

Bisdemethoxycurcumin in combination with α -PD-L1 antibody boosts immune response against bladder cancer

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Abstract: Curcumin was recently discovered to strengthen immune response through multiple mechanisms. Cytotoxic CD8⁺ T-cells play a critical role in modulating anticancer immune response, but is severely restricted by T-cell exhaustion. Bladder carcinomas express PD-L1 and can abrogate CD8⁺ T-cell response. Thus, we hypothesized that bisdemethoxycurcumin, a natural dimethoxy derivative of curcumin, may provide a favorable environment for T-cell response against bladder cancer when used in combination with α -PD-L1 antibody. Immuno-competent C56BL/6 mouse models bearing subcutaneous or lung metastasized MB79 bladder cancer were established to validate this conjecture. We found that bisdemethoxycurcumin significantly increased intratumoral CD8⁺ T-cell infiltration, elevated the level of IFN- γ in the blood, and decreased the number of intratumoral myeloid-derived suppressor cells. Furthermore, α -PD-L1 antibody protected these amplified CD8⁺ T-cells from exhaustion, and therefore facilitated the secretion of IFN- γ , granzyme B, and perforin through these CD8⁺ T-cells. As a result, this combination treatment strategy significantly prolonged survival of intraperitoneal metastasized bladder cancer bearing mice, suggesting that bisdemethoxycurcumin in combination with α -PD-L1 antibody may be promising for bladder cancer patients.

Keywords: bladder cancer, immunotherapy, bisdemethoxycurcumin, PD-L1, combination therapy, metastasis

Introduction

Bladder cancer, the second most common genitourinary malignancy, is threatening human beings by its invasive and metastatic behavior. Conventional adjuvant chemotherapies such as combinations of platinum, gemcitabine, and ifosfamide usually fail to show survival advantages. Alternative nonsurgical therapeutic strategies are critical to improve patient survival. The immune system plays a critical role in controlling tumor growth, but malignancies, including bladder cancer, can escape immune attack through multiple pathways.^{1,2} Any method that can improve immune response against bladder cancer will provide opportunities for better outcome, especially for patients in whom first-line chemotherapy failed to control tumor progression.³

In immune cells, CD8⁺ T-cell is one of the most effective cytotoxic cells in mediating tumor-specific attack. However, there are suffocating restraints in CD8⁺ T-cell-based immunotherapy, such as immune checkpoint PD-1/PD-L1 axis, and the tumor immunosuppressive microenvironment including local Treg cells. Pathology studies have proven that most bladder cancer cells express PD-L1,^{4,5} which can profoundly impair the effects of cytotoxic CD8⁺ T-cells (CTLs). In addition, tumors can develop an immunosuppressive microenvironment via different pathways,

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and consequently decrease the amount of tumor infiltrating lymphocytes (TILs). These two factors in combination, can result in suppressed CTL homing and weakened CTL function, and finally abrogate the spontaneous immune attack against bladder cancer.

Recently, antibodies for PD-1/PD-L1 pathway displayed favorable results in strengthening the activity of CD8⁺ T-cells in clinical trials for bladder cancer.^{6,7} If there is a method to improve the immune response via immune checkpoint independent ways, it can improve the effects of checkpoint inhibitors. Concerning the immunosuppressive environment, intratumoral Treg cells and myeloid-derived suppressor cells (MDSCs) play critical roles. Curcumin, a widely studied component of turmeric, has been proven to facilitate immune response against different solid malignancies via multiple pathways.^{8–10} However, poor bioavailability has limited the application of curcumin.¹¹ Thus, analogs of curcumin, such as bisdemethoxycurcumin (BDMC), may be promising drugs for clinical application.¹² Given the fact that curcumin can mediate immune response, we conjectured that BDMC could promote immune attack against bladder cancer, and the effects could be strengthened when used in combination with PD-1/PD-L1 antibody.

In this study, we tried to validate our hypothesis that BDMC treatment and PD-1/PD-L1 blockade can promote spontaneous immune response against bladder cancer. We established immunocompetent C56BL/6 mouse models bearing subcutaneous (s.c.) or metastatic bladder cancer, and then treated them with BDMC and/or α -PD-L1 antibody. We discovered that low dose BDMC, in combination with α -PD-L1 antibody, displayed favorable effects in facilitating immune response and significantly prolonged mouse survival.

Materials and methods

Ethics approval

The ethics committees from Shanghai Ninth People's Hospital and Shanghai Yueyang Hospital have approved this animal study, and the approval was obtained prior to the commencement of the study. All animal studies were performed following the guidelines and regulations of the animal care committees of Shanghai Ninth People's Hospital and Yueyang Hospital.

Animals, cells, and chemicals

Female C57BL/6 mice aged 7–8 weeks were purchased from Shanghai experimental animal center and maintained properly.

The MB49 mouse bladder carcinoma cells were maintained in Dulbecco's Modified Eagle's Medium with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine,

1 mmol/L sodium pyruvate, and 100 U/mL penicillin-streptomycin.

BDMC (Sigma-Aldrich Co., St Louis, MO, USA) was dissolved in dimethyl sulfoxide at 20 mM stock solution. Working concentrations were freshly prepared before use. Anti-mouse PD-L1 antibody was purchased from Bio X Cell (West Lebanon, NH, USA) and diluted in PBS.

Tumor models

To establish s.c. bladder cancer models, 1×10^6 MB49 cells in 100 μ L PBS were injected in the right shaved flank. For lung metastasis models, 2×10^5 MB49 cells in 100 μ L PBS were administrated intravenously in the tail vein. One week post-tumor inoculation (day 7), the mice were randomized into different groups, each having at least ten mice per group as follows: vehicle control (PBS), BDMC single-drug treatment (BDMC), α -PD-L1 antibody single-drug treatment (α -PD-L1), and combined treatment (combination). All treatments were then started. BDMC was administrated intravenously at 3 mg/kg body weight every 3 days for 2 weeks (metastasis models) or 4 weeks (s.c. models). α -PD-L1 antibody was administrated intraperitoneally three times a week at 200 μ g for 2 weeks (metastasis models) or 4 weeks (s.c. models). Tumor volume was measured with caliper and calculated with the following formula: volume = (length \times width²)/2. Survival rate and mouse body weight were monitored.

Sample processing

For mice to be prepared for enzyme-linked immunosorbent assay (ELISA) and flow cytometry analysis, five mice from each group were anesthetized by intraperitoneal administration of ketamine (9 mg/mL in saline) and xylazine (0.9 mg/mL in saline). Peripheral blood was collected from the tail when the mice were warmed up by heating lamp. Then the mice were sacrificed, and the spleens, s.c. tumor tissues, lungs, and TDLNs were harvested. The spleens were mashed, received red blood cell lysis, and passed through 40 μ m cell strainers. Also, lymph nodes were mashed and passed through strainers. Tumors and lungs were disaggregated by razors and incubated at 37°C for 1 hour in Roswell Park Memorial Institute 1640 medium with collagenase type IV (2 mg/mL, Sigma-Aldrich Co.), DNase (0.1 mg/mL, Sigma-Aldrich Co.), hyaluronidase (0.1 mg/mL, Sigma-Aldrich Co.), and bovine serum albumin (0.2 mg/mL, Sigma-Aldrich Co.). Cell suspensions were passed through 100 μ m cell strainers to remove aggregates. All the cells mentioned previously were then washed with staining buffer (Biolegend, San Diego, CA, USA) and were ready for flow antibody incubation.

ELISA

Serum IFN- γ level was tested by ELISA (R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions.

Flow cytometry

The following antibodies were purchased from Biolegend: anti-CD3 (clone 17A2), anti-CD45 (clone 30-F11), anti-CD4 (clone GK1.5), anti-CD8 α (clone 53-6.7), anti-IFN- γ (clone XMG1.2), anti-CD11b (clone M1/70), and anti-Gr-1 (clone RB6-8C5). The following conjugated antibodies were obtained from eBioscience (San Diego, CA, USA): anti-granzyme B (clone NGZB) and anti-PD-1 (clone J43). Live cells were determined by 7AAD viability staining (Biolegend). For cell surface markers, cells were stained with antibodies at room temperature for 30 minutes. For intracellular antigen detection, an intracellular staining kit containing fixation/permeabilization reagents from eBioscience was used. Flow cytometry analyses were performed on BD LSRFortessa X-20 (BD Biosciences, San Jose, CA, USA).

All gating strategies were determined by fluorescence minus one. Flowjo 10 was used to analyze the flow data.

Statistical analysis

Survival curves were illustrated using Kaplan–Meyer method. Statistical analysis was determined by one-way analysis of variance with Dunn's multiple comparison. All the analysis was performed on GraphPad Prism 5. All column data were presented as mean \pm standard error of the mean. $P < 0.05$ was regarded as statistically significant.

Results

Combination treatment controlled tumor growth and prolonged mouse survival

We evaluated the volumes of s.c. tumors and monitored the survival of mice receiving different treatment regimens in both s.c. tumor and metastasis models. Low dose BDMC alone did not delay tumor growth (Figure 1A) in s.c. tumor models, but did have a trend in prolonging survival in both models, although the effect was modest (Figure 1B and C).

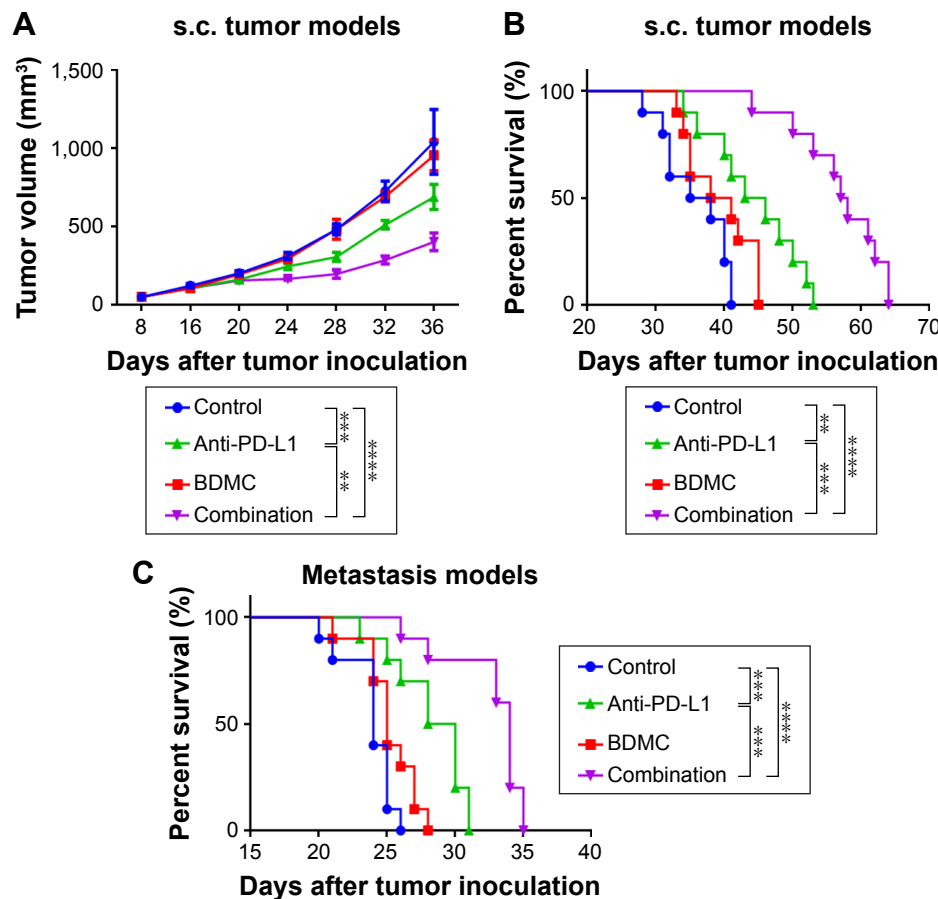


Figure 1 Combined treatment of BDMC and α -PD-L1 antibody inhibited tumor progression and significantly increased survival of mice bearing s.c. and metastatic bladder cancer.

Notes: (A) Tumor volumes were measured by caliper. (B) The survival curves of mice bearing s.c. bladder cancer were monitored. (C) The survival curves of mice with metastatic bladder cancer. $n = 10/\text{group}$. $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Data were presented as mean \pm SEM.

Abbreviations: BDMC, bisdemethoxycurcumin; s.c., subcutaneous; SEM, standard error of the mean.

In addition, BDMC treatment did not reduce the body weight of mice (data not shown). α -PD-L1 antibody alone, however, not only shrank the tumor, but also prolonged survival of mice. For mice receiving phosphate-buffered saline (PBS), the median survival of s.c. tumor models and lung metastasis models was 36.5 days and 24 days, respectively; while in α -PD-L1 antibody treatment alone groups, the median survival was 44.5 days ($P=0.0033$) and 29 days ($P=0.0007$), respectively. Furthermore, combined treatment displayed much stronger benefit both in controlling tumor progression and prolonging survival. On day 36, the median volume of tumors in the control group was 1,040 mm³, while it was 402 mm³ in the combination treatment group ($P<0.0001$), ie, combined therapy significantly delayed tumor growth. The median survival in combination group in s.c. tumor models and metastasis models was 57.5 days ($P<0.0001$) and 34 days ($P<0.0001$), respectively. The combination strategy of BDMC and α -PD-L1 antibody illustrated much more powerful antitumor effects than single drug treatment.

BDMC increased the number and activity of CD8⁺ T-cells

To reveal the effects of BDMC on lymphocytes, we analyzed the phenotypes of splenic lymphocytes and cells in tumor-draining lymph nodes (TDLNs) from different groups

in both models. In splenocytes, BDMC significantly increased the proportion of CD8⁺ T-cells (Figure 2A–D) in both models. This elevated number of CD8⁺ cells indicated that BDMC helped in CD8⁺ T-cell survival. In TDLNs from s.c. tumor models, again, an increased number of CD8⁺ T-cells were discovered in BDMC treated mice, while PD-L1 antibody had no effect on the number of T-cells (Figure 3A and B). These elevated CD8⁺ T-cells (by BDMC) expressed a higher level of IFN- γ (Figure 3C and D) and granzyme B (Figure 3E and F) than that in control or α -PD-L1 antibody alone groups, indicating that BDMC not only elevated the number of CD8⁺ T-cells in the TDLNs, but also strengthened their antitumor activity. Furthermore, serum IFN- γ level was measured. Unsurprisingly, BDMC elevated serum IFN- γ level in both s.c. and lung metastasis models (Figure 3G and H), demonstrating that BDMC significantly strengthened immune response.

CD8⁺ T cells were more infiltrated but restricted to exhaustion after BDMC treatment

In both models, flow cytometry results showed a significantly larger number of CD8⁺ TILs in BDMC treated mice when compared with PBS control mice. BDMC alone resulted in a four-fold (s.c. model) (Figure 4A and B) or three-fold (lung metastasis model) (Figure 5A and B) increase of intratumoral

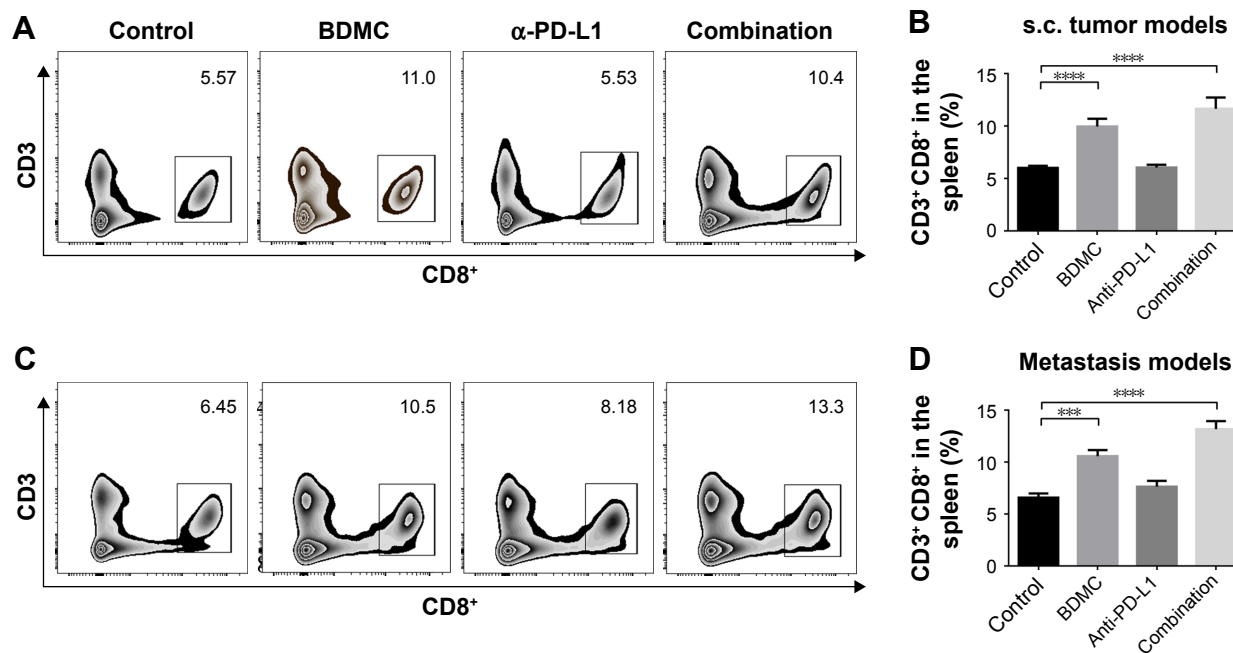


Figure 2 Combination treatment of BDMC and α -PD-L1 antibody increased the proportion of CD8⁺ T-cells in the spleen of mice bearing s.c. and metastatic bladder cancer.

Notes: (A) Representative data of proportions of CD8⁺ T-cells in s.c. tumor models. (B) Pooled data of proportions of CD8⁺ T-cells in the spleen in different groups in s.c. models. n=5/group. (C) Representative data of proportions of CD8⁺ T-cells in metastasis models. (D) Pooled data of proportions of CD8⁺ T-cells in the spleen in different groups in metastasis models. n=5/group. *** $P<0.001$, and **** $P<0.0001$. Data were presented as mean \pm SEM.

Abbreviations: BDMC, bisdemethoxycurcumin; s.c., subcutaneous; SEM, standard error of the mean.

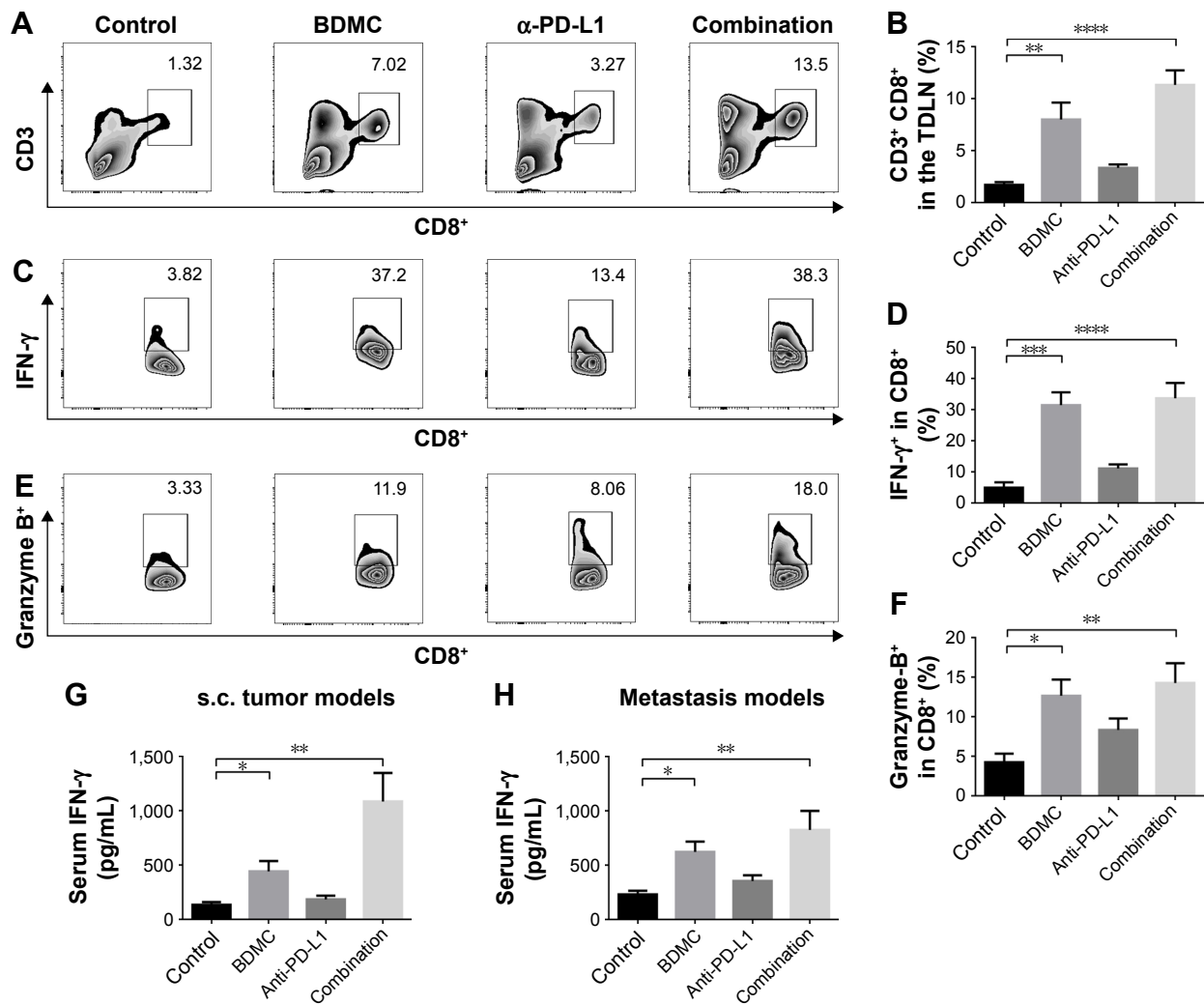


Figure 3 Combined treatment significantly strengthened CD8⁺ T-cells in TDLNs.

Notes: Representative data (A) and pooled data (B) of proportions of CD8⁺ T-cells in TDLNs in different groups. Representative data of proportions of IFN-γ⁺ (C) and granzyme B⁺ (E) cells in CD8⁺ cells from TDLNs. Pooled data of frequencies of IFN-γ⁺ (D) and granzyme B⁺ (F) cells in CD8⁺ cells in TDLNs from different groups. ELISA assay also revealed that BDMC treatment significantly elevated serum IFN-γ level in s.c. tumor models (G) and metastasis models (H). n=5/group. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. Data were presented as mean ± SEM.

Abbreviations: TDLNs, tumor-draining lymph nodes; ELISA, enzyme-linked immunosorbent assay; BDMC, bisdemethoxycurcumin; s.c., subcutaneous; SEM, standard error of the mean.

CD8⁺ T-cells. However, in both models, most of these intratumoral CD8⁺ T-cells were PD-1 positive (Figure 4C and D; Figure 5C and D), while very few of them expressed IFN-γ (Figure 4E and F; Figure 5E and F). This status was also seen in PBS control mice, which indicated that although more CD8⁺ CTLs infiltrated the tumor after low dose BDMC treatment, these cells failed to present their antitumor ability. The exhausted status of intratumoral CD8⁺ T-cells may account for the limited anticancer effect of BDMC treatment alone.

α-PD-L1 antibody boosted T-cell response

We validated the role α-PD-L1 antibody played in both s.c. and lung metastasis models. Although α-PD-L1 antibody alone did not display a statistically significant benefit for

CD8⁺ T-cells in the spleen, it did have favorable effects in CTLs in TDLNs. α-PD-L1 antibody elevated the secretion of IFN-γ (Figure 3C and D) and granzyme B (Figure 3E and F) by CD8⁺ T-cells. In tumor tissues from both models, although there was no statistically significant elevation in the number of tumor infiltrated CD8⁺ T-cells, α-PD-L1 antibody down-regulated the expression of PD-1 on intratumoral CD8⁺ T-cells (Figure 4C and D; Figure 5C and D) and elevated the level of IFN-γ in these cells (Figure 4E and F; Figure 5E and F), indicating that α-PD-L1 antibody alone protected the function of intratumoral effector T-cells.

In mice receiving combined treatment of BDMC and α-PD-L1 antibody, the immune response was markedly triggered. As mentioned above, BDMC had positive effects

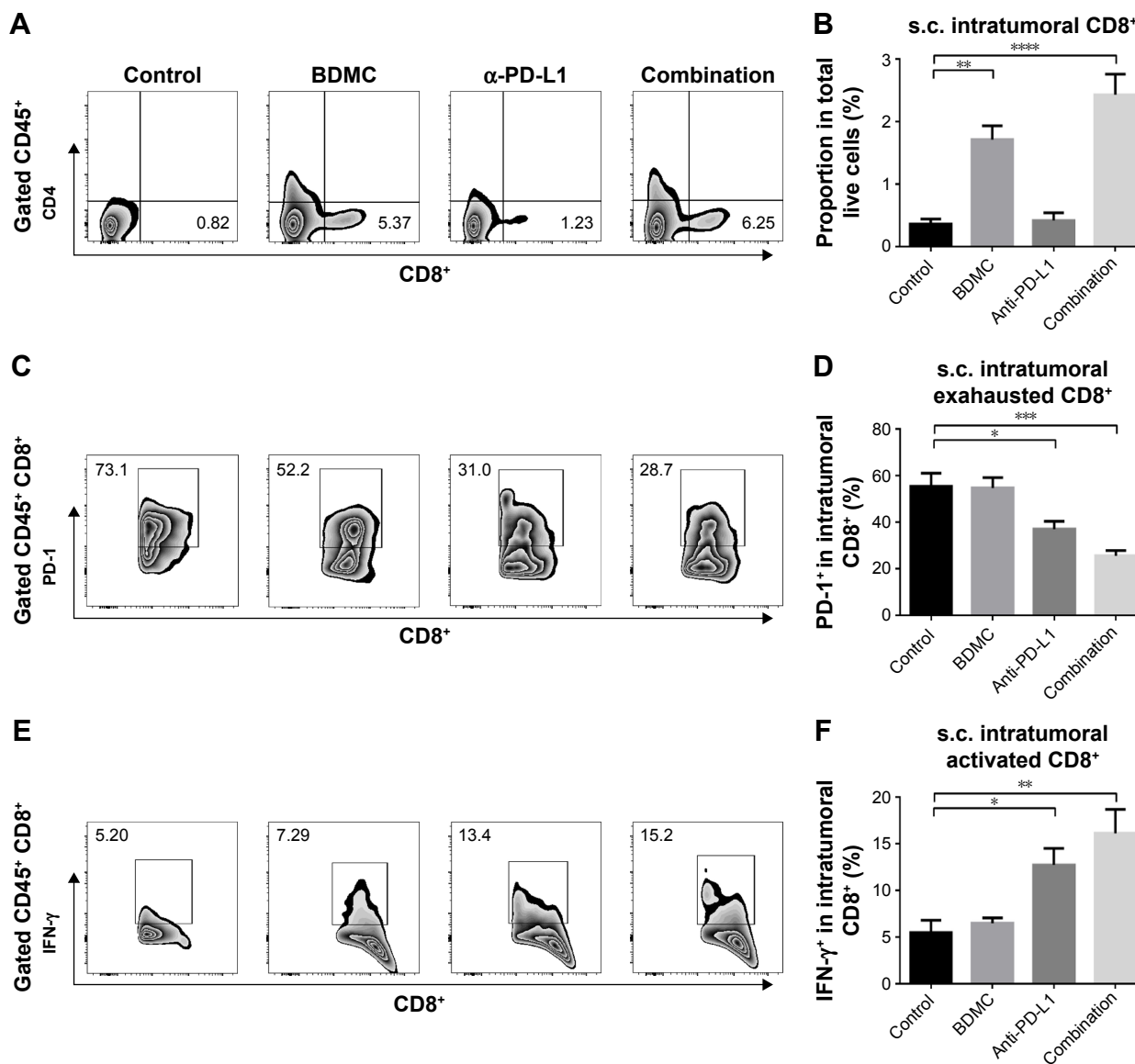


Figure 4 Combination treatment elevated the number and activity of intratumoral CD8⁺ T-cells in s.c. tumors. **Notes:** Representative data (A) and pooled data (B) of proportions of CD8⁺ T-cells in tumors from different groups. Representative data of proportions of PD-1⁺ (C) and IFN-γ⁺ (E) cells in intratumoral CD8⁺ cells. Pooled data of frequencies of PD-1⁺ (D) and IFN-γ⁺ (F) cells in intratumoral CD8⁺ cells from different groups. n=5/group. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. Data were presented as mean ± SEM. **Abbreviations:** s.c., subcutaneous; SEM, standard error of the mean.

on CD8⁺ T-cell and activation, and α-PD-L1 antibody protected the intratumoral CD8⁺ T-cells from exhaustion. In the combination group, the number of CD8⁺ T-cells in TDLNs from s.c. models was significantly improved (Figure 3A and B), along with improved functioning of CD8⁺ T-cells (Figure 3C–F). In addition, a profoundly elevated secretion of IFN-γ by intratumoral CD8⁺ T-cells was discovered, which was much higher than that in BDMC or α-PD-L1 antibody treatment alone group (Figure 4E and F; Figure 5E and F). Furthermore, the proportion of exhausted T-cells in the combination group was much lower than in BDMC alone group (Figure 4C and D; Figure 5C and D).

Combination treatment increased the number of intratumoral CTLs, facilitated immune response, and protected effector T-cells from exhaustion.

Combination treatment reduced the proportion of MDSCs in s.c. models

MDSCs are highly immune suppressive in the tumor microenvironment. We analyzed the intratumoral MDSCs in both models. BDMC treatment alone had a trend in controlling the number of MDSCs, but α-PD-L1 antibody treatment and combined treatment significantly decreased the number of MDSCs in the tumor (Figure 6A and B) in s.c. models.

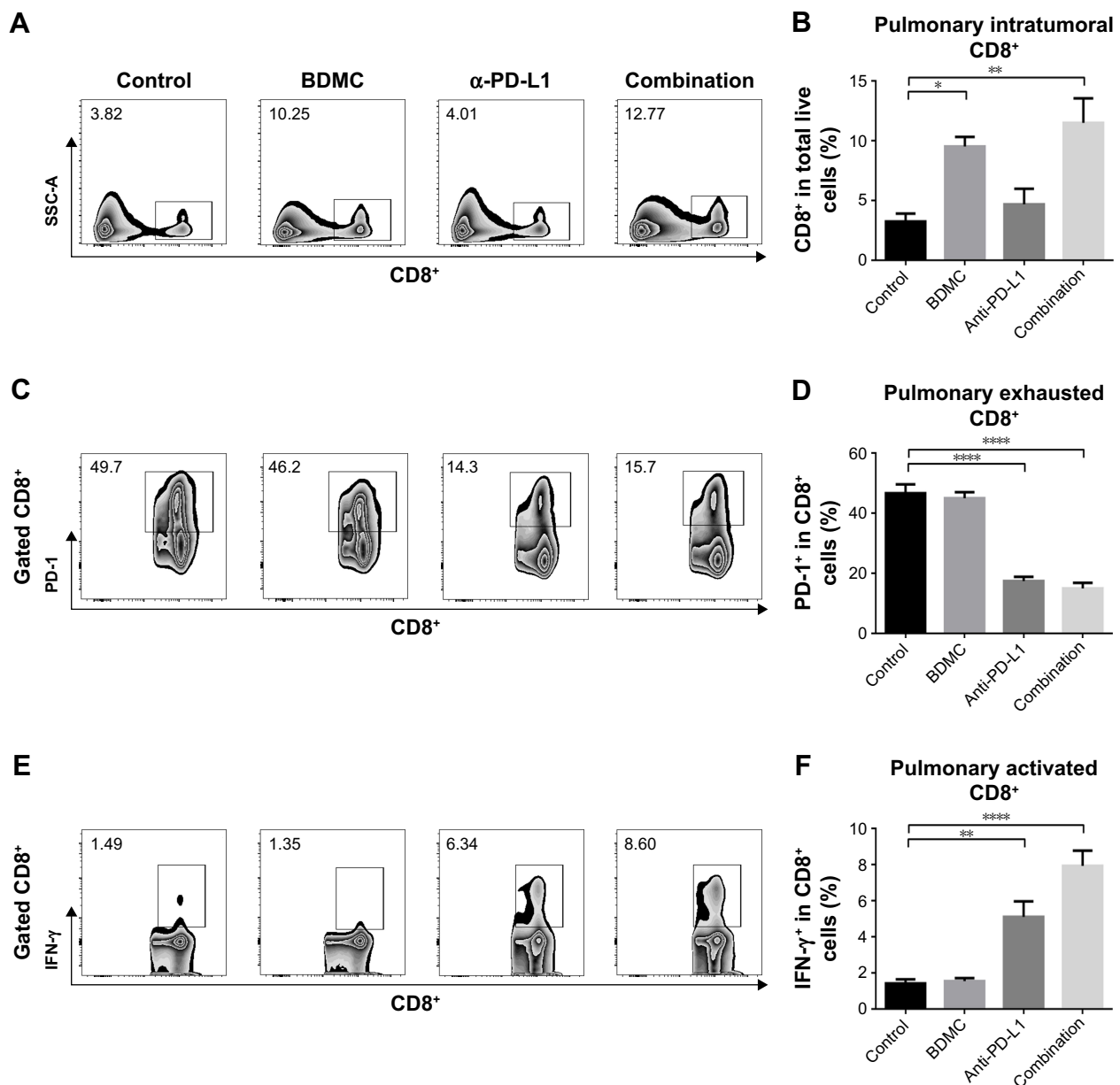


Figure 5 Combination treatment boosted immune response in pulmonary metastasized bladder cancer.

Notes: Representative data (A) and pooled data (B) of proportions of CD8⁺ T-cells in tumors from different groups. Representative data of proportions of PD-1⁺ (C) and IFN-γ⁺ (E) cells in pulmonary intratumoral CD8⁺ cells. Pooled data of frequencies of PD-1⁺ (D) and IFN-γ⁺ (F) cells in intratumoral CD8⁺ cells from different groups. n=5/group. *P<0.05, **P<0.01, and ****P<0.0001. Data were presented as mean ± SEM.

Abbreviation: SEM, standard error of the mean.

In lung metastasis models, however, comparatively fewer MDSCs were discovered in the lung. No statistically significant difference was seen among groups (data not shown).

Discussion

CD8⁺ T-cells play critical roles in immune attack against tumors. However, the underlying mechanisms affecting T-cell activation, differentiation, function, and survival are still largely unknown. Any method which can facilitate T-cell activity will undoubtedly help in cancer immunotherapy.

Curcumin, and its analogs, were recently validated as a potential immune stimulator.^{8,10} However, the underlying mechanisms by which curcumin modulates immune response are still elusive. It has been demonstrated that curcumin could facilitate immune response by preventing T-cell apoptosis and altering the tumor microenvironment.^{13–15} Besides, the clinical application of curcumin has been restricted because of its poor bioavailability.¹¹ In addition, malignancies, especially solid tumors, can weaken immune attack by cytotoxic T-cells through multiple pathways. PD-1/PD-L1 pathway

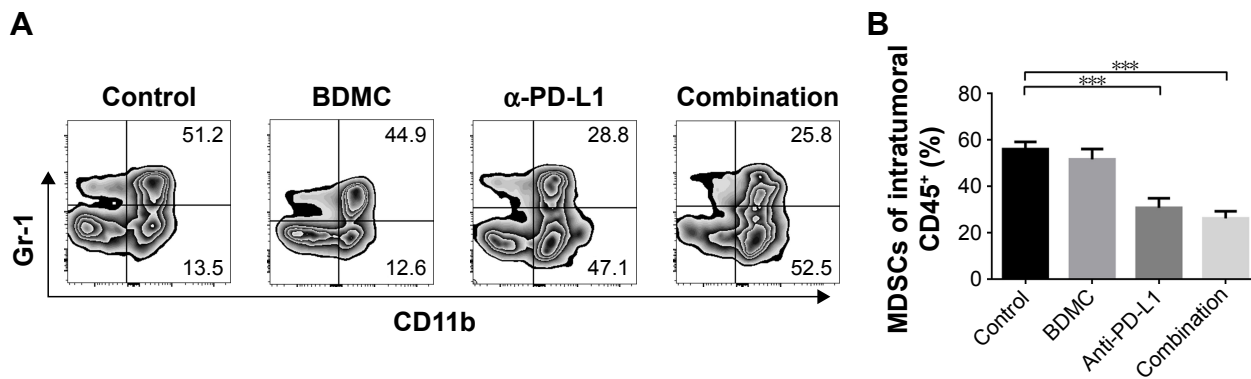


Figure 6 Combination treatment inhibited MDSC proliferation in s.c. tumor models.

Notes: Representative data (A) and pooled data (B) of frequencies of CD11b⁺ Gr-1⁺ MDSCs in intratumoral CD45⁺ cells (calculated from CD11b⁺ Gr-1⁺/CD45⁺). n=5/group. ***P<0.001. Data were presented as mean ± SEM.

Abbreviations: MDSC, myeloid-derived suppressor cell; s.c., subcutaneous; SEM, standard error of the mean.

plays one of the most important roles in inhibiting immune response.^{16,17} Without controlling the immune checkpoint modulators, T-cell-mediated antitumor immune response will be severely impaired.

In the current study, we evaluated the combination treatment of BDMC, an analog of curcumin which has elevated bioavailability and stability, and α-PD-L1 antibody for bladder cancer in immunocompetent mouse models. We demonstrated that mice receiving low-dose BDMC treatment had strengthened T-cell response. Elevated levels of IFN-γ and granzyme B secretion were monitored. However, although experiments on peripheral lymphocytes and the number of intratumoral T-cells revealed favorable results, single-agent treatment of BDMC still had modest effects in prolonging mouse survival and intratumoral T-cell activity. In tumor tissues, most of the infiltrated T-cells displayed an exhausted phenotype, which may be correlated with the expression of PD-L1 on bladder cancer cells. On the other hand, the single-agent administration of α-PD-L1 antibody maintained the activity of CD8⁺ T-cells, while the number of infiltrated T-cells was still far from satisfactory. Thus, the combination treatment of both BDMC and α-PD-L1 antibody successfully boosted immune response against bladder cancer, and significantly prolonged survival. CTLs, regardless of whether in the TDLNs or in the tumors, secreted significantly higher levels of IFN-γ and granzyme B, and consequently controlled tumor progression. Besides, potent immune suppressor cells, MDSCs, were also inhibited after BDMC treatment. We observed decreased expression of PD-1 on intratumoral CD8⁺ T-cells, which was not seen in single-agent treated mice. We conjectured that it might be associated with the decreased number of intratumoral MDSCs.¹⁸

Clinical trials for bladder cancer using different kinds of immunotherapies such as adoptive cell transfer and

PD-1/PD-L1 antibodies are ongoing. However, solid tumors escape immune response through different mechanisms, including immune suppressive local environment and immune checkpoint ligands/receptors. Any method which can facilitate immune response will be valuable for cancer immunotherapy. BDMC can be used in combination with other therapies, and theoretically reach at least additive, or even synergistic effects. The authors are looking forward to the future, in which cocktail immunotherapy, combining adoptive cell transfer, adjuvants such as BDMC, and checkpoint inhibitors, can achieve the most powerful anticancer effects. Of note, the potential side effects of BDMC and related combination treatment are not negligible. As an analog of curcumin, BDMC shares most of the side effects of curcumin, including allergies, gastrointestinal problems such as nausea and diarrhea, etc.^{11,12} However, these side effects are usually mild and subside fast.¹²

In conclusion, this is the first study demonstrating that BDMC treatment in combination with α-PD-L1 antibody can suppress bladder cancer progression in vivo and prolong mouse survival. Combination treatment boosted immune response by stimulating CD8⁺ T-cell activity and suppressing MDSCs. Our results suggest that the combination of BDMC and α-PD-L1 antibody may be a promising therapeutic regimen for treatment of bladder cancer patients.

Disclosure

The authors report no conflicts of interest in this work.

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