

The potential antitumor effects of combining intravesical therapy with recombinant bacillus calmette-guérin and an anti-PD-1 inhibitor in bladder cancer

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Title:

The Potential Antitumor Effects of Combining Intravesical Therapy with Recombinant Bacillus Calmette-Guérin and an Anti-PD-1 Inhibitor in Bladder Cancer

Running Title:

Recombinant BCG and Anti-PD-1 in Bladder Cancer

Authors:

Jung Hoon Kim^{1*}, Sejung Maeng^{2*}, Joongwon Choi¹, Chung Un Lee¹, Yong Seong Lee¹, Mirinae Kim², Se Young Choi², In Ho Chang²

¹Department of Urology, Chung-Ang University Gwangmyeong Hospital, Chung-Ang University College of Medicine, Gwangmyeong, Gyeonggi-do, Republic of Korea

²Department of Urology, Chung-Ang University Hospital, Chung-Ang University College of Medicine, Seoul, Republic of Korea

*These two authors contributed equally to this work as co-first authors.

ORCID IDs

Jung Hoon Kim: 0000-0002-0947-876X

Joongwon Choi: 0000-0001-5978-8179

Chung Un Lee: 0000-0002-3030-0405

Yong Seong Lee: 0000-0003-0611-4554

Sejung Maeng: 0000-0002-0730-0300

Mirinae Kim: 0000-0003-4199-8105

Se Young Choi: 0000-0002-4615-0966

In Ho Chang: 0000-0003-0240-1310

Corresponding author:

In Ho Chang, MD, PhD

Department of Urology, Chung-Ang University Hospital, Chung-Ang
University College of Medicine

102, Heukseok-ro, Dongjak-gu, Seoul, 06973, Republic of Korea

Tel: +82-2-6299-1819, Fax: +82-2-6294-1406, E-mail: caucih@cau.ac.kr

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Abstract

Background: Recombinant Bacillus Calmette-Guérin (rBCG) is a genetically modified BCG that enhances therapeutic efficacy and safety in patients with bladder cancer. Pembrolizumab is an immune checkpoint inhibitor (ICI) that is used to treat bladder cancer. We aimed to evaluate the effectiveness of the combination of rBCG and pembrolizumab *in vitro* and *in vivo* bladder cancer models.

Methods: We investigated the antitumor effects of rBCG-*dltA* and pembrolizumab in a bladder cancer-on-a-chip (BCOC) model, an organoid three-dimensional cell culture tool. An orthotopic mouse model was employed to assess the *in vivo* effects of the combination therapy. To overcome limitations arising from differences between species, an *in vivo* study was conducted using a mouse PD-1 inhibitor instead of pembrolizumab.

Results: The T24 bladder cancer cell line showed reduced viability when rBCG-*dltA* and pembrolizumab were used in the BCOC model. Monocyte migration was also significantly greater than that in the other groups. In the orthotopic bladder cancer model, the combination treatment inhibited the growth of bladder cancer.

Conclusions: Intravesical therapy with rBCG-*dltA* and pembrolizumab may be effective in treating bladder cancer. This study suggests that the combination of rBCG and ICIs may be an alternative strategy for treating patients with BCG-unresponsive bladder cancer.

Keywords: Bacillus Calmette-Guérin; Bladder cancer; Pembrolizumab; three-dimensional cell culture; Organ-on-a-chip devices

Background

According to Global Cancer Statistics 2020, bladder cancer is the 10th most commonly diagnosed malignant tumor in the world (1). It is the second most common type of urinary tract cancer after prostate cancer. Although surgical management of bladder cancer has remained relatively unchanged, the field has witnessed significant advancements in therapeutic agents over recent years. Recently, traditional and innovative therapeutic agents, such as chemotherapy and immune checkpoint inhibitors (ICIs), have been integrated to manage both non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). In addition to ICIs, novel intravesical approaches using antibody-drug conjugates have shown promising results (2).

Intravesical immunotherapy with Bacillus Calmette-Guérin (BCG) is a common therapeutic modality for patients with bladder cancer. The European Association of Urology (EAU) guidelines strongly recommend intravesical BCG after transurethral tumor resection in patients with intermediate- and high-risk bladder cancer (3). BCG prevents disease progression in NMIBC by up to 27% and reduces the recurrence rate by 32% (4). Despite these therapeutic benefits, a significant portion of patients still experience disease recurrence, highlighting the limitations of

current treatment protocols. Furthermore, BCG therapy is associated with various side effects such as hematuria, dysuria, urinary tract infections, and BCG sepsis (5). Owing to the collapse of the global supply chain, BCG shortages are an emerging issue. Various studies have been conducted to overcome the BCG unresponsiveness, increase its effectiveness, and decrease its side effects including intravesical immune-activating therapy (6, 7). Recently, novel intravesical immune-activating agents were developed to stimulate potent local immune responses with minimized toxicity (8).

Recombinant BCG (rBCG) is a modified version of BCG. Compared with conventional BCG treatment, genetically modified BCG products aim to increase the efficiency of immunotherapy and reduce complication rates. rBCGs have been developed to induce specific immune responses or include foreign antigens (9). rBCG has been shown to induce the overexpression of cytokines to a greater extent than conventional BCG treatment. Bioengineered 3D tumor models are used to construct tumor microenvironments (TMEs) that play key roles in tumor growth, drug resistance, and metastasis (10). The cancer-on-a-chip model better emulates the TME, cancer architecture, and flow dynamics (11). Bladder cancer-on-a-chip (BCOC) is a form of organ-on-a-chip. It is a layered structure of cell blocks printed via 3D bioprinting (12).

Pembrolizumab is a highly selective monoclonal antibody against PD-1 that blocks the interaction between PD-1 and its ligands. Pembrolizumab

is an effective ICI that has antitumor activity in patients with bladder cancer (13). Compared with systemic chemotherapy, pembrolizumab has shown significant overall survival gains for 3 months (14). The antitumor effects of pembrolizumab are achieved not only through systemic administration but also through intravesical instillation. A phase I clinical trial of intravesical pembrolizumab and BCG treatment reported safe and feasible results in patients with BCG-unresponsive NMIBC (15).

We hypothesized that the combination of rBCG and pembrolizumab would enhance immunological reactions more than the conventional BCG immunotherapy. In this study, we used a high-throughput BCOC model to evaluate the immunotherapeutic effects of rBCG and pembrolizumab.

Methods

1. Cell lines and reagents of the BCOC Model

To evaluate the combined antitumor effect of rBCG and pembrolizumab, we employed a high-throughput BCOC system, composed of four parallel modules driven by a syringe pump (12, 16). The 3D bioprinted BCOC blocks consisted of the T24 bladder cancer cells, MRC-5 fibroblasts, Jurkat T lymphocytes, THP-1 monocytes, and human umbilical vein endothelial cell line cells (HUVEC), cultured using standard protocols provided by the suppliers: Korean Cell Line Bank (Seoul, Republic of Korea) and Lonza (Basel, Switzerland). MBT2-luc murine bladder cancer cells were

generously provided by Dr. Sang-Jin Lee (National Cancer Center, Republic of Korea). Commercially available *Mycobacterium bovis* BCG was obtained from OncoTice (Merck Sharp, Kenilworth, NJ, USA). BCG was prepared at a multiplicity of infection (MOI) of 30 (1.8×10^6 cells/mL) and stored at -80°C until use. The GelMA prepolymer mixture used was Gel4Cell (Innoregen, Daegu, Republic of Korea). The rBCG strain was engineered to express the *dltA* gene, cloned into a pMV306 vector using *Hind* III and *Sal*I restriction enzymes, following previously described protocols (17).

2. Live/dead cell viability and THP-1 cell migration assay

Cell viability in the 3D structures was assessed on the first and third days following the administration of rBCG-*dltA* and pembrolizumab (Keytruda; Merck Sharp and Dohme Ireland, Carlow, Ireland). Prior to staining with fluorescent live and dead solutions (Thermo Fisher, Waltham, MA, USA), each 3D cell construct was washed three times with Dulbecco's phosphate-buffered saline. A fluorescence microscope (Leica DMI8; Leica, Germany) was used to observe the cell morphology, and three independent samples were analyzed.

THP-1 monocytes were transformed into macrophages by incubation with 25 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The differentiated cells were seeded into the bottom layer of the chip at a density of 2×10^4 cells/20 μL , and the THP-1 cells were visualized via an Olympus CKX41 inverted microscope (Olympus,

Tokyo, Japan) and counted in three randomly selected fields, and the average was calculated.

3. Animal studies

A syngeneic orthotopic bladder cancer mouse model was established using immunocompetent female C3h/HeN mice (12, 18). MBT2-luc bladder cancer cells (1×10^6 cells in 50 μ L phosphate-buffered saline) were instilled into the bladders. After a mouse bladder cancer model was established, the mice were divided into four groups. Four groups of mice, consisting of five mice each, received either PBS, rBCG-*dltA*, anti-mPD-1, or a combination of both directly into the bladder through a catheter twice weekly. Intravesical instillation was performed using a 24-gauge catheter (Becton Dickinson, USA) under aseptic conditions. rBCG-*dltA* and anti-mPD-1 were administered intravesically according to the established protocol, with the solution retained in the bladder for approximately 2 hours before spontaneous voiding.

To ensure species-specific immunological compatibility, different PD-1 inhibitors were employed in the *in vitro* and *in vivo* experiments. Human pembrolizumab was used in the BCOC experiments containing human-derived cell lines. For the animal experiments, a murine-specific anti-PD-1 antibody was used because pembrolizumab does not cross-react with the mouse PD-1 receptor. Bladder cancer progression was monitored via bioluminescence imaging (BLI), and body weight measurements were taken twice weekly for 2 weeks. The BLI data were obtained and analyzed

via the Living Image software, CleVue version 3.1.3.2054 (Vieworks, Anyang, Republic of Korea). A schematic of the treatment protocol is shown in Figure 1.

Prior to tissue collection, all animals underwent anesthesia utilizing an inhalation anesthesia system. The anesthetic agent was Ifran Liquid for Inhalation (Isoflurane; Hana Pharm, Seoul, Korea). Anesthesia was initiated within a chamber utilizing a gas mixture of nitrogen and oxygen in a 1:1 ratio, with isoflurane administered at a concentration of 2–3%. After approximately 5 min of induction, the animals were transitioned to a nose cone connected to the anesthesia system to sustain isoflurane anesthesia during the tissue harvesting process. Following the collection of samples, the animals were euthanized via cervical dislocation while still under deep anesthesia. This approach was chosen to minimize animal suffering and to ensure a humane endpoint.

4. Hematoxylin and eosin (H&E) staining

Bladder cancer tissues were harvested from an orthotopic bladder cancer mouse model and fixed in 4% paraformaldehyde solution (Biosesang, Yongin, Republic of Korea) at 4°C for 24 h. Following standard processing, tissues were dehydrated, embedded in paraffin, and sectioned at 4 µm thickness. Sections were deparaffinized, rehydrated, and stained with H&E using conventional protocols. The stained slides were imaged via an image scanner (Pannoramic MIDI; 3DHISTECH, Budapest, Hungary) for histological evaluation.

5. Statistical analysis

Experimental data were obtained from a minimum of three independent experiments. The Statistical Package for Social Sciences (SPSS) version 25.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as mean $\pm$ SEM. Statistical significance among multiple groups was determined using one-way ANOVA followed by Tukey's post-hoc test for pairwise comparisons. A p -value < 0.05 was considered statistically significant.

6. Ethics approval

The procedures and animal care were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (Approval No. A2021020, date: Jan 09, 2023, Seoul, Republic of Korea) and performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. In addition, this study was conducted in accordance with the ARRIVE guidelines.

Results

1. Cellular viability after treatment with rBCG-*dltA* with or without pembrolizumab in BCOCs

Cell viability was assessed at two different time intervals, on the first and third days, for each treatment group. In the live/dead staining assay, the T24 cancer cell line in the combination group of rBCG-*dltA* and

pembrolizumab exhibited markedly decreased viability, whereas MRC-5 cells and HUVECs maintained a normal level of viability (Figure 2A). The merged image represents the relative proportions of living (green) and dead (red) cells. The images clearly indicate a higher rate of T24 cell death in the combination group. The fluorescence intensity of the T24 cells (mean $\pm$ standard error [SE], %) was significantly lower in the combination group (22.24 ± 5.01) than in the control group (Figure 2B). The MRC-5 cells and HUVECs did not significantly differ between the groups. Compared with both the rBCG-*dltA* and pembrolizumab groups, the combination treatment group demonstrated superior effectiveness in inhibiting tumor growth ($p < 0.05$).

2. Effects of chemotaxis following the administration of rBCG-*dltA* and/or pembrolizumab

The chemotactic response of monocytic THP-1 cells across the membrane was evaluated in the control, rBCG-*dltA* and pembrolizumab groups (Figure 3A). Few migrated cells were observed in the control group, whereas treatment with rBCG-*dltA* or pembrolizumab increased THP-1 cell migration, with the combination treatment showing the most pronounced accumulation. Cell migration was visualized by crystal violet staining, and scale bars represent 100 μm in each image. The average migration number (mean $\pm$ SE, %) of THP-1 cells was significantly greater in the combination group ($10,540 \pm 1,551$, $p < 0.01$) than in the control group (554.7 ± 354.6) (Figure 3B).

3. Evaluation of combination treatment in an orthotopic mouse model of bladder cancer (*in vivo* study)

To assess the inhibition of tumor growth in the combination treatment group, we compared the BLI intensities in an orthotopic mouse model of bladder cancer (Figure 4A). Following the implantation of bladder cancer cells on day 0, all experimental mice were randomly allocated to the four groups and subjected to BLI. The imaging signal intensities were assessed by calculating the average of each measurement (Figure 4B). At the time of randomization, there was no statistically significant difference in signal intensity between the groups. The mean $\pm$ standard error (SE) of the signal intensity, as measured by BLI, in the combination group ($9.44 \pm 1.36 \times 10^{10}$) was significantly lower than that in both the control group ($1.69 \pm 1.47 \times 10^{11}$, $p < 0.05$) and the anti-mPD-1 group ($1.88 \pm 3.46 \times 10^{11}$, $p < 0.05$) on day 10. Compared with the individual treatment groups, the combined treatment group presented a significantly decreased BLI signal ($p < 0.05$). Following the completion of the *in vivo* experiment, the bladders were extracted from the mice. The presence of the tumors within the bladder was visually verified via H&E staining (Figure 4C). H&E staining of excised bladders confirmed that the reduction in BLI signal intensity corresponded to a genuine decrease in tumor burden. Notably, the combination group exhibited a markedly reduced tumor area compared to the other groups, providing histological evidence of the synergistic antitumor efficacy.

4. Evaluation of drug toxicity and safety

Weight measurements were performed on the mice in each experimental group to assess the potential toxicity of the treatment regimens prior to BLI (Figure 5). No notable differences in weight changes were observed between the groups.

Discussion

The present study demonstrates that the combination of rBCG-*dltA* and PD-1 blockade exerts a synergistic antitumor effect in both *in vitro* and *in vivo* bladder cancer models. Using our previously validated 3D bioprinted BCOC platform (12, 16), we observed that co-administration of rBCG-*dltA* and pembrolizumab significantly reduced bladder cancer cell viability and enhanced THP-1 monocyte migration compared with either monotherapy. These results indicate that rBCG-*dltA*-induced immune activation and PD-1 inhibition cooperate to augment cytotoxic immune responses within the tumor microenvironment (TME).

Recreating a physiologically relevant TME has become a major goal of recent cancer research. 3D cell culture systems and organ-on-a-chip (OOC) technologies have been increasingly applied across multiple cancer types to mimic cellular interactions, extracellular matrix dynamics, and immune infiltration (19). Building upon these advances, our high-throughput BCOC model effectively reproduces the bladder TME by incorporating tumor,

stromal, endothelial, and immune cells within a dynamic microfluidic system. This platform enables dynamic assessment of immune responses to intravesical therapies and provides a physiologically relevant system for preclinical drug testing.

In recent years, multiple immunotherapeutic strategies combining ICIs with other modalities have been investigated to improve outcomes in bladder cancer. Among these, PD-1 and PD-L1 inhibitors have shown particular relevance, as PD-L1 expression in bladder tumors has been linked to both cancer progression and BCG unresponsiveness (20). Combination approaches pairing PD-1/PD-L1 blockade with chemotherapeutic agents, cancer vaccines, or other immunomodulators have demonstrated enhanced immune activation in preclinical and clinical studies. For example, the use of a dendritic-cell vaccine together with PD-1 antibodies significantly increased antitumor immune responses in animal models (21). Other combinations under investigation include ICIs with CTLA-4 blockade, chemotherapy, radiotherapy, and targeted therapies, each aiming to augment antitumor efficacy (22).

Previous immunotherapy studies for bladder cancer have primarily focused on evaluating the systemic efficacy of ICIs administered intravenously (23-25). However, this approach does not fully reflect the localized immune responses occurring within the bladder following intravesical therapy. In contrast, our BCOC allows simultaneous assessment of both intravesical drug effects and immune interactions in a

controlled microenvironment. By integrating rBCG-*dltA* and PD-1 blockade in this 3D platform, it captures the dynamic interplay between tumor, stromal, and immune cells exposed to locally delivered agents. This provides a unique advantage for studying the localized immunomodulatory mechanisms of combination intravesical therapies and optimizing strategies for BCG-unresponsive bladder cancer.

The present study has two defining features. First, we used a recombinant strain rBCG-*dltA* instead of conventional BCG. The *dltA* gene confers resistance to cationic antimicrobial peptides (AMPs) through D-alanylation of lipoteichoic acids, increasing the bacterial surface charge and reducing AMP binding (26, 27). AMPs are secreted by urothelial and immune cells as part of innate defense mechanisms and can limit BCG survival and immune stimulation. By overcoming this barrier, rBCG-*dltA* achieves greater persistence and sustained antigen presentation in the bladder, which may potentiate the immune effects of PD-1 blockade.

Second, the combination of intravesical rBCG-*dltA* and anti-mPD-1 in the orthotopic mouse model produced a stronger antitumor response than either treatment alone, without causing significant systemic toxicity. Serial bioluminescence imaging showed a marked reduction in tumor signal intensity in the combination group, and subsequent histological examination of the excised bladder after mouse sacrifice confirmed a substantial decrease in actual tumor size on hematoxylin and eosin (H&E) staining. These results demonstrate that the combined therapy not only

reduced tumor viability *in vivo* but also led to a tangible reduction in bladder tumor volume at the tissue level. This finding provides strong histopathological evidence that the observed bioluminescent signal reduction reflects genuine tumor regression rather than transient metabolic suppression.

This study acknowledges several limitations. Although the synergistic antitumor effects of combining rBCG-*dltA* with PD-1 blockade were clearly demonstrated in both the BCOC and the orthotopic mouse model, the detailed immunological mechanisms underlying this interaction remain to be fully elucidated. Cytokine alterations induced by rBCG-*dltA* have been characterized in our previous work, which showed increased expression of IL-6, TNF- α , and IFN- γ ; therefore, cytokine profiling was not repeated in the present study to avoid redundancy. Future investigations will focus on comprehensive immunophenotyping of infiltrating immune cells, including CD8⁺ and CD4⁺ T cells and macrophage subtypes, using flow cytometry and immunohistochemistry to clarify the mechanistic basis of the observed synergy. Additionally, the histological evaluation via H&E staining was primarily conducted to validate the macroscopic tumor regression and correlate it with the BLI signal intensity. Consequently, a detailed immunophenotyping and spatial quantification of tumor-infiltrating immune cells were not performed in this histological analysis, which remains a limitation for a deeper understanding of the local immune landscape.

While the 3D bioprinted BCOC platform provides a physiologically relevant system that mimics the bladder tumor microenvironment, it still lacks key elements of the *in vivo* setting, such as a functional vasculature and a complete adaptive immune system, limiting its ability to assess systemic immune dynamics. Furthermore, the use of immortalized commercial cell lines ensures experimental reproducibility but does not fully reflect the genetic and phenotypic heterogeneity of patient-derived tumors. These constraints may affect the evaluation of long-term immune interactions, pharmacokinetics, and metastatic behavior.

Systemic toxicity was evaluated based on body-weight changes, which showed no abnormalities. However, serum biochemical analyses were not conducted; therefore, potential systemic toxicity cannot be fully excluded. As this study represents an early proof-of-concept evaluation, future investigations will include detailed hematological and biochemical assessments once the therapeutic protocol is fully established and optimized.

Despite these limitations, the BCOC remains a useful and reproducible preclinical platform for studying localized immune responses to intravesical therapies. Future studies will aim to incorporate patient-derived tumor cells and autologous immune components into the BCOC system to more accurately model the complexity and heterogeneity of human bladder cancer, ultimately bridging the gap between *in vitro* and *in vivo* immunotherapy research.

Conclusions

This research illustrates the potential therapeutic efficacy of the combination of rBCG-*dltA* and pembrolizumab in the treatment of bladder cancer. Utilizing a BCOC platform alongside an *in vivo* murine model, we identified synergistic antitumor effects resulting from this combinatorial strategy. This study provides preclinical evidence suggesting that the combination of rBCG and ICIs could serve for managing BCG-unresponsive bladder cancer.

List of abbreviations

Recombinant Bacillus Calmette-Guérin (rBCG); bladder cancer-on-a-chip (BCOC); non-muscle-invasive bladder cancer (NMIBC); muscle invasive bladder cancer (MIBC); Bacillus Calmette-Guérin (BCG); three-dimensional (3D); tumor microenvironments (TMEs); immune checkpoint inhibitor (ICI)

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care

and Use Committee of Chung-Ang University (Approval No. A2021020, January 09, 2023, Seoul, Republic of Korea) and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the ARRIVE guidelines. This study did not involve human participants or human tissue; therefore, consent to participate is not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data needed to evaluate the conclusions are presented in the paper. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

JHK: conceptualization, funding acquisition, writing-original draft, and writing-review & editing. JC: Data curation. CUL: investigation. YSL: supervision and investigation. SM: Data curation, formal analysis, visualization, and writing-original draft. MK: data curation, validation, and methodology. SYC: supervision and validation. IHC: conceptualization, supervision, funding acquisition, and writing-review & editing.

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Figure Legends

Figure 1. Schematic of the drug treatment protocol for an orthotopic mouse model of bladder cancer. After 10 to 14 days of intravesical instillation of MBT2-luc cells, the mice were divided into four groups: the control, rBCG-*dltA*, anti-mPD-1, and combination groups. The target drugs were administered a total of 4 times every 3–4 days, and the experiments were terminated on the 10th day of administration.

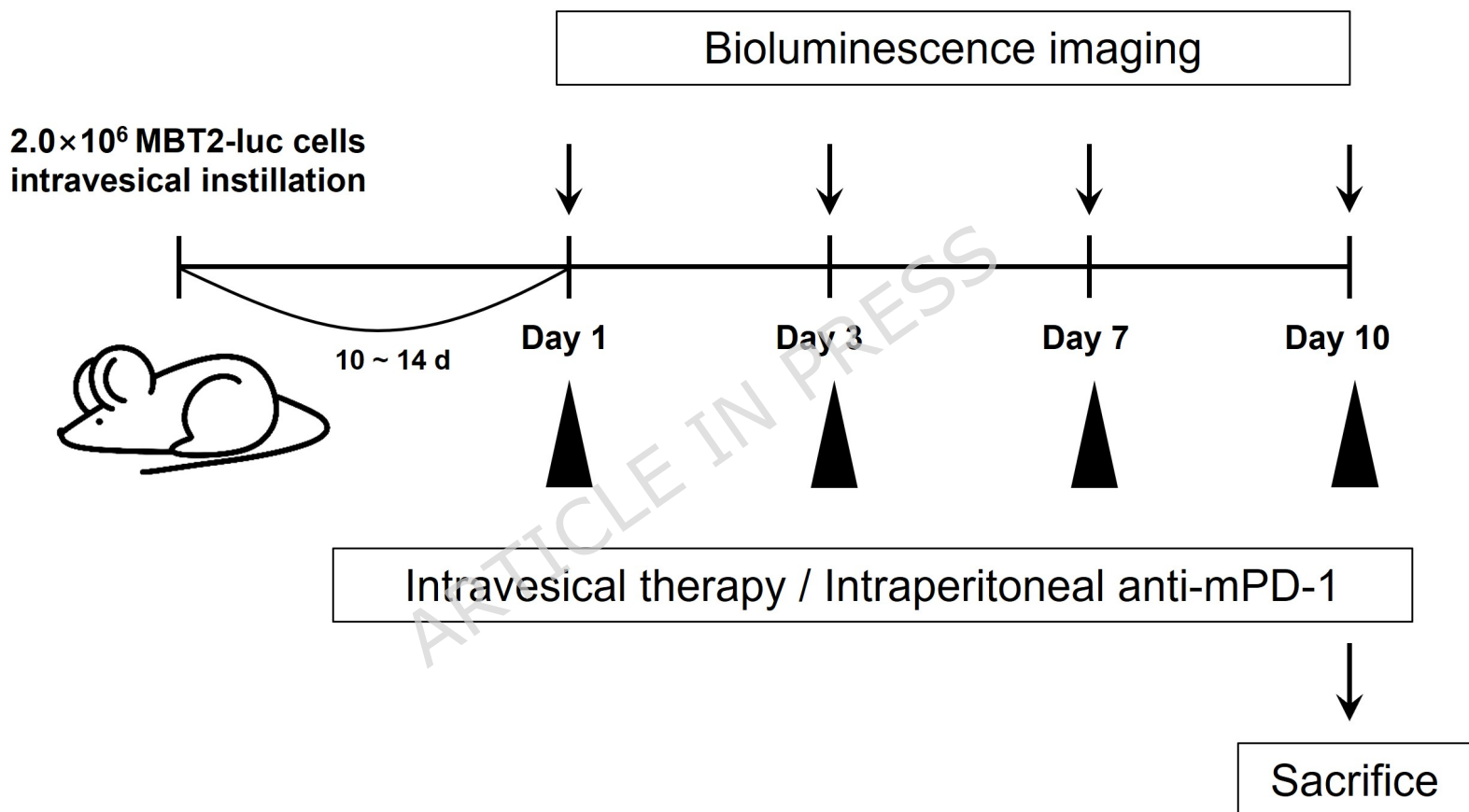
Figure 2. Cell viability of 3D bioprinted bladder cancer-on-a-chip model. (A) Live/dead staining on the 3rd day after drug treatment. T24 bladder cancer cell blocks in the rBCG-*dltA* and pembrolizumab combination group had lower cell survival rates than those in the other groups did. Noncancerous cell lines, such as HUVECs and MRC-5 cells, survived well in all groups. The scale bar at the lower right corner represents 200 μm . (B) According to the results of the quantitative cell density test, T24 cancer cells were affected by rBCG-*dltA* and pembrolizumab. The group with the lowest cell density was the combination-treated group. $*p < 0.05$ vs. control.

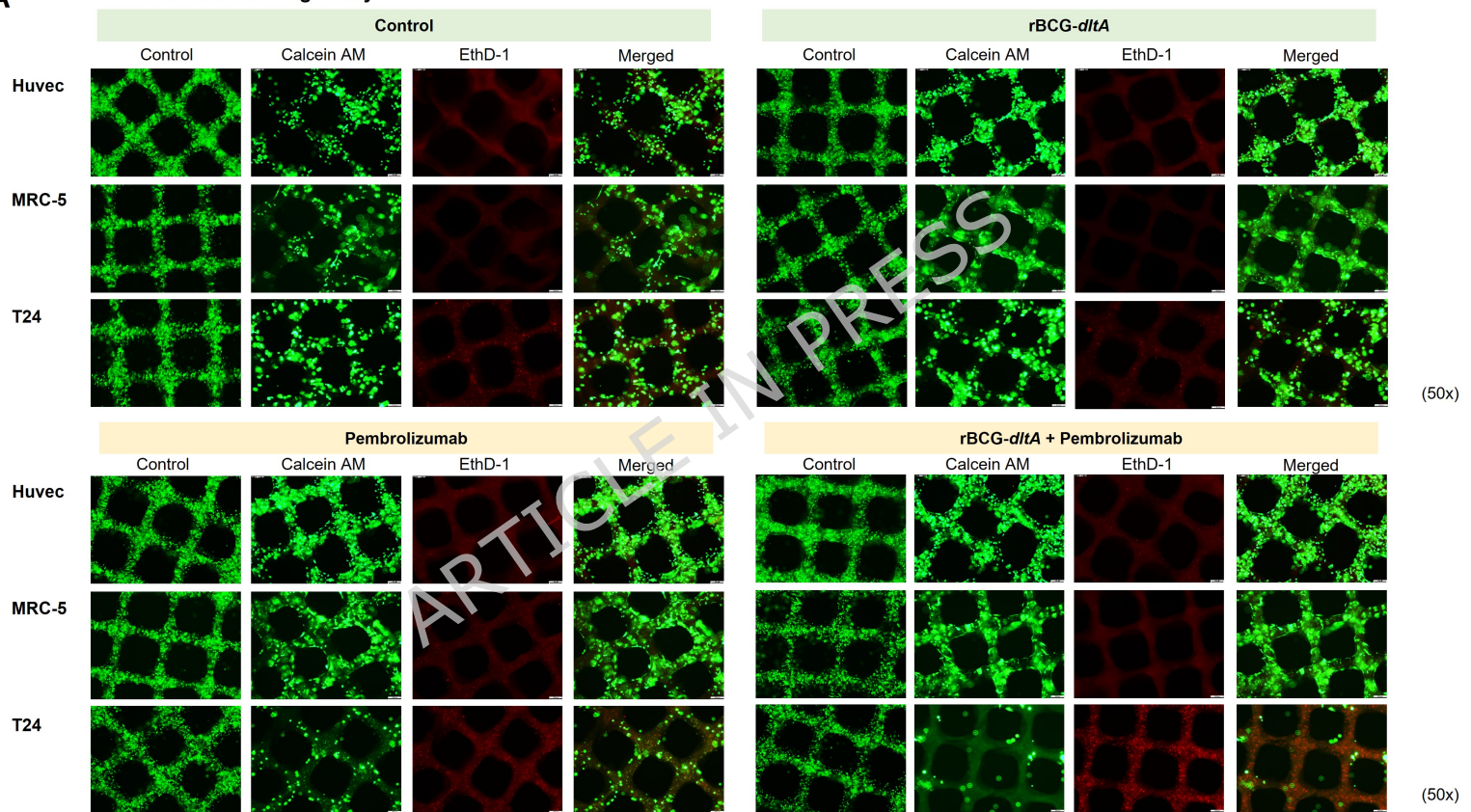
Figure 3. Chemotactic behavior of monocytic THP-1 cells within a chip model featuring a permeable membrane of bladder cancer cells. (A) Migration and distribution patterns of THP-1 cells in response to the treated agents. Blue-stained, monocytic THP-1 cells are shown migrating

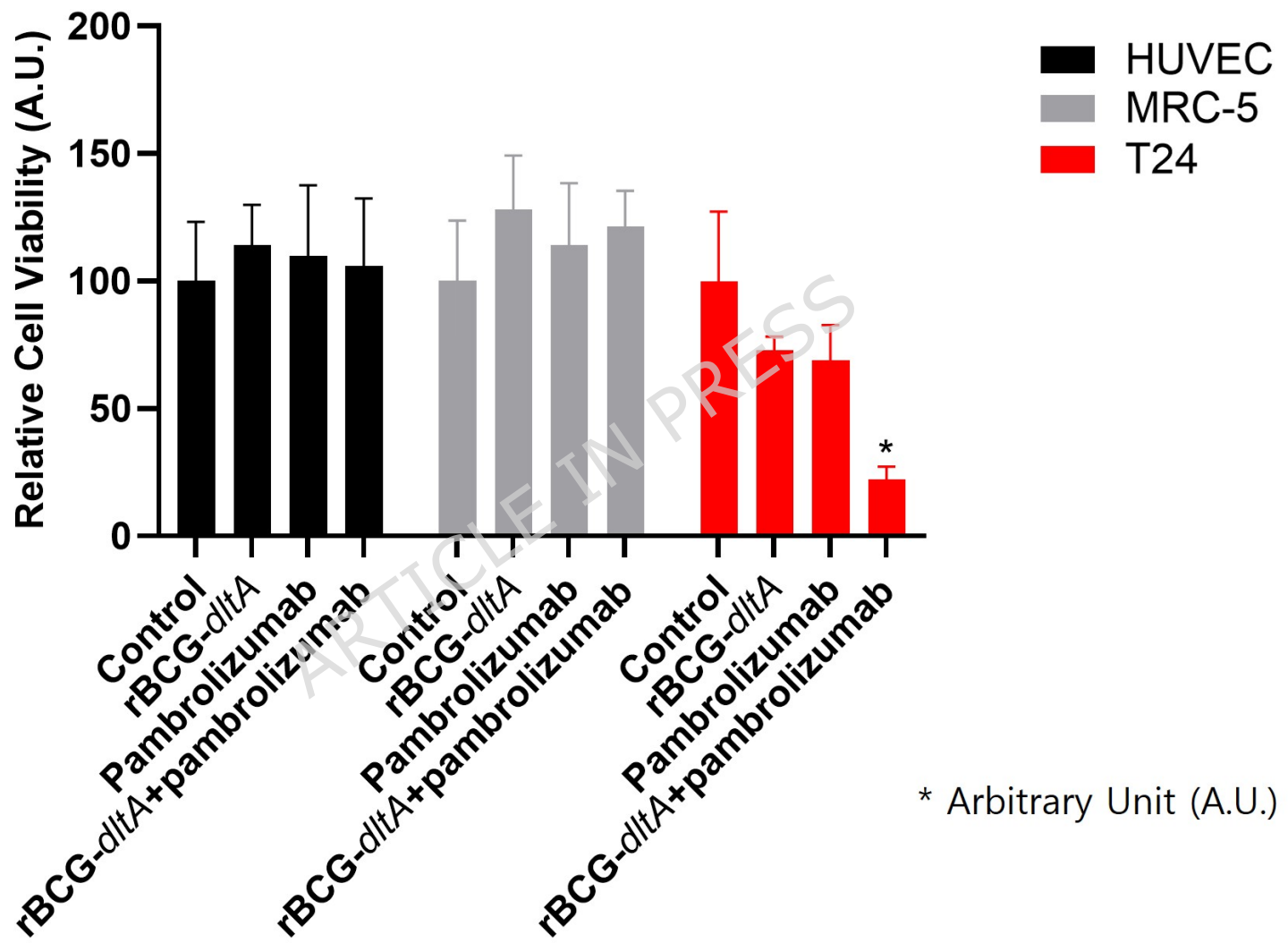
through the porous membrane toward bladder cancer cells after different treatments at 50× and 100× magnifications. (B) Quantitative analysis of the migration rates of THP-1 cells.

Figure 4. Therapeutic efficacy of the combination of rBCG-*dltA* and anti-mPD-1 in an orthotopic mouse model of bladder cancer. (A) Illustration of bioluminescence intensity demonstrating that the volume of bladder cancer decreased in the combination therapy group compared with the other treatment groups. (B) On the 10th day, quantitative analysis of signal intensity revealed a significant decrease in the rBCG-*dltA* and pembrolizumab combination group. (C) Bladder images from each group were stained with hematoxylin and eosin (H&E) stain following the sacrifice of the mice. The combination group exhibited smaller overall tumor area.

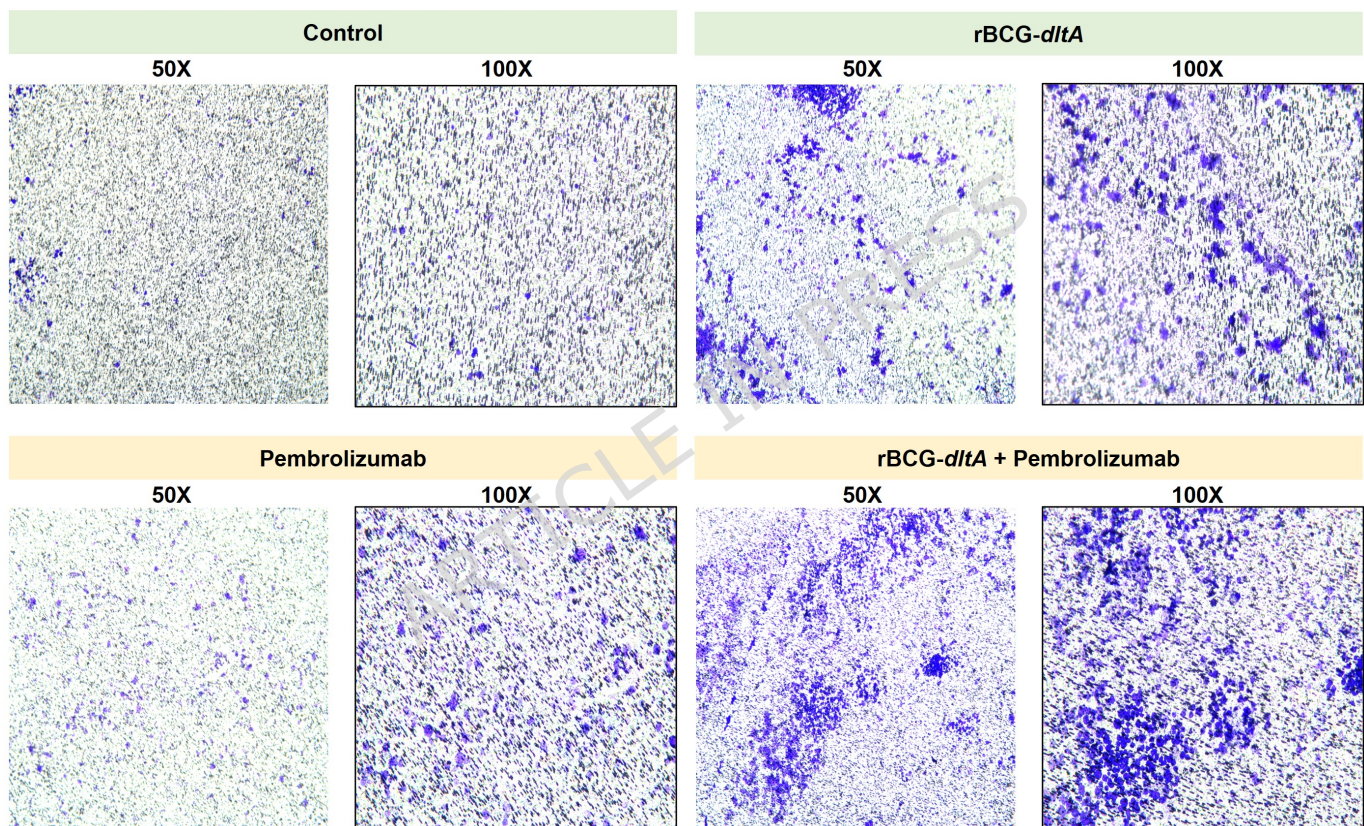
Figure 5. Estimation of body weights in each treatment group. A small amount of weight loss was observed in all four groups; however, there was no significant weight loss compared with that in the control group.



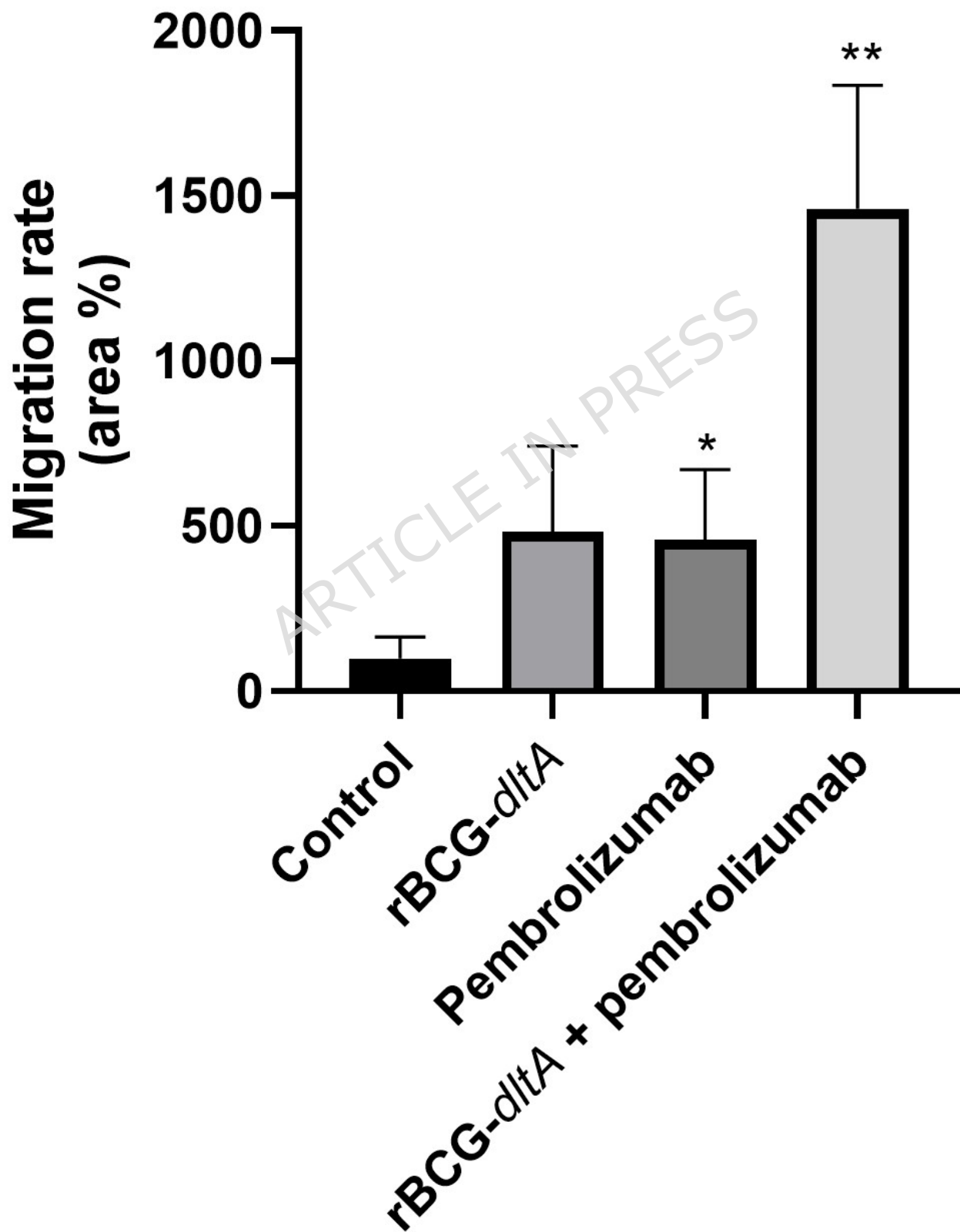
A Live & Dead Staining Assay

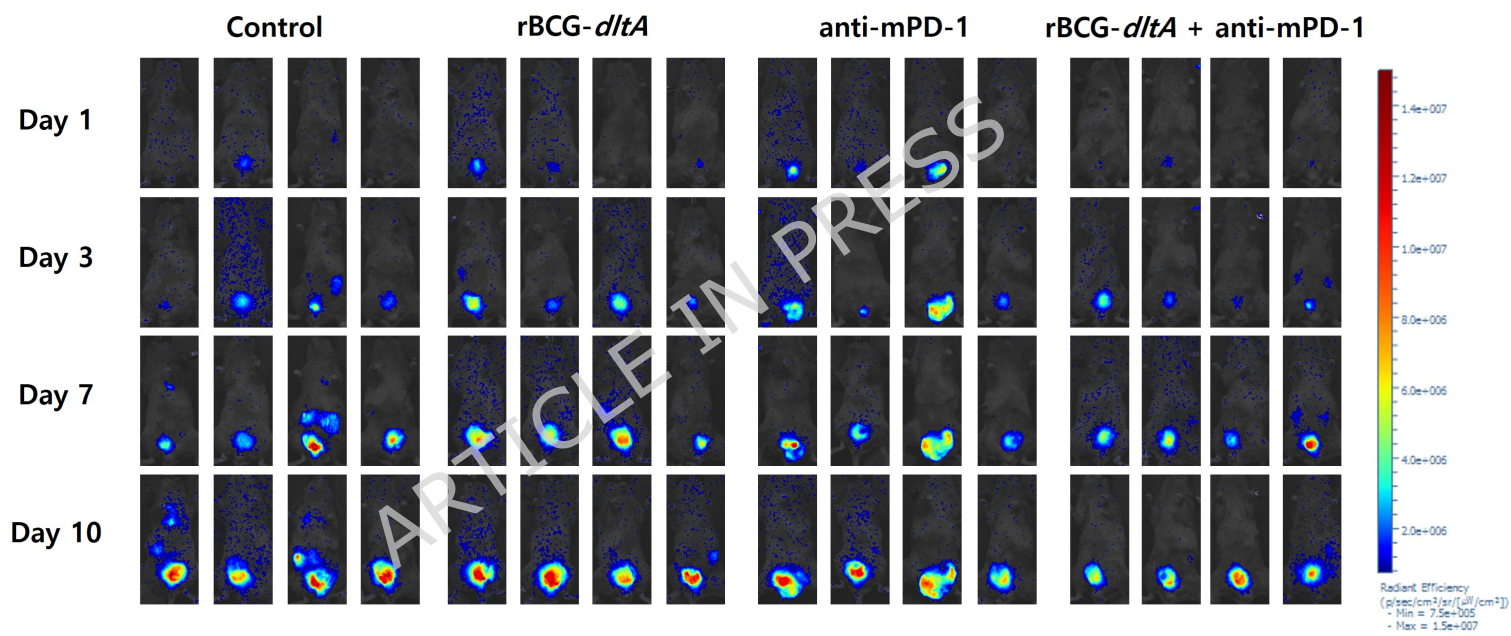
B

A

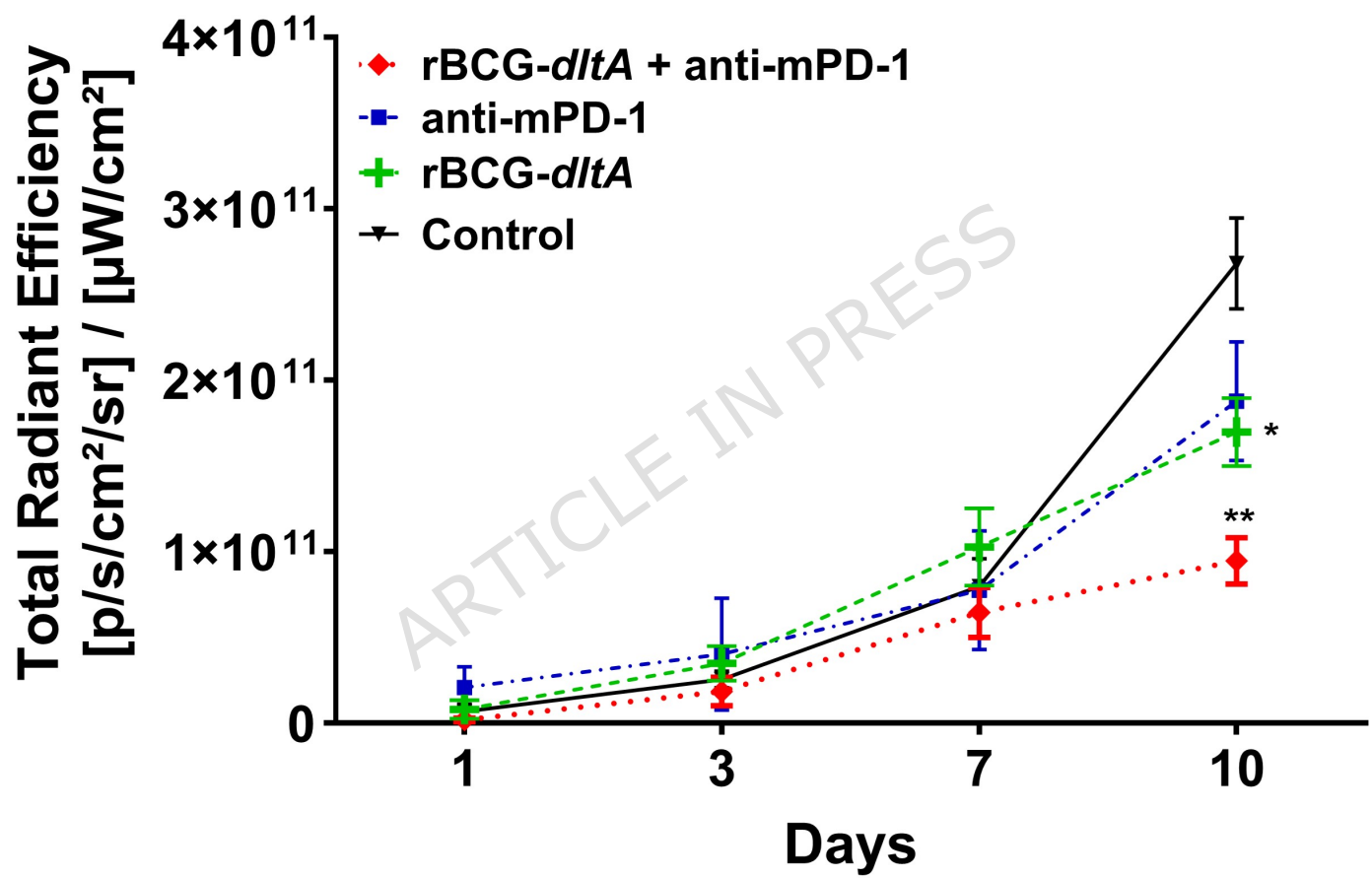


Scale bar, 100um

B

A

B



C

