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Enhanced anti-tumor immunity of vaccine combined with anti-PD-1 antibody in a murine bladder cancer model

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Purpose: Programmed cell death protein 1 (PD-1) and ligand programmed death ligand 1 (PD-L1) are important immune-suppressive regulators in the tumor microenvironment. A vaccine-induced immune effect on tumor cells is blunted by the immunosuppressive tumor microenvironment. Therefore, we hypothesized that a dendritic cell (DC) vaccine combined with anti-PD-1 (αPD-1) antibodies could elicit a synergistic anti-tumor immunity in bladder cancer.

Materials and Methods: We produced a model of subcutaneous transplantation in C3H/HeJ mice by transplanting murine MBT-2 bladder cancer cells. DCs were isolated from normal C3H/HeJ mice, followed by stimulation against MBT-2 lysate before injection. Two weeks later of MBT-2 inoculation, αPD-1 and stimulated DCs were injected two times at one-week interval intraperitoneally and intravenously, respectively. Tumor-infiltrating immune cells and splenocytes were analyzed using flow cytometry. T-cell-mediated anti-tumor responses were measured by interferon (IFN)-γ ELISPOT and lactate dehydrogenase assays.

Results: The mice treated with DC+αPD-1 showed a significant decrease in tumor volume compared to the DC-treated mice and IgG-treated group. Survival of the DC+αPD-1–treated group was improved compared with that of the IgG-treated mice. IFN-γ secretion from splenocytes against tumor cells was significantly increased in the DC+αPD-1 group compared with that of αPD-1–treated mice. The frequency of $CD8^+$ and $CD4^+$ T-cells in spleens was statistically increased in the DC+αPD-1–treated mice compared to those receiving monotherapy (DC- or αPD-1–treated group).

Conclusions: Our results support the hypothesis that the combination therapy of a DC vaccine and αPD-1 antibodies could enhance the anti-tumor immune response against bladder cancer.

Keywords: Anti-programmed cell death protein-1 antibody; Bladder cancer; Dendritic cells; Immunotherapy; Vaccine

INTRODUCTION

Bladder cancer is the most common cancer involving the urinary system, and one-fourth of patients will experience muscle-invasive disease and either present with or later develop metastases [1,2]. Various treatment options have been developed for patients with bladder cancer such as surgery, intravesical therapy, chemotherapy, radiation therapy, and

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immunotherapy. Systemic chemotherapy is the standard treatment for inoperable locally advanced or metastatic stage [2]. Although each therapeutic method has been continuously developed and tested in clinical studies, there is still limited success in improving outcomes for patients with locally advanced or metastatic bladder cancer, considering that the five-year survival rate is about 15% with contemporary regimens [2]. Immunotherapy has been applied to improve outcomes in bladder cancer patients. As a first immunotherapy approach, Bacillus Calmette–Guérin (BCG) has been confirmed to have positive effects on cancers since the 1950s; it has been associated with promising clinical outcomes, and intravesical BCG immunotherapy remains the gold standard for treatment of non–muscle-invasive bladder cancer [3,4]. Immune checkpoint inhibitors have shown promise in several cancers, including melanoma, non–small cell lung cancer, head and neck cancer, and bladder cancer [4,5]. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) inhibits T-cell activation at the initial activation site; therefore, several clinical studies using anti-CTLA-4 antibodies have been conducted in various types of cancer patients with or without chemotherapy [6,7]. Programmed cell death protein 1 (PD-1) is upregulated in activated T lymphocytes and inhibits T-cell function upon binding to its ligand programmed death ligand 1 (PD-L1). Several studies have shown that bladder cancer cells express PD-L1, in which increased PD-L1 level has been correlated with tumor stage and reduced overall survival, suggesting that bladder cancer cells may escape T-cell-mediated anti-tumor immune response by PD-1/PD-L1 signaling [8,9]. In addition, increased resistance to BCG therapy is associated with PD-L1 expression of tumor cells [10]. Recently, five PD-1/PD-L1 inhibitors (pembrolizumab, nivolumab, atezolizumab, durvalumab, and avelumab) have been approved by the US Food and Drug Administration (FDA) for patients who have progressed during or after platinum-based therapy based on clinical trial results, which showed only 15% to 25% overall response rate [11]. Despite these approvals by the FDA, there is still a need for developing novel immunotherapies that enhance anti-tumor activity and prolong survival in patients with metastatic bladder cancer. Moreover, even bladder cancer patients with high PD-L1 expression or tumor mutation burden often did not respond to PD-1/PD-L1 antibody treatment [12].

Dendritic cells (DCs) have been used as vaccine platforms to induce anti-tumor cytotoxic T lymphocytes due to their proficiency at antigen cross-presentation [13]. In preclinical studies, DC vaccine showed anti-tumor immunity against bladder cancer [14-16]. Some of these DC-based vaccines are now in clinical trials in patients with bladder cancer and other types of cancers (NCT04184232, NCT03406715). Unfortunately, vaccination against established malignancies has mostly shown limited clinical benefits [17]. Tumor cell eradication could be impacted by immune-suppressive protein overexpression, such as that of checkpoint receptor signaling (CTLA-4, PD-1/PD-L1) and immunomodulatory cellular subsets (regulatory T-cells and myeloid-derived suppressor cells) [17]. Therefore, ongoing trials using DC-based vaccines are evaluating the use of combined immunotherapies to favor DC activation and stimulate T-cell functions to overcome tumor immune evasion [18]. However, no study has investigated the anti-tumor effect of anti-PD-1 (αPD-1) with DC vaccines for bladder cancer [19,20]. Therefore, we investigated the DC vaccine in combination with αPD-1 antibody to determine if this approach could elicit a synergistic anti-tumor immunity in bladder cancer.

MATERIALS AND METHODS

1. Animal model generation, treatment, and cell line

We produced a bladder cancer model by subcutaneous injection with 1×10^6 MBT-2 cells (murine bladder cancer cells) in eight-week-old C3H/HeJ mice from Jackson Laboratory (Bar Harbor, ME, USA). The MBT-2 cell line was kindly provided by Dr. Kyung Seok Han (Severance Hospital, Yonsei University, Seoul, Korea) [21].

The tumor volume (V) was monitored twice a week and estimated by measuring tumor size: $V=(L\times W^2)/2$, where L represents the length and W is the width of tumor. Around two weeks after MBT-2 injection, the average tumor volumes of mice reached around 400 mm^3 and then 200 μg of αPD-1 (Affymetrix Ebioscience, San Diego, CA, USA) and/or 1×10^7 of activated DCs were injected two times at one week, respectively. αPD-1 was administered into the peritoneal cavity, and DC was injected intravenously. The experimental group (n=38) was randomly divided into five groups: saline-treated normal mice (without MBT-2 injection, n=4), immunoglobulin G (IgG)-treated controls (with MBT-2 injection, n=10), DC-treated (with MBT-2 injection, n=8), αPD-1–treated (with MBT-2 injection, n=8), and DC+αPD-1–treated (with MBT-2 injection, n=8) group. Body weight, tumor size, and survival measurements were obtained every 2 days from day 0 to day 19 (Fig. 1), samples were collected at day 34, followed by anesthesia using a Zoletil 50 (40 mg/kg)–rompun (10 mg/kg) mixture. All experimental procedures for our animal studies were approved by the Committee for the Care and Use of Laboratory Animals of Catholic Kwandong University College of Medicine (no. CKU02-2015-007) and performed

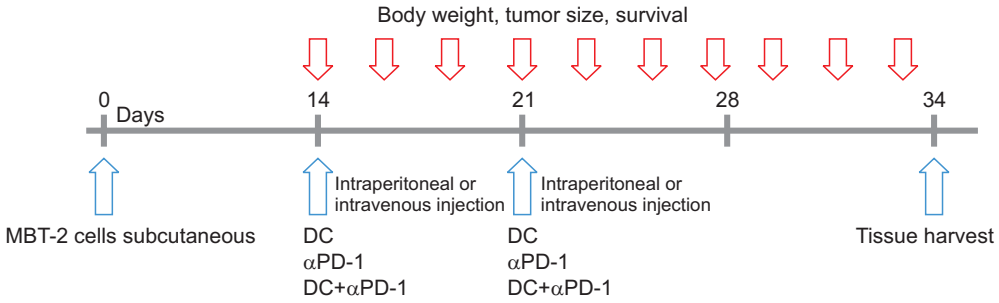

Fig. 1. Schematic diagram of the treatment protocol. Around two weeks after MBT-2 injection, a total of 1×10^7 dendritic cells (DCs) were injected intravenously, and 200 μg of anti-programmed cell death protein 1 (αPD-1) antibodies were injected intraperitoneally. Body weight, tumor size, and survival measurement were every 2 days (red arrows). Mice were sacrificed and the samples were collected (day 34).

in accordance with the Committee's Guidelines and Regulations for Animal Care.

2. Activated DC generation

DCs were isolated from bone marrow of normal C3H/HeJ mice and cultured with granulocyte-macrophage colony stimulating factor (GM-CSF, 20 ng/mL) and interleukin-4 (IL-4, 500 ng/mL) in RPMI-1640 (Gibco, Carlsbad, CA, USA) (R10) at 37°C in an incubator containing 5% CO_2. Growth medium with GM-CSF and IL-4 was changed every 2 to 3 days. Eight days after induction, DCs were stimulated against MBT-2 lysate (10 μg/mL) for 4 hours to generate tumor lysate-pulsed DCs before injection. The phenotypic characterization of generated tumor lysate-pulsed DCs was confirmed by FACS analysis using DC surface expression molecules of CD11c (Cat No: 553802, BD Bioscience, San Jose, CA, USA), CD40 (Cat No: 553790, BD Bioscience), CD80 (Cat No: 553768, BD Bioscience), H-2Dk (Cat No: 110307, BioLegend, San Diego, CA, USA), CD86 (Cat No: 553691, BD Bioscience), MHCII (Cat No: sc-32247, SantaCruz Biotechnology, Dallas, TX, USA), and DEC205 (Cat No: 138214, BioLegend) (Supplementary Fig. 1).

3. Primary splenocytes preparation

Immediately after spleens were harvested on the day of sacrifice (day 34), splenocytes were isolated by density gradient centrifugation using Lymphosep reagent (Biowest, Liverside, MO, USA) according to the manufacturer's instructions. Collected splenocytes were suspended in HBSS (GE healthcare, Pittsburgh, PA, USA) and then directly used for flow cytometry.

4. Tumor cell isolation

Isolated fresh tumor tissues from mice bladder cancers were sliced into 1 to 3 mm^3 pieces and then incubated at 37°C for 1 hour in HBSS (GE Healthcare) with 1.5 mg/mL collagenase I and IV (Worthington Biochem, Lakewood, NJ, USA) and 200 U/mL DNase I (Worthington Biochem). Single cells were obtained using a 100-μm nylon cell strainer (Sigma-Aldrich, Saint Louis, MO, USA). Collected cells were then washed with HBSS and used directly for experiments.

5. Lactate dehydrogenase assay

To detect the anti-tumor effect of splenocytes on MBT-2 as target cells, 1×10^4 of MBT-2 was co-incubated with splenocytes (effector cells) at a ratio of 1 to 5 for 12 hours. After centrifugation, lactate dehydrogenase (LDH) in the medium was measured using an LDH cytotoxicity detection kit (TaKaRa Bio, Otsu, Japan), in accordance with the manufacturer's protocol.

6. Interferon γ assay

To detect the cytokine activity of splenocytes, 1×10^6 of MBT-2 cells were incubated with 5×10^6 of splenocytes under RPMI containing 10% FBS (Gibco, Carlsbad, CA, USA) and 100 U/mL IL-2 (R&D Systems, Minneapolis, MN, USA). The cell culture supernatant was collected 48 hours later. Quantitative determination of interferon (IFN)-γ was performed using Quantikine ELISA (R&D Systems) and estimated under a microplate at 450 nm with a correction wavelength of 540 nm.

7. Flow cytometry

Tumor-infiltrating immune cells and splenocytes were analyzed using flow cytometry. Anti-CD45-BV421 (BD bioscience), anti-CD8-FITC (BioLegend), and anti-CD4-FITC (BD Bioscience) antibodies were used to characterize the cells (1×10^6) using the FACSVerseTM system (BD Bioscience, Piscataway, NJ, USA) in accordance with the manufacturer's protocol.

8. Statistical analysis

Kaplan–Meier survival curves were generated from the data tabulated using Microsoft Excel (Microsoft Corp., Red-

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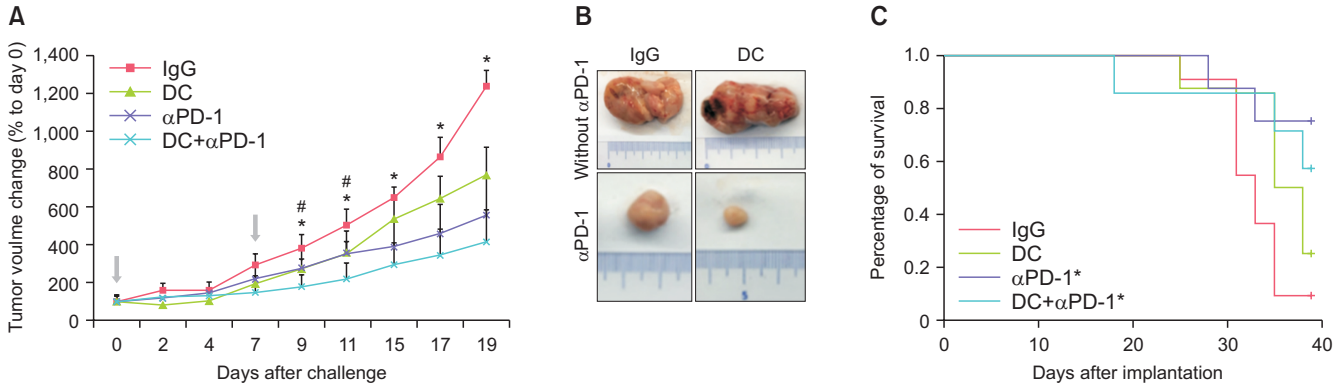

Fig. 2. Effect of stimulated dendritic cell (DC) and anti-programmed cell death protein 1 (αPD-1) antibodies in a bladder tumor mouse model. (A) Tumor size was estimated every three days for 20 days. The gray arrows mean the injection time of DC, αPD-1, or DC+αPD-1. *p<0.05 (immunoglobulin G [IgG] vs. DC+αPD-1), #p<0.05 (DC vs. DC+αPD-1). (B) Representative images show bladder tumors from experimental groups. (C) Kaplan–Meier survival curves. *p<0.05 (IgG vs. αPD-1, IgG vs. DC+αPD-1).

mond, WA, USA) and analyzed using SPSS for Microsoft Windows, version 22.0 (IBM Corp., Armonk, NY, USA). Statistical analysis of multiple groups was performed using one-way ANOVA followed by Tukey's multiple comparison tests with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data are presented as mean±standard error of the mean. $p<0.05$ was considered statistically significant.

RESULTS

1. PD-1 antibody and stimulated DC present synergistic anti-tumor effects

To confirm the anti-tumor effect of DC vaccine with αPD-1 antibody *in vivo*, we generated a bladder tumor mouse model using the MBT-2 mouse bladder tumor cell line. Stimulated DC and αPD-1 antibodies were injected twice every seven days to enhance T-cell immunity (Fig. 1). After the second injection of αPD-1 and DC, significant differences in tumor volume were observed between the IgG-treated group and other groups, such as DC, αPD-1, and DC+αPD-1. At day 9 and 11 after challenge, the DC+αPD-1 group showed a significant decrease in tumor volume compared to the IgG-treated and DC-treated groups (Fig. 2A). Additionally at day 19 after challenge, the DC+αPD-1 group showed a significant decrease in tumor volume compared to the IgG-treated group (Fig. 2A). Moreover, one complete remission (CR) case was observed in DC+αPD-1–treated group (1/8=13.0%), but such CR did not occur in other groups.

When cancer tumor size reached 3,500 mm^3, we sacrificed all mice. Survival of the DC+αPD-1–treated group as well as the αPD-1 group was improved compared with that of the IgG-treated group (Fig. 2C). This suggested that DC+αPD-1 treatment efficiently inhibited tumor growth, although there were no significant differences in survival between the DC+αPD-1 group and the αPD-1 group.

No noticeable body weight decrease was recorded during the study in any of the groups; in fact, body weight increased in all groups. This observation supported the toxicity of DC+αPD-1 treatment was not increased compared to the monotherapy. Tumor growth increases the weight of a mouse (Fig. 3A). Spleen weight was measured at time of sacrifice and analyzed as spleen/body weight ratio (Fig. 3B). In controls, the spleen/body weight ratio significantly increased but was attenuated with DC+αPD-1 treatment. Splenomegaly in oncogenesis animal model is known to be related to an immune-suppressive reaction [22]. Based on these results we next investigate whether the combination of DC with αPD-1 could enhance the anti-tumor immune response.

2. PD-1 antibody and stimulated DC accelerates bladder cancer cell death through T-cell response

IFN-γ is a cytokine that has multiple immunoregulatory effects and is known to activate cytotoxic T-cells (CTLs) [17]. Therefore, we investigated IFN-γ secretion under each set of experimental conditions. IFN-γ secretions from splenocytes against tumor cells were significantly highest in the DC+αPD-1 group, intermediate in the DC-treated group, and lowest in the αPD-1 or IgG-treated group (Fig. 4A). Similar trends were observed for the cytotoxicity assessed with LDH assay. Cytotoxicity against MBT-2 cells was significantly higher in the splenocytes of the DC+αPD-1 group compared to controls (Fig. 4B). The frequency of $CD8^+$ and $CD4^+$ T-cells in spleens was statistically increased in the DC+αPD-1–treated mice compared to the DC- or αPD-1–treated groups (Fig. 5A, B). In tumor tissues, DC+αPD-1–treated mice showed in-

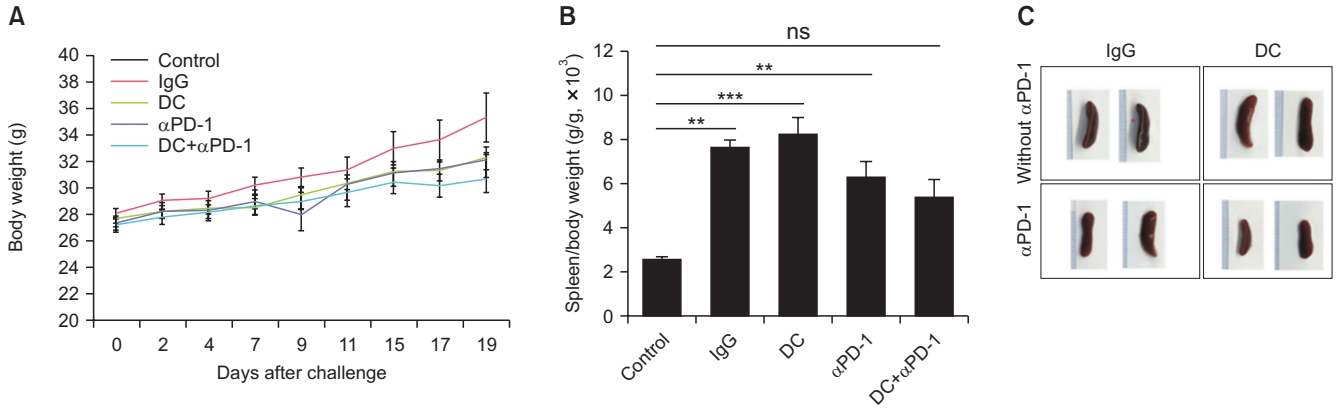

Fig. 3. Effects of stimulated dendritic cell (DC) and anti-programmed cell death protein-1 (αPD-1) antibodies on body weight and spleen weight in a bladder tumor mouse model. (A) Body weight was estimated every three days for 20 days. (B) Spleen/body weight ratio was measured 20 days after DC and αPD-1 antibody challenge. (C) Representative images show spleens from experimental groups. Control, saline-treated normal mice; IgG, immunoglobulin G; ns, not significant. **p<0.01, ***p<0.001.

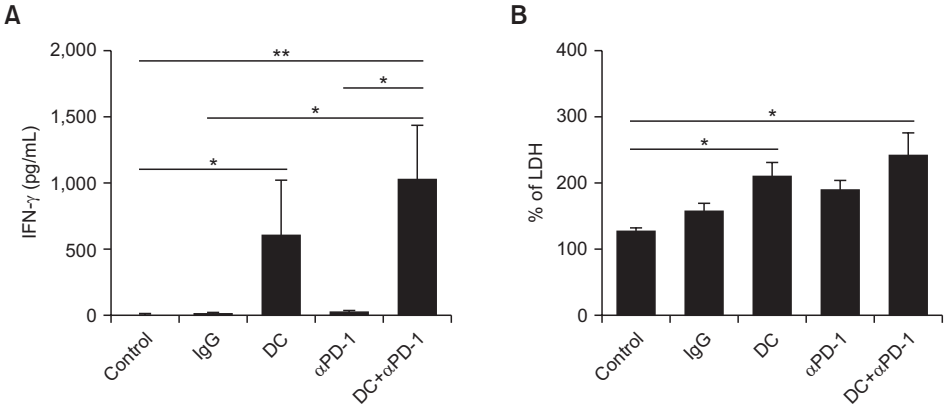

Fig. 4. Effect of stimulated dendritic cell (DC) and anti-programmed cell death protein-1 (αPD-1) antibodies in primary cultured splenocytes. Splenocytes were activated by interleukin-2 for 48 hours and then co-cultured with MBT-2 cells. (A) Interferon γ (IFN-γ) was measured by an ELISA assay from cell culture media (n=4–6 of each group, *p<0.05, **p<0.01). (B) Lactate dehydrogenase (LDH) activity was measured from cell culture media (n=5–7 of each group, *p<0.05 vs. control). LDH was normalized to the untreated MBT-2 cells. Control, saline-treated normal mice; IgG, immunoglobulin G.

creased $CD4^+$ T-cells, while the αPD-1–treated group revealed an increasing number of $CD8^+$ T-cells (Fig. 5C, D). These results show that DC combined with αPD-1 could enhance T-cell–mediated cancer immunity in comparison to monotherapy using each treatment.

DISCUSSION

Immune checkpoint inhibitors have been approved by the FDA for treatment of metastatic bladder cancer [23]. However, a large majority of bladder cancer patients does not respond to αPD-1 inhibitors as monotherapy. Combination treatments have been evaluated to improve the efficacy of αPD-1 agents with anti-CTLA-4 drugs, chemotherapy and radiotherapy, targeted therapies, or other immunomodulatory agents [23].

In this study, we showed that the combination of αPD-1 and DC significantly enhanced anti-tumor immune response in bladder cancer mouse models, and that there was an increase in $CD4^+$ and $CD8^+$ cells in spleens compared to controls. There was one CR in DC+αPD-11–treated group. In addition, the combination treatment significantly increased IFN-γ secretion and cytotoxicity of splenocytes, although PD-1 antibody treatment alone did not have this effect. DCs, which are the most potent antigen-presenting cells, are a major regulator of T-cell–mediated cancer immunity. Recent investigation has suggested that conventional DCs (cDCs) 2 primarily activate $CD4^+$ T-cells, and that cDC1 mainly activate $CD8^+$ T-cells [24]. It is reasonable to assume that tumor lysate-pulsed DCs in our study might prime T-cells and induce cancer-specific T-cell response. Research studies have shown that DC activation can stimulate T-cells; therefore, a

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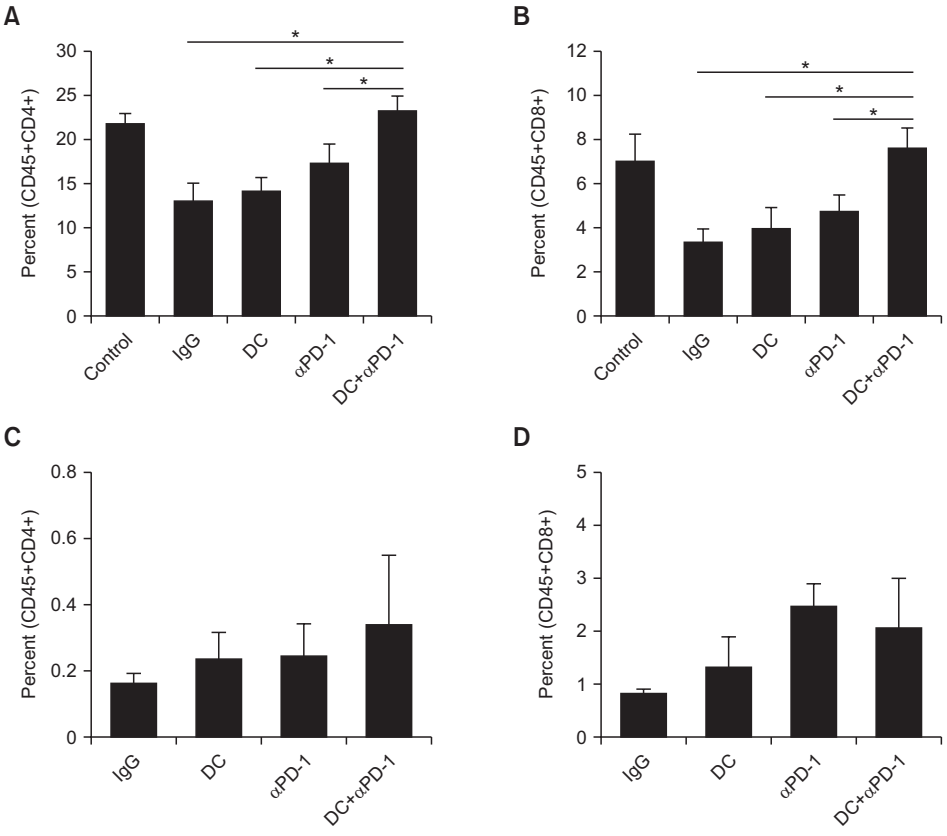

Fig. 5. Immune cell infiltration into spleen and tumors following dendritic cell (DC) and anti-programmed cell death protein-1 (αPD-1) antibody. (A, B) Flow cytometry was performed using splenocytes obtained from spleens (n=3–7 of each group). (C, D) Flow cytometry was conducted using tumor cells obtained from tumor tissue (n=3–7 of each group). Control, saline-treated normal mice; IgG, immunoglobulin G. *p<0.05.

DC-based vaccine has been generated using common antigens such as HER-2/neu or human cancer specific antigens [25,26]. Because bladder cancer has been reported to undergo frequent genetic alterations, a DC vaccine generated from personalized tumor antigens is expected to be a new type of therapeutic approach. Experimental results from Nishiyama et al. [26] using an autologous DC vaccine pulsed with the tumor-specific epitope peptides showed good potential for developing personalized treatments for bladder cancer. Four HLA-A24$^+$ patients with advanced MAGE-3$^+$ bladder cancers were treated with autologous DC pulsed with MAGE-3 epitope peptides. Three of four patients showed significant reductions in the size of lymph node metastases and/or liver metastasis [26].

As a new type of combination therapy with checkpoint inhibitors, activated DC with αPD-1 antibodies has been reported to enhance therapeutic effects of the anti-cancer agent in hepatocellular carcinoma. PD-1 antibodies and DCs pulsed with exosomes derived from tumor cells enhanced the efficacy of sorafenib compared to monotherapy [27]. For the HER2$^+$ mammary carcinoma model, PD-1 antibody administered sequentially with HER2 peptide-pulsed DCs delayed tumor growth compared to the DC or PD-1 antibody alone [28]. These results are similar to our results showing that combination therapy with PD-1 antibody and DC vaccines showed enhanced therapeutic effects in a bladder cancer mouse model. Moreover, Park et al. [29] demonstrated that PD-1 negatively modulates the survival of DC in PD-1 knock-out mouse model. Lim et al. [30] found that DCs with PD-1 expression suppressed cytokine secretions such as IL-2 and IFN-γ and proliferation of CD8$^+$ T-cells. Furthermore, myeloid cell-specific PD-1 ablation leads to enhanced T-effector memory cells with anti-tumor immunity, suggesting that the PD-1 antibody directly affects the functionality of myeloid cells, including DC [31]. These results show that the PD-1 antibody may be useful for enhancing DC activity as well as T-cell activity in the immune-suppressive tumor microenvironment. Taken together, these findings suggest that DC vaccination may be clinically useful in combination with αPD-1 antibody for tumor control in cancer patients. Considering that only a fraction of bladder cancer patients benefit from immune checkpoint blockade, this hypothesis warrants continued study in the setting of clinical trials.

The limitation of our study was that we investigated one murine bladder cancer model, MBT-2. Different mice strains might have different immune status [32]. Consequently, repeat of this study with other murine mouse models could lead to different results. MB49, which is a commonly used murine bladder cancer model, shows enhanced anti-tumor effects of immunotherapy combined with chemotherapy

[33]. On the other hand, standard chemotherapy reduces the anti-tumor effect of immune checkpoint inhibitors in MBT-2 bladder cancer. In addition, radiation led to upregulation of immune stimulatory molecules CD80, MHC I, and Fas on MB49, but not MBT- 2 cells [34]. These previous results emphasized that the effects of combination with conventional treatments, such as chemotherapy and radiotherapy, and αPD-1 antibody on anti-tumor activity depend on murine model. Further study is needed to evaluate anti-tumor effects of combination of αPD-1 and DC in other bladder cancer models.

The therapeutic sequence of DC and αPD-1 must be considered. We treated DC+αPD-1 simultaneously, but it is possible that sequenced treatment could lead to different results. Furthermore, analysis of other immune cells (e.g., NK cell, macrophage) and immune modulating proteins (e.g., JAK/STAT) in tumor and of immune-related cytokines (e.g., IFN-γ, TGF-β) in serum could provide additional insights into anti-tumor immunity of DC combined with αPD-1 antibody (Supplementary Fig. 2). Lastly, identification and characterization of MHC-associated tumor antigens that can prime antigen-specific $CD8^+$ T-cells from tumor lysate-pulsed DCs would promote DC-based vaccine development as well as the discovery of immunogenic peptides for tumor immunotherapy [35].

CONCLUSIONS

Taken together, our study results support the combination of αPD-1 and DC-mediated immunotherapy as an approach to enhance IFN-γ production and anti-tumor immunity by antigen-specific T-cells. The combination of αPD-1 antibodies and DC vaccines may pave the way to improve the clinical response for patients with bladder cancer.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Hyun Chang. Data acquisition: all authors. Statistical analysis: all authors. Data analysis and interpretation: Hyun Chang and Soyeon Lim. Drafting of the manuscript: all authors. Critical revision of the manuscript: all authors. Obtaining funding: Hyun Chang. Administrative, technical, or material support: Soyeon Lim and Jun-Hee Park. Supervision: Hyun Chang. Approval of the final manuscript: all authors.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.4111/icu.20220031.

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