phosphate-buffered saline; Mock, non-recombinant BCG with empty plasmid; MOI, multiplicity of infection. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 vs. control, #p<0.05 and ##p<0.01 vs. mock BCG 30 MOI.
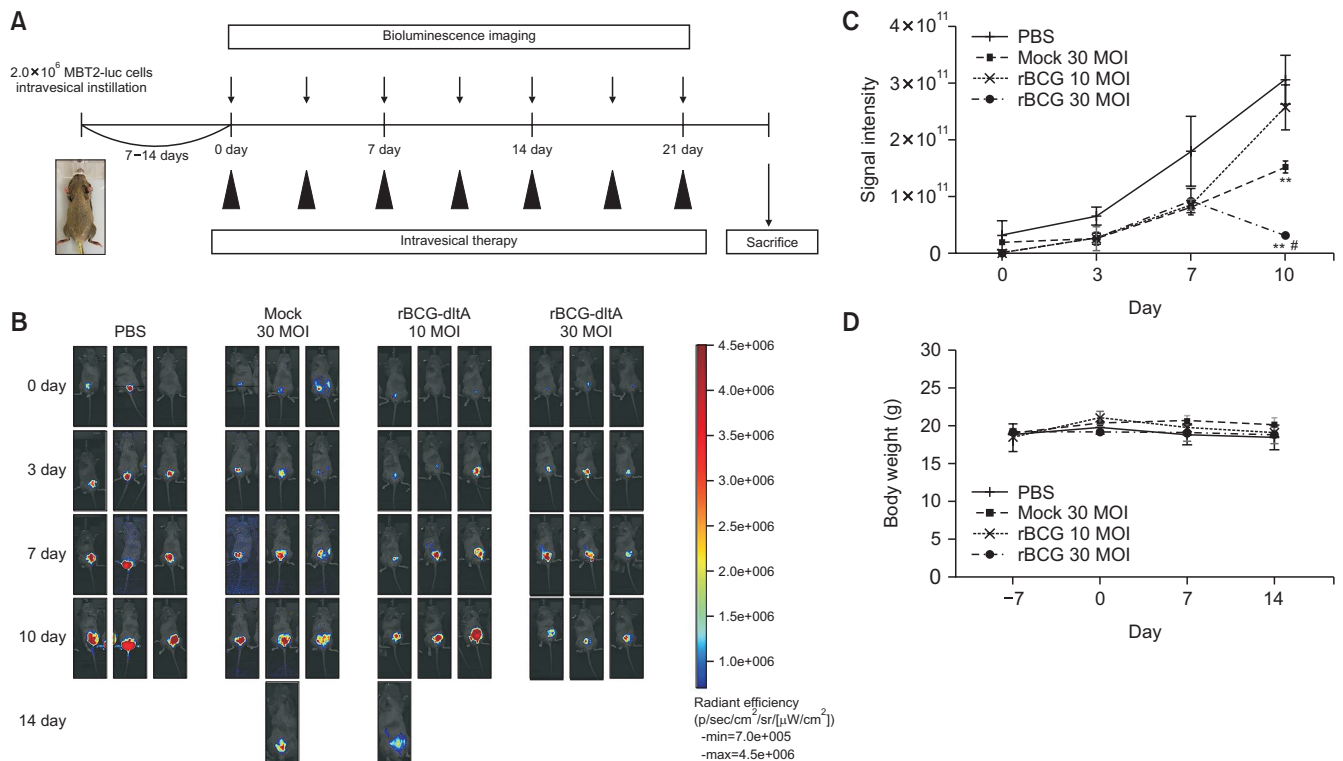
**Fig. 5.** *In vivo* anti-tumor effects of recombinant Bacillus Calmette-Guérin (rBCG)-dltA and body weight changes in mouse model. (A) Intravesical instillation in orthotopic bladder cancer mouse model. (B) *In vivo* imaging of mouse with bioluminescent signals. (C) Quantitative comparison of tumor volume changes calculated with signal intensity. (D) Body weight of each treatment group. Data are presented as the mean±standard error of the mean (n=3–5, per group). PBS, phosphate-buffered saline (control); Mock, non-recombinant BCG with empty plasmid; MOI, multiplicity of infection. **$p<0.01$ vs. control, #$p<0.05$ vs. mock BCG 30 MOI.

cer model and injected mock BCG or rBCG-dltA into the mouse's bladder (Fig. 5A). On the 7th day (designated on day 0) after tumor transplantation, all mice that produced BLI were divided into 4 groups randomly (control, mock BCG 30 MOI, rBCG-dltA 10 MOI, and rBCG-dltA 30 MOI). No significant difference in BLI intensity between groups was observed after randomization. On the tenth day, all groups showed an increasing trend in BLI intensity; BLI intensity (mean±standard error, $\times10^9$) were significantly lower in the rBCG-dltA 30 MOI group than in the control (32.5±1.9 vs 257.3±82.6, $p<0.01$) and mock BCG 30 MOI (32.5±1.9 vs. 152.3±10.7, $p<0.05$) groups (Fig. 5).

To evaluate adverse effects of rBCG-dltA, we measured the body weight of the mice during the experiments (Fig. 5D). Significant weight changes were not identified in all groups, indicating the safety of rBCG-dltA. The longest survival period in the control group was 16 days, and there was no significant difference of 14 days in each rBCG-dltA concentration group. Detailed date of death data is presented in Supplementary Fig.

## DISCUSSION

In our study, rBCG-dltA 30 MOI demonstrated better immune and anticancer effects than mock BCG 30 MOI, and several possibilities for its use as a treatment for BUBC have been shown. rBCG-dltA 10 MOI had an effect similar to that of mock BCG 30 MOI in *ex vivo* models in T24 cell line. In the 253J cell line, rBCG 10 MOI showed a greater decrease in the number of 253J cells compared to mock BCG.

The migration rates of THP-1 cells were increased (Fig. 3), it is estimated that the migration of THP-1 cells increased due to the chemotactic effect caused by the increased cytokine. However, rBCG 10 MOI in mouse *in vivo* study had less effect of lowering signal intensity than Mock BCG, unlike rBCG 30 MOI. Through further research, it is necessary to check whether the lower volume of rBCG (10 MOI) has better anti-tumor activity *in vivo*.

The advantage of rBCG-dltA is that it increases the efficiency of BCG, with the original characteristics of BCG, which is currently known to be the most effective in NMIBC. In addition, since rBCG has higher affinity in normal urothelium, may have the potential toxicity. We compared mice weights every week between the four groups

to indirectly identify toxicity, and statistically compared the number of days of death, no significant difference in death or weight was observed. However, further research to find direct evidence of toxicity should continue. Interestingly, all mice groups showed similar intensities/growth of tumor until the seventh day and started diverging after that day. Further, the current treatment trend is combination therapy, such as BCG plus pembrolizumab, and we believe that there is a possibility of replacing BCG with rBCG-dltA in the future [18].

BCG has a long incubation period and needs expensive specialized equipment, causing easy bacterial contamination. These factors eventually led to the closure of the global BCG supplier Sanofi Laboratory in 2012, which has led to a difficulty in obtaining BCG supplies because Merck Company is the biggest supplier of oncoTICE BCG in the world [19]. Although there are several other producers of BCG for bladder cancer treatment, they produce different substrains of BCG and have lower production capacity.

While there have been various studies on drugs such as oportuzumab monatox, rAd-IFNa/Syn3, and pembrolizumab to replace BCG in BUBC [20], most drugs demonstrated a worse outcome than conventional BCG. Valrubicin, an FDA-approved drug, provided inappropriate cancer control and showed a 13% CRR after one year of treatment [21]. Expert panels of workshop by American Urological Association and FDA argued that clinically meaningful drugs as those with at least a response rate of at least 40%–50% in the first six months and 30% of continuous response rate in 18–24 months [22]; drugs such as oportuzumab monatox or rAd-IFNa/Syn3 do not meet the above criteria.

The cell surface of most gram-positive bacteria contains wall lipoteichoic acid and teichoic acid backbone [23]. The dltA protein is required for the D-alanylation of lipoteichoic acids, which play an essential role in the cell walls of BCG. D-alanyl esterification of triclosan can increase positive charges, reducing the effectiveness of AMPs [24].

The cancer-on-a-chip model is actively studied in various oncological fields [25,26]. Generally, preclinical drug development evaluates the efficacy and safety of *in vivo* and *in vitro* drug candidates, using animal studies and 2D or 3D cell cultures. On the other hand, 2D and 3D laboratory cell culture plates lack the systemic nature of living cells [26]. The cancer-on-a-chip model can be characterized by not only a non-relevance of ethical issues, but also cost effectiveness and better simulation of human nature.

The current trend for bladder cancer is the combination therapy of immune checkpoint inhibitors (ICIs), but there is no suitable method to evaluate the merged effect in one chamber [27]. So far, BCOC research with microfluidic system has shown that only one drug can be tested at a time; therefore, there could be differences in the TME due to differences in terms of time, temperature, or the physician and which results in reducing the reliability of the interpretation of the results for the control group and the experimental group [28,29]. To overcome this, our research team produced a BCOC with a microfluidic system to study four chips at the same time. High-throughput BCOC has the advantage of evaluating the therapeutic effect of ICI-combination drugs instead of BCG, and it is possible to reduce errors occurring during the experiment by simultaneously using four BCOC models. In addition, our BCOC is structurally the simplest among the microfluidic BCOCs reported so far, which may have advantages in reproducibility [28,29].

The BCOC models presented in this study implement the human TME using 3D bio-printing technology and a microfluidic system. TME is crucial for cell proliferation, progression, and metastasis and can determine the malignant phenotype of bladder cancer [28]. Understanding TME is useful in predicting the effects of BCG and ICIs in bladder cancer and is currently useful in evaluating various treatments of BUBC. Novel drugs that use the bladder cancer microenvironment are under development and awaiting FDA approval [30].

Our study has several limitations. First, Phase III human clinical trials of rBCG have not been implemented worldwide. Rentsch et al. [2] have only published their results from Phase I and a Phase I/II single-arm trial of rBCG expressing the Listeria monocytogenes, listeriolysin gene in recurrent NMIBC patients. The efficacy of rBCG in humans has to be proven with prospective trials. Second, direct evidence of rBCG is less counteracted by AMP should be revealed through further research.

If human bladder cancer tissues are investigated on BCOC, it would be possible to choose a more appropriate drug with good responsivity and could provide patient-customized treatment. We make an animal model using a cell line as a first step. The subsequent study is to create harvested-cell-based cancer-on-a-chip by a metastatic animal model. The final goal would be the drug screening by harvesting an individual's cancer cell and creating a cancer on-a-chip with a human sample.

## CONCLUSIONS

In conclusion, rBCG-dltA has the potential to have better anti-tumor activity and immunomodulatory effects than BCG. Anti-tumor activity was confirmed in *ex vivo* and *in*

*vivo*, and further studies are needed for the detailed immunomodulatory effect of rBCG *in vivo*. Further research of rBCG about toxicity and efficacy of rBCG 10 MOI *in vivo* is also needed to verify the effect of low dose rBCG. Furthermore, the BCOC with a microfluidic high-throughput system reflected the bladder TME.

## CONFLICTS OF INTEREST

The authors have nothing to disclose.

## AUTHORS' CONTRIBUTIONS

Research conception and design: In Ho Chang and Tae Young Jung. Data acquisition: Su Jeong Kang, Mirinae Kim, and Young Wook Choi. Statistical analysis: Sejung Maeng and Se Young Choi. Drafting of the manuscript: Joongwon Choi. Critical revision of the manuscript: Jung Hoon Kim. Obtaining funding: In Ho Chang, Tae Young Jung, and Joongwon Choi. Administrative, technical, or material support: Sung-Hwan Kim. Supervision: In Ho Chang and Tae Young Jung. Approval of the final manuscript: In Ho Chang.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found via https://doi. org/10.4111/icu.20220293.

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