

RESEARCH PAPER

## Combination therapy with L-arginine and $\alpha$ -PD-L1 antibody boosts immune response against osteosarcoma in immunocompetent mice

Xiaojun He<sup>a</sup>, Haiqing Lin<sup>a</sup>, Li Yuan<sup>b</sup>, and Binghao Li<sup>c</sup>

<sup>a</sup>Department of Oorthopaedics, The Second Hospital of Jiaxing, The Second Affiliated Hospital of Jiaxing University, Jiaxing, China; <sup>b</sup>Department of Epidemiology, School of Public Health, Fudan University, Shanghai, China; <sup>c</sup>Department of Oorthopaedics, Institute of Orthopaedic Research, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China

### ABSTRACT

L-arginine supplementation was recently proved to promote the function of immune cells, especially T-cells, by facilitating T-cell proliferation, differentiation and survival *in vivo*. Cytotoxic CD8<sup>+</sup> plays a crucial role in modulating anti-cancer response mediated by the immune system, but was restricted by exhaustion. Thus, we hypothesized that L-arginine, in combination with  $\alpha$ -PD-L1 antibody, may provide a favored environment for T-cell response against osteosarcoma. Immunocompetent BALB/c mouse models bearing orthotopic and metastatic osteosarcoma were established to validate this conjecture. We found that L-arginine significantly elevated the number of splenic CD8<sup>+</sup> T-cells, the level of serum interferon- $\gamma$ , and CD8<sup>+</sup> T-cell infiltration. Furthermore,  $\alpha$ -PD-L1 antibody protected these amplified CD8<sup>+</sup> T-cells from exhaustion, and therefore strengthened the secretion of interferon- $\gamma$ , granzyme B and perforin by these T-cells. As a result, this combination treatment strategy significantly prolonged survival of osteosarcoma bearing mice, suggesting that L-arginine supplementation in combination with  $\alpha$ -PD-L1 antibody may be a promising method for osteosarcoma patients.

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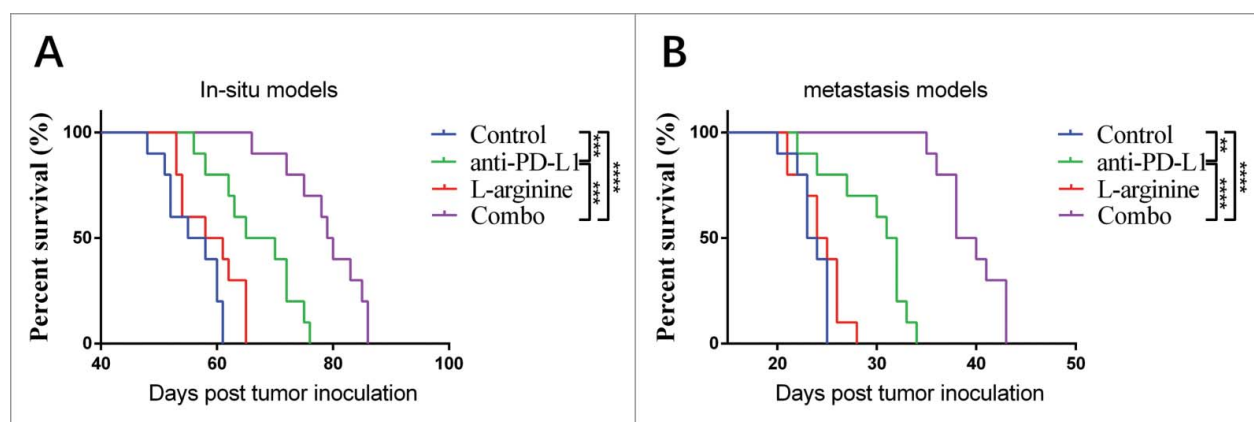
### Introduction

Immunotherapy is a promising therapeutic strategy against osteosarcoma, a highly aggressive malignant tumor characteristic of forming early lung metastasis and bad prognosis. Conventional therapeutic strategy consists surgical resection, chemotherapy, with or without radiotherapy.<sup>1</sup> However, once the tumor relapse or behave as a chemo-resistant phenotype, conventional none-surgical therapy can hardly make efforts in efficiently prolonging the survival. Thus, there is an urgent need to establish an alternative regimen, such as immunotherapy, for osteosarcoma.<sup>2</sup>

CD8<sup>+</sup> T-cell is one of the most effective killer cells in mediating antitumor immune response. Efforts have been made to utilize CD8<sup>+</sup> T-cells in adoptive cell transfer immunotherapy against osteosarcoma.<sup>3–5</sup> However, there are still some suffocating restraints in CD8<sup>+</sup> T-cell-based immunotherapy, such as immune checkpoint, and the tumor immunosuppressive microenvironment. Pathology study has revealed that most of osteosarcoma cells express PD-L1,<sup>6</sup> which can dramatically suppress the effects of CD8<sup>+</sup> cytotoxic cells (CTLs). Besides, tumors can develop an immunosuppressive environment via different ways, and consequently significantly decrease the amount of tumor infiltrating lymphocytes (TILs).<sup>7</sup> These 2 factors in combination can result in depressed CTL homing and impaired CTL function, and finally abrogate the spontaneous immune attack against osteosarcoma.

Recently, antibodies for PD-1/PD-L1 pathway displayed inspiring effects in strengthening the activity of CD8<sup>+</sup> T-cells in clinical trials for quite a few kinds of solid malignancies. Considering the expression of PD-L1 on osteosarcoma cells, it is reasonable to evaluate the effect of  $\alpha$ -PD-L1 antibody in osteosarcoma.<sup>8</sup> Concerning the immunosuppressive environment, L-arginine and arginase (type I and II) were discovered to play critical roles.<sup>9,10</sup> L-arginine is an important amino acid for human beings. However, L-arginine is usually scarce in the tumor environment as the tumor cells may over-express arginase,<sup>11,12</sup> which leads to T-cell dysfunction and apoptosis. Besides, degradation of L-arginine results in reduced expression of CD3, and consequently impairs T-cell responsiveness.<sup>13,14</sup> Thus, following these theories, L-arginine supplementation was recently proved to enhance T-cell survival and antitumor activity.<sup>15,16</sup>

In this study, we tried to validate our hypothesis that L-arginine supplementation and PD-1/PD-L1 pathway blockade can facilitate spontaneous immune response against osteosarcoma. We established immunocompetent mouse models using BALB/c mice bearing *in situ* osteosarcoma or metastasized osteosarcoma, and then treated them with L-arginine and/or  $\alpha$ -PD-L1 antibody. We discovered that L-arginine supplementation, in combination with  $\alpha$ -PD-L1 antibody, displayed encouraging effects in promoting immune response and significantly prolonged the survival.



**Figure 1.** Combination treatment with L-arginine and  $\alpha$ -PD-L1 antibody significantly increased survival in mice bearing *in situ* and metastatic osteosarcoma. (A) The survival curves of mice bearing *in situ* osteosarcoma were monitored. (B) The survival curves of mice with metastatic osteosarcoma.  $n = 10/\text{group}$ . \*\*\* $P < 0.01$ , \*\*\*\* $P < 0.001$ , and \*\*\*\*\* $P < 0.0001$ .

## Results

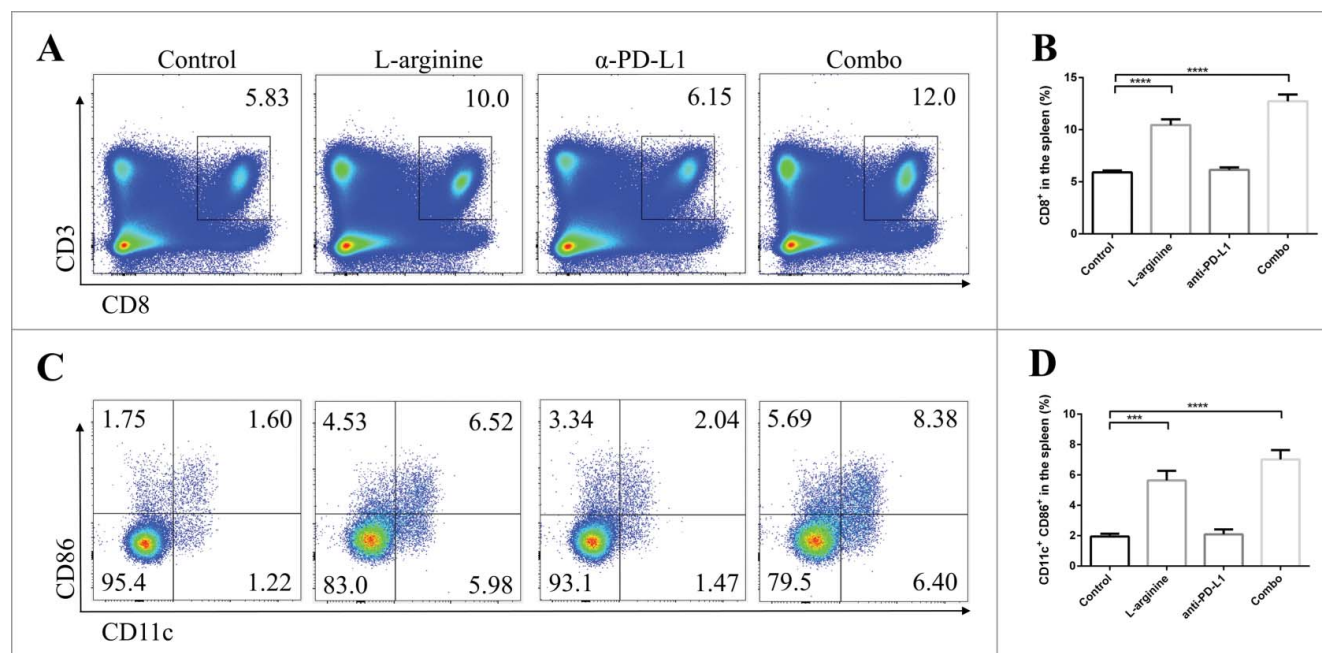
### Combination therapy significantly improved survival in osteosarcoma tumor bearing

We monitored the survival of mice underwent different treatment regimens in both *in situ* and metastasis models. L-arginine supplementation alone didn't show statistically significant survival benefit for osteosarcoma bearing mice (Fig. 1A, B).  $\alpha$ -PD-L1 antibody, however, significantly prolonged mice survival. For mice receiving PBS, the medium survival of *in situ* models and metastasis models were 56.5 d and 23.5 days, respectively; while in  $\alpha$ -PD-L1 antibody treatment alone groups, the medium survival were 67.5 d ( $P = 0.0004$ ) and 31.5 d ( $P = 0.011$ ), respectively.

Moreover, combination treatment revealed even stronger benefit. The medium survival in combo group were 79.5 d ( $P < 0.0001$ ) and 39 d ( $P < 0.0001$ ), respectively. Although L-arginine supplementation alone didn't result in prolonged survival, the combination treatment with L-arginine and  $\alpha$ -PD-L1 antibody displayed much more powerful antitumor abilities than  $\alpha$ -PD-L1 antibody treatment alone.

### L-arginine supplementation increased the number and activity of CD8<sup>+</sup> T-cells

We analyzed the phenotype of splenocytes and lymphocytes in tumor-draining lymph nodes (TDLNs) from different groups in *in situ* models. In splenic lymphocytes, L-arginine

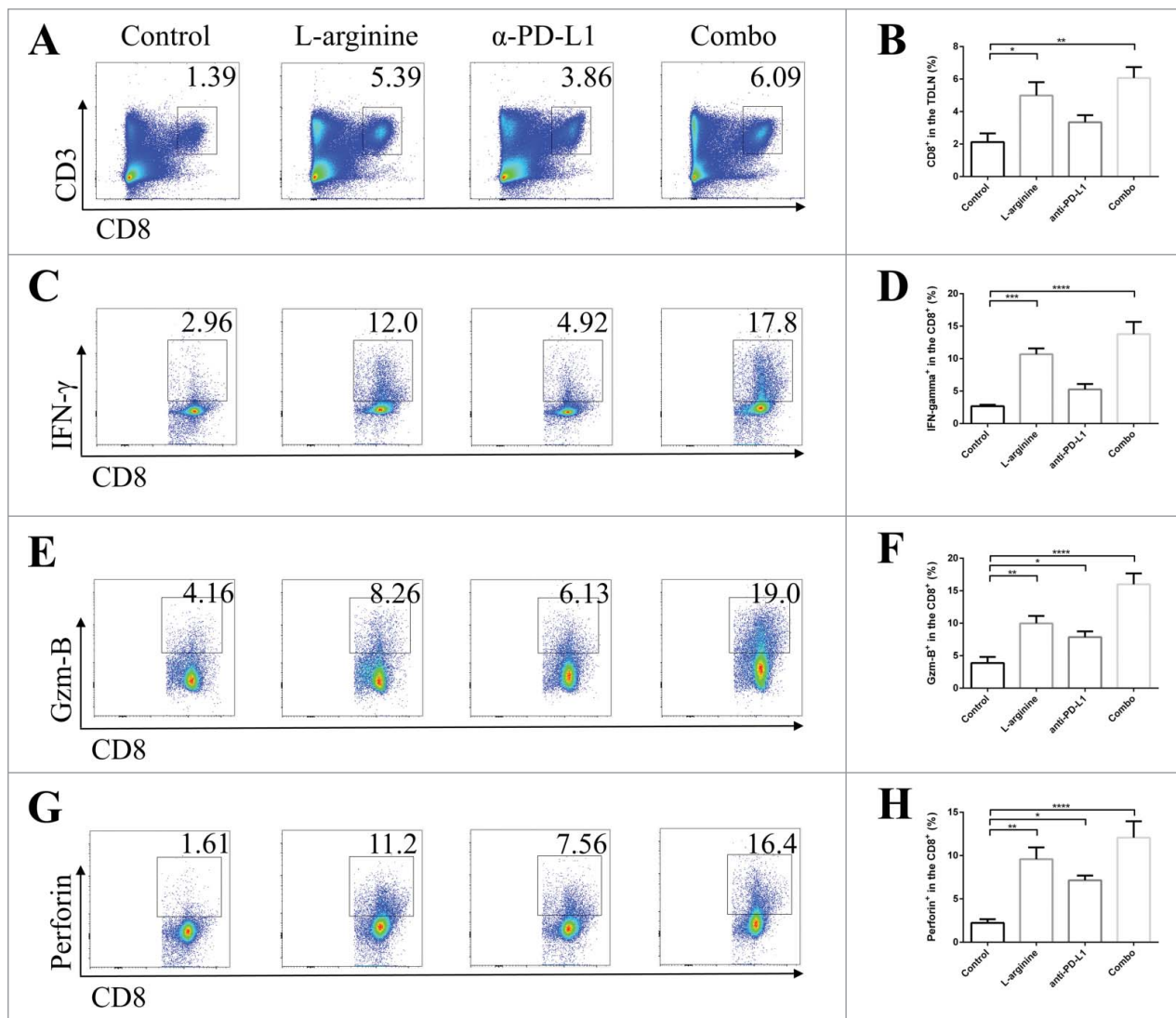


**Figure 2.** Combination treatment with L-arginine and  $\alpha$ -PD-L1 antibody increased the proportion of CD8<sup>+</sup> T-cells and CD86<sup>+</sup> CD11c<sup>+</sup> dendritic cells in the spleen of mice bearing *in situ* osteosarcoma. (A) Representative data of proportions of CD8<sup>+</sup> T-cells. (B) Pooled data of proportions of CD8<sup>+</sup> T-cells in the spleen in different groups.  $n = 5/\text{group}$ . (C) Representative data of proportions of mature dendritic cells. (D) Pooled data of proportions of mature dendritic cells in the spleen in different groups.  $n = 5/\text{group}$ . \*\*\* $P < 0.001$ , and \*\*\*\*\* $P < 0.0001$ . Data were presented as mean  $\pm$  SEM.

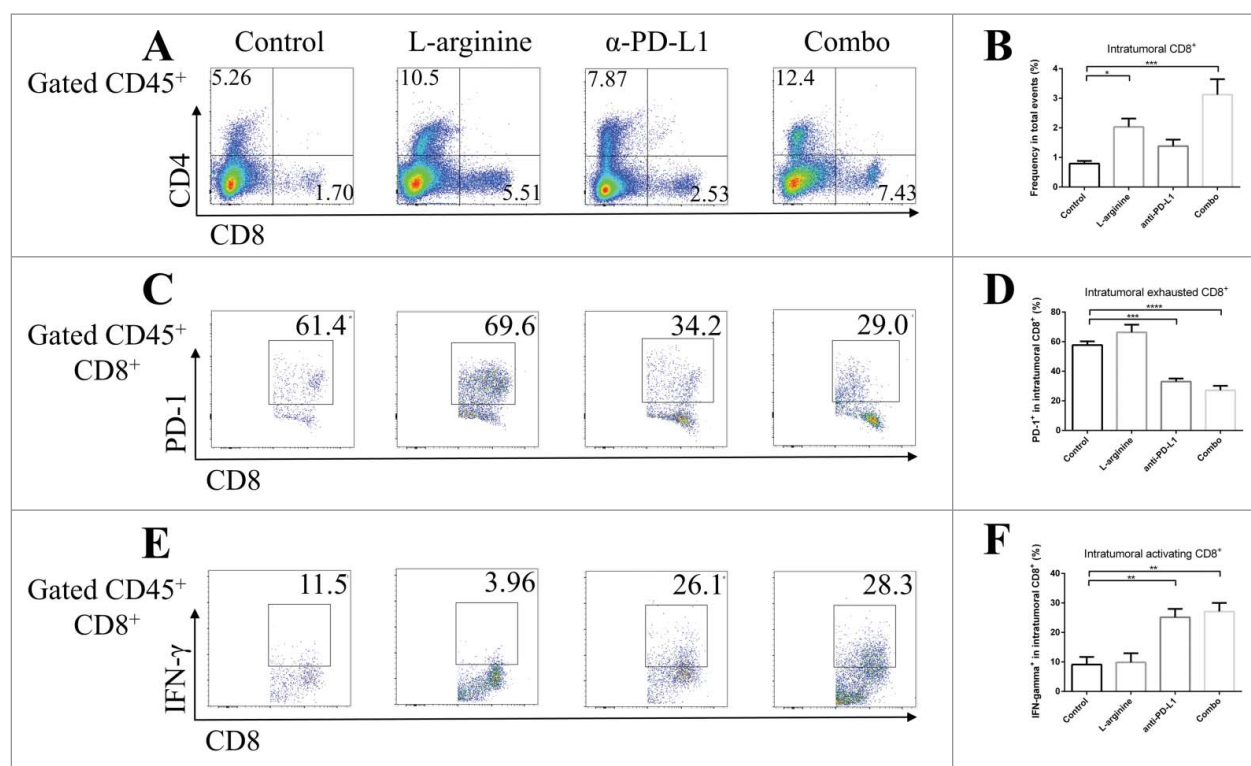
supplementation treatment significantly increased the proportion of CD8<sup>+</sup> T-cells (Fig. 2A, B). Although these splenic CD8<sup>+</sup> T-cells are usually regarded as rest cells, the elevated number of CD8<sup>+</sup> T-cells indicated that L-arginine helped in CD8<sup>+</sup> T-cell survival. In addition, more mature dendritic cells (DCs) expressing CD11c and CD86 were found in L-arginine supplementation mice (Fig. 2C, D). In TDLNs, elevated number of CD8<sup>+</sup> T-cells were discovered in L-arginine treated mice (L-arginine alone and combo group) (Fig. 3A, B), and these CD8<sup>+</sup> T-cells displayed higher level of IFN- $\gamma$  (Fig. 3C, D), Granzyme B (Fig. 3E, F), and perforin (Fig. 3G, H) than that in control or  $\alpha$ -PD-L1 antibody treatment alone groups, indicating that L-arginine not only increased the number of CTLs in the TDLN, but also improved the activity of them. Moreover, serum IFN- $\gamma$  level was evaluated by ELISA. Unsurprisingly, L-arginine treatment elevated serum IFN- $\gamma$  level (Fig. S1A, B) in both *in situ* and metastasis models, indicating that L-arginine significantly strengthened immune response.

### L-arginine supplementation elevated the number of TILs but severely restricted to T-cell exhaustion

In both *in situ* models and metastasis models, flow cytometry analyses showed significantly elevated number of TILs in L-arginine treated mice when compared with vehicle control mice. L-arginine supplementation alone achieved a 3-fold (*in situ* model) (Fig. 4A, B) or over 2-fold (metastasis model) (Fig. 5A, B) increase of intratumoral CD8<sup>+</sup> T-cells. However, most of these intratumoral CD8<sup>+</sup> T-cells were PD-1 positive (Fig. 4C, D), while very few of them expressed IFN- $\gamma$  (Fig. 4E, F), which indicated that although more CD8<sup>+</sup> CTLs were successfully homing to the tumor site after L-arginine supplementation, these cells failed to present their antitumor ability. Although L-arginine treatment seemed to have a trend in elevating the proportion of PD-1<sup>+</sup> cells in CD8<sup>+</sup> T-cells (Fig. 4C, D; Fig 5C, D), there was no statistical significance when compared with that in control mice. The exhausted phenotype of CD8<sup>+</sup> TILs may



**Figure 3.** Combined treatment significantly elevated the number and activity of CD8<sup>+</sup> T-cells in tumor-draining lymph nodes. (A) Representative data of proportions of CD8<sup>+</sup> T-cells in TDLNs. (B) Pooled data of proportions of CD8<sup>+</sup> T-cells in TDLNs in different groups. Representative data of proportions of IFN- $\gamma$ <sup>+</sup> (C), granzyme-B<sup>+</sup> (E), and perforin<sup>+</sup> (G) cells in CD8<sup>+</sup> cells from TDLNs. Pooled data of frequencies of IFN- $\gamma$ <sup>+</sup> (D), granzyme-B<sup>+</sup> (F), and perforin<sup>+</sup> (H) cells in CD8<sup>+</sup> cells in TDLNs from different groups. n = 5/group. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , and \*\*\*\*  $P < 0.0001$ . Data were presented as mean  $\pm$  SEM.



**Figure 4.** Combined treatment significantly elevated the number and activity of CD8<sup>+</sup> T-cells in orthotopic tumors. (A) Representative data of proportions of CD8<sup>+</sup> T-cells in tumor. (B) Pooled data of proportions of CD8<sup>+</sup> T-cells in tumor in different groups (calculated from CD8<sup>+</sup>/total events). Representative data of proportions of PD-1<sup>+</sup> (C) and IFN- $\gamma$ <sup>+</sup> (E) cells in intratumoral CD8<sup>+</sup> cells. Pooled data of frequencies of PD-1<sup>+</sup> (D) and IFN- $\gamma$ <sup>+</sup> (F) cells in intratumoral CD8<sup>+</sup> 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  $\pm$  SEM.

account for the modest antitumor effect of L-arginine supplementation alone as was shown in Fig. 1.

#### $\alpha$ -PD-L1 antibody treatment strengthened intratumoral T-cell response

We tried to validate the role  $\alpha$ -PD-L1 antibody played in both models. Although  $\alpha$ -PD-L1 antibody treatment alone didn't show significant benefit for peripheral CD8<sup>+</sup> T-cells, it made *bona fide* effects in CTLs in TDLNs; elevated secretion of granzyme-B (Fig. 3E, F) and IFN- $\gamma$  (Fig. 3G, H) by CD8<sup>+</sup> T-cells were found in  $\alpha$ -PD-L1 antibody treated mice. In tumor tissues from both models, albeit no statistically significant elevation in the number of intratumoral CD8<sup>+</sup> T-cells,  $\alpha$ -PD-L1 antibody seemed to down-regulate the expression of PD-1 on CD8<sup>+</sup> T-cells (Fig. 4C, D and Fig. 5C, D) and increased the level of IFN- $\gamma$  by CD8<sup>+</sup> T-cells (Fig. 4E, F and Fig. 5E, F) than control mice.

In mice receiving combination treatment with L-arginine and  $\alpha$ -PD-L1 antibody, the immune response was profoundly triggered. As mentioned above, L-arginine helped in CD8<sup>+</sup> T-cell survival and activation, and  $\alpha$ -PD-L1 antibody protected the intratumoral CTLs from exhaustion. Dramatically increased secretion of IFN- $\gamma$  by intratumoral CD8<sup>+</sup> T-cells was discovered in combo group, much higher than in L-arginine alone group (Fig. 4E, F and Fig. 5E, F). Furthermore, the proportion of exhausted cells (PD-1 positive) in intratumoral CD8<sup>+</sup> T-cells was much lower than L-arginine alone group (Figs. 4C, D and 5C, D). Combination treatment increased CTL infiltration, facilitated anticancer immune response and protected effective cells from exhaustion.

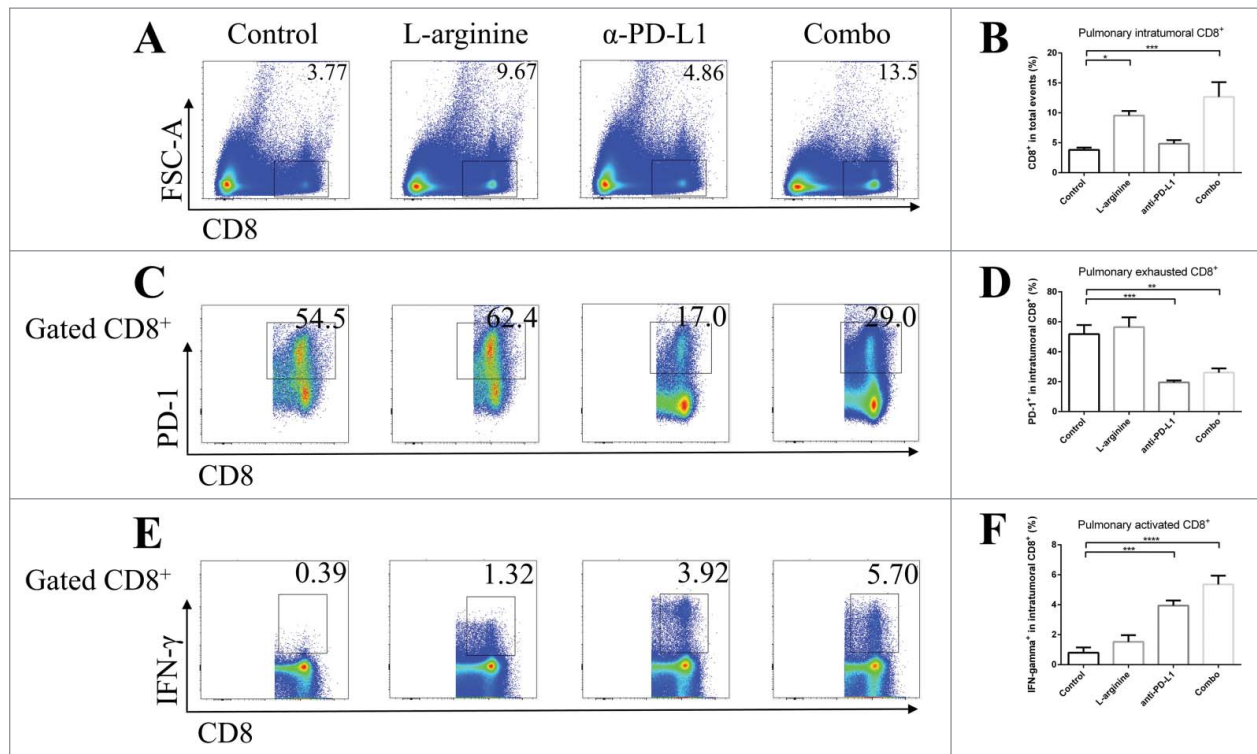
#### Combination treatment inhibited the proliferation of myeloid-derived suppressive cells

Myeloid-derived suppressive cells (MDSCs) are powerful immune suppressive roles in the tumor microenvironment. We analyzed intratumoral MDSCs in both *in situ* models and metastasis models. L-arginine treatment alone had a trend in inhibiting the proliferation of MDSC, but  $\alpha$ -PD-L1 antibody and combination treatment reduced the frequencies of MDSC within the tumor (Fig. 6A, B) in orthotopic models. Study on MDSCs in metastasis models didn't reveal changes in population. We also analyzed MDSCs in the spleen and TDLNs in both models, but no statistical difference was found (data not shown).

#### Discussion

CD8<sup>+</sup> cytotoxic T-cells play crucial roles in cellular immune response against tumor. Numerous studies are focusing using CD8<sup>+</sup> T-cells for cancer immunotherapy, including osteosarcoma.<sup>17-19</sup> 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.

L-arginine, regarded as a nutritional agent in the past, was recently identified to be an important immune stimulator.<sup>15</sup> T-cells activation consumes large amount of L-arginine, and the underlying mechanism was nicely demonstrated.<sup>15</sup> Given the fact that arginase-mediated L-arginine depletion profoundly suppress T-cell immune response,<sup>20,21</sup> L-arginine supplementation is

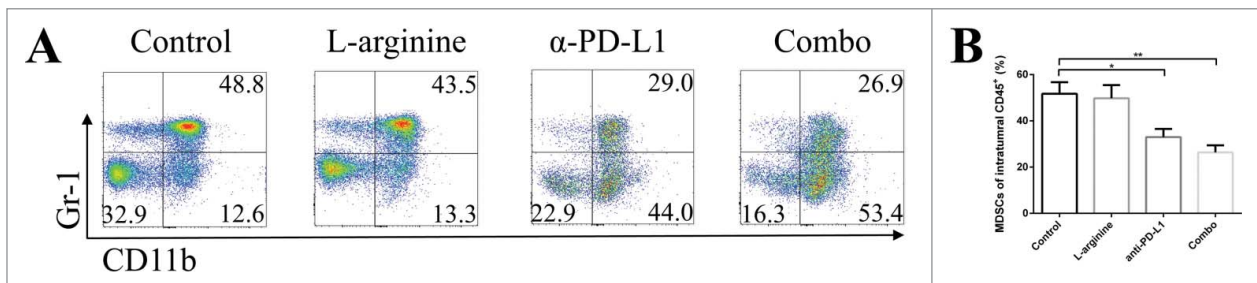


**Figure 5.** Combined treatment promoted immune response in pulmonary metastasized osteosarcoma. (A) Representative data of frequencies of CD8<sup>+</sup> T-cells in metastasized osteosarcoma. (B) Pooled data of proportions of CD8<sup>+</sup> T-cells in tumor in different groups (calculated from CD8<sup>+</sup>/total events). Representative data of proportions of PD-1<sup>+</sup> (C) and IFN- $\gamma$ <sup>+</sup> (E) cells in pulmonary intratumoral CD8<sup>+</sup> cells. Pooled data of frequencies of PD-1<sup>+</sup> (D) and IFN- $\gamma$ <sup>+</sup> (F) cells in intratumoral CD8<sup>+</sup> cells from different groups.  $n = 5/\text{group}$ . \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , and \*\*\*\*  $P < 0.0001$ . Data were presented as mean  $\pm$  SEM.

a good idea to feed the T-cells, and to inhibit tumor growth. However, malignancies, especially solid tumors, can escape from or impair immune attack by multiple mechanisms. PD-1/PD-L1 pathway was one of the most important role in inhibiting immune response.<sup>22,23</sup> Without controlling the immune checkpoint, the effect of T-cell-mediated anticancer response will be severely weakened.

In the current study, we evaluated the combination treatment with L-arginine and  $\alpha$ -PD-L1 antibody for osteosarcoma. We demonstrated that mice receiving L-arginine treatment showed significantly strengthened T-cell activity. Elevated levels of IFN- $\gamma$ , Granzyme B, and perforin secretion were detected. However, although assays on peripheral blood and lymphocytes revealed promising results, single-agent treatment with L-arginine still made modest effects in prolonging mouse survival. In tumor tissues, the strengthened T-cells were

severely restricted by immune checkpoint, which may be correlated with the expression of PD-L1 on osteosarcoma cells. The administration of  $\alpha$ -PD-L1 antibody protected the intratumoral T-cells from exhaustion, while the number of T-cells were still limited. Thus, the combination treatment with both L-arginine, the “fertilizer,” and  $\alpha$ -PD-L1 antibody, the “protector,” successfully promote immune response and prolonged mice survival. Cytotoxic CD8<sup>+</sup> T-cells, no matter in the TDLNs or in tumor tissues, secreted significantly higher levels of IFN- $\gamma$ , Granzyme B, and perforin, and consequently control tumor promotion. Moreover, immune suppressor roles, MDSCs, were also inhibited after L-arginine treatment. In addition, we observed decreased expression of PD-1 on intratumoral CD8<sup>+</sup> T-cells, which was not seen in single-agent treated mice. We conjectured that it might be associated with decreased number of intratumoral MDSCs. Although L-arginine supplementation



**Figure 6.** L-arginine plus  $\alpha$ -PD-L1 antibody inhibited MDSC proliferation in orthotopic osteosarcoma models. (A) Representative data of frequencies of CD11b<sup>+</sup> Gr-1<sup>+</sup> MDSCs in intratumoral CD45<sup>+</sup> cells. (B) Pooled data of proportions of MDSCs in tumor in different groups (calculated from CD11b<sup>+</sup> Gr-1<sup>+</sup>/CD45<sup>+</sup>).  $n = 5/\text{group}$ . \*  $P < 0.05$  and \*\*  $P < 0.01$ . Data were presented as mean  $\pm$  SEM.

showed a trend in strengthening PD-1 expression on CD8<sup>+</sup> T-cells, there was no statistical significance. Also, we didn't find any evidence that L-arginine had the possibility in promoting T-cell exhaustion.

Clinical trials for osteosarcoma using different kinds of immunotherapies such as adoptive cell transfer and PD-1/PD-L1 antibodies are ongoing.<sup>3</sup> However, the response rates, no matter using HER-2 specific chimeric antigen receptor T-cells or immune checkpoint inhibitors, are still far from that in leukemias. Solid tumors escape from immune response via multiple ways, including immune suppressive microenvironment and overexpressed immune checkpoint ligands/receptors. L-arginine, a nutritional agent, can be used in combination with other therapies, and theoretically reach at least an additive, or even synergistic effects. The authors can expect that cocktail immunotherapy, combining adoptive cell transfer, adjuvants such as L-arginine, and checkpoint inhibitors, may achieve most powerful anticancer effects.

In conclusion, this is the first study demonstrating that L-arginine supplementation in combination with  $\alpha$ -PD-L1 antibody can suppress osteosarcoma growth *in vivo* and prolong survival. Combination treatment stimulated immune response by triggering CD8<sup>+</sup> T-cell activity and suppressing MDSCs. Our results suggest that L-arginine and  $\alpha$ -PD-L1 antibody may be a promising therapeutic strategy for treatment of osteosarcoma patients.

## Materials and methods

### Animals, cell line, and reagents

Female BALB/c mice age 4–5 weeks were purchased from Shanghai experimental animal center and maintained in gnotobiotic animal facility properly in Zhejiang Chinese Medical University. All animal procedures were following protocols approved by the animal care and use committee from our institutions.

The K7M2 mouse osteosarcoma cell line was obtained from the Cell Bank of Chinese Academy of Science in Shanghai. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin.

L-arginine was purchased from Sigma-Aldrich and diluted with PBS before use. Anti-mouse PD-L1 was obtained from Bio X Cell (West Lebanon, NH, USA) and diluted in PBS.

### Tumor models and treatment

To establish *in situ* osteosarcoma models,  $1 \times 10^6$  K7M2 cells in 20  $\mu$ l PBS was injected into tibia medullary cavity. For metastasis models,  $5 \times 10^5$  cells in 100  $\mu$ l PBS was administrated via the tail vein. One week after tumor inoculation (Day 7), the mice were randomized into 4 different treatment groups, each having at least 10 mice per group as follows: vehicle control (PBS), L-arginine single-agent treatment (L-arg),  $\alpha$ -PD-L1 antibody single-agent treatment ( $\alpha$ -PD-L1), and combination treatment (Combo). L-arginine was oral administrated daily at 2 g/kg body weight for 2 weeks (metastasis models) or 4 weeks (*in situ* models).  $\alpha$ -PD-L1 antibody was injected *i.p.* 3 times a week

at 200  $\mu$ g for 2 weeks (metastasis models) or 4 weeks (*in situ* models). Survival rate was monitored.

### Sample processing

For mice to be prepared for ELISA and flow cytometry analysis, 5 mice from each group were anesthetized by *i.p.* administration of Ketamine (9 mg/ml in saline) and Xylazine (0.9 mg/ml in saline). Peripheral blood was collected via cardia puncture when the mice were anesthetized. Then the spleens, *in situ* tumor tissues, the lungs and lymph nodes were harvested. The spleens were mashed, underwent red blood cell lysis, and passed through 40  $\mu$ m cell strainers. Similarly, lymph nodes were mashed and passed through 40  $\mu$ m cell strainers. For tumor tissues, the tumors were disaggregated by razors and incubated at 37°C for 2 hours in DMEM with collagenase type IV (2 mg/ml, Sigma), DNase (0.1 mg/ml, Sigma), and hyaluronidase (0.1 mg/ml, Sigma). Cell suspensions were passed through 70  $\mu$ m cell strainers to remove aggregates. All the cells mentioned above were washed with cell staining buffer (Biolegend, San Diego, CA, USA) and then ready for antibody incubation.

### ELISA

Serum IFN- $\gamma$  concentration was evaluated by ELISA (R&D Systems, 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 $\alpha$  (clone 53-6.7), anti-IFN- $\gamma$  (clone XMG1.2), anti-CD11b (clone M1/70), and anti-Gr-1 (Clone RB6-8C5). Conjugated antibodies obtained from eBioscience were as follows: anti-granzyme B (clone NGZB), anti-perforin (clone eBioOMAK-D), and anti-PD-1 (clone J43). Live cells were determined by LIVE/DEAD<sup>®</sup> staining (ThermoFisher). 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 (BD Biosciences). For splenocytes and cells from lymph nodes,  $1 \times 10^5$  events were collected for each sample. For intracellular lymphocytes,  $6 \times 10^6$  events for each tumor sample were collected to obtain enough data. All gating strategies were determined by FMO (fluorescence minus one). Flowjo 10 was used to analyze the data.

### Statistical analysis

Survival curves were illustrated using Kaplan-Meyer method. Statistical analysis was determined by one-way ANOVA with Bonferroni's multiple comparison. All the analysis was performed on GraphPad Prism 6. Data were presented as mean  $\pm$  SEM.  $P < 0.05$  was regarded as statistical significant.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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