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The Interplay Between Regular T Cells and Immunotherapy in Cervical Cancer

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Abstract

Background: Immune checkpoint blockade inhibitors have aroused great expectation on many types of tumor eradication. However, the therapeutic effect of anti-PD-L1 treatment on cervical cancer is unsatisfactory and the potential antagonist is not very clear. Here, we investigated the therapeutic effect of anti-PD-L1 in cervical tumor mouse model and identified the potential threats for anti-PD-L1 therapeutic efficacy.

Results: we found that PD-L1 had a moderate expression in human and mouse cervical tumor cell lines and clinical samples compared to other tumor types and para-tumor tissue. Interestingly, our results showed that the anti-PD-L1 treated mice were dichotomously divided into responsive and unresponsive group even with the same genome background C57BL/6 syngeneic tumor model. The unresponsive tumors showed less immune cell infiltration and higher Tregs population induced immunosuppression activity than the responsive ones. Furthermore, we found that anti-PD-L1 autonomously upregulated Tregs proliferation and frequency in multiple immune organs, and, most importantly, Tregs depletion more significantly depressed the tumor growth rate and tumor weight than either anti-PD-L1 or anti-CD25 alone. Finally, we observed that the upregulating effector CD8⁺ T cell is associated with the better therapeutic effect of anti-PD-L1 therapy post Tregs depletion.

Conclusion: In conclusion, anti-PD-L1 therapy upregulates Tregs frequency and proliferation in tumor model, and the depletion of Tregs may be a useful adjuvant strategy for anti-PD-L1 therapy in the immunotherapy of cervical cancer.

Introduction.

Cervical cancer, as the second most gynecological tumors with high incidence and high

mortality among women, severely threatens women's health all around the world [1, 2]. One of the most important reason for the increasing trend of cervical cancer is the higher frequency exposure to human papillomavirus (HPV) caused by the bad sexual habits, such as early beginning of sexual activities, multiple partners, no use of condoms [3]. Currently, the main therapy for cervical cancer includes radiotherapy, chemotherapy, surgery and targeted therapy [4]. However, both radiotherapy and chemotherapy caused serious side-effects, such as hair loss, nausea, anorexia and diarrhea, which undermines the quality of patient life. Besides, the common total radical hysterectomy and bilateral pelvic lymphadenectomy surgery may be futile for patients with metastasis or at advance stage [5]. Although EGFR and COX-2 mediated targeted therapy have been used for the treatment of cervical cancer, the survival rate and prognosis of cervical patients were not significantly improved [6, 7].

The initiation and development of cervical cancer are associated with the immunosuppression on CD4⁺ and CD8⁺ T cells caused by HPV infection. Recently, immune checkpoint blockade (ICB) inhibitors, such as PD-1, PD-L1 and CTLA-4, have been intensely studied in many solid tumors, and many clinical trials have shown the long-lasting improved prognosis of patients, especially in melanoma and lung cancer [8-10]. As for cervical cancer, anti-CTLA-4 showed little clinical efficacy in patients with recurrence or metastasis [11]. Previous studies showed that tumoral PD-L1 expression was observed in 72% cervical and vulvar squamous carcinomas (SCC) and 95% cervical intraepithelial neoplasias (CINs) [9, 12]. Meanwhile, Meng et al. reported that 61% (59/97) of the patients exhibited PD-1 expression in the tumor stroma of cervical cancer [13]. Pembrolizumab (anti-PD-1) also had been approved by FDA for advanced cervical cancer, and the clinical studies have demonstrated that pembrolizumab demonstrated antitumor activity and exhibited a safety profile in patients with programmed death ligand 1-positive advanced cervical cancer [14, 15]. Even so, the overall response rate was only 14.3%-17%, and 75% patients experienced treatment related adverse events, such as rash and pyrexia [14, 16]. Therefore, the therapeutic efficiency of anti-PD-L1 treatment is urgent to be improved in advanced cervical cancer.

Regulatory T cells (Tregs), defined by CD3⁺CD4⁺CD25⁺FOXP3⁺, plays an important role in immune escape and thus undermines the therapeutic efficacy of immunotherapy in various tumor types. Previous studies showed that TNFR2⁺ Tregs increased in tumors of cervical cancer patients, and Foxp3⁺ tumor infiltrating immune cells in the central tumor area might be a biomarker for risk stratification in cervical cancer patients [17-19]. In contrast, Simone Punt et al. reported that a high total number of Tregs were significantly correlated with improved disease-specific and disease-free survival in cervical adenocarcinoma [20]. However, the effect of anti-PD-L1 on Tregs levels and functions is not clear in cervical cancer. Here, we constructed syngeneic cervical tumor mouse model to explore the interact between anti-PD-L1 treatment and Tregs cell and investigated the threats undermining the effect of anti-PD-L1 therapy.

Methods.

Cells and reagents

U14 and Hela cell lines are purchased from ATCC, and were maintained in DMEM supplemented with 10% FBS + 1% penicillin/streptomycin antibiotics.

Protein extraction from cells and tissue.

For U14 and Hela cell lines, the collected cells were washed by cold PBS for two times. 2×

106 cells were treated by RAPA buffer on ice for 30 min. Then, the lysis solution was centrifuged for 15 min at 12000g at 4°C. Then, the protein concentration was measured by BSA method. The loading sample was made for 1µg/µl and loaded for 10µl in SDS-PAGE gel. For tumors, the tumor tissue was sufficiently cut into pieces with sterile scissors, and digested with collagenase and hyaluronate for 1h at 37°C. The collected single-cell suspension was lysate and quantified as mentioned for the protein extraction of U14 and HeLa cell lines.

***In vivo* tumor progression and immunotherapy models.**

3×10⁶ logarithmic growth phase U14 cells were transplanted subcutaneously into the flanks of 5-to 6-week-old C57BL/6 female mice. 3-4 mice were assigned for every group. PBS, Anti-PD-L1 or anti-CD25 were injected every two days. The tumor size was measured seven days post tumor challenge with a caliper every 2–3 days, and tumor volume was calculated by width² × length × 0.5. Mice were sacrificed according to the animal welfare requirement at the endpoint (The maximum tumor less than 20 mm in diameter). All animal protocols were approved by Shanghai JiaoTong University Institutional Animal Care and Use Committee.

RNA extraction and RT-PCR

The cells were harvested and washed for two time with cold PBS. 1ml Trizol reagent was added in 2 x 10⁶ cells and sufficiently suspended. The total RNA was extract according established protocol. In quantitative PCR (q-PCR), the reverse transcription of 1.5µg total RNA were conducted by using SuperScript III First-Strand Synthesis System. The harvested cDNA was diluted for five times by ddH₂O. The SYBR Green PCR Master Mix (Applied Biosystems) was used for qPCR, and three repeats were assigned in a Real-Time PCR System (Applied Biosystems). All used primers for qPCR are listed as follow: mouse-PD-L1: forward-5'- GCTCCAAAGGACTTGTACGTG-3'; reverse-3' -TGATCTGAAGGGCAGCATTTC-5' ; human-PD-L1: forward-5'-GCTGCACTAATTGTCTATTGGGA-3'; reverse-3'-AATTCGCTTGTAGTCGGCACC-5'; mouse-GAPDH : forward-5'-GAAGGTCGGTGTGAACGGAT-3' ; reverse-3'-TGATGGGCTTCCCGTTGATG-5'; human-GAPDH: forward-5'-CGGATTTGGTCGTATTGGG-3' ; reverse-3'-CTCGCTCCTGGAAGATGG-5'; mouse-Ki67: forward-5'-ATCATTGACCGCTCCTTTAGGT-3'; reverse-3'-GCTCGCCTTGATGGTTCCT-5'.

Immunohistochemistry

Tumor and spleen tissue samples were carefully extracted and immediately fixed in 4% paraformaldehyde overnight at room temperature. The fixed tissues were embedded in standard paraffin wax to product 5-µm sections for HE and immunohistochemistry assay. In brief, the tissue sections were deparaffinized in xylene for 3 times (10min/time) and rehydrated via an ethanol gradient (100%, 95%, 80%, 75%, 50%). After antigen retrieval with pH 6.0 citrate buffer, sections were incubated in a 0.3% H₂O₂ solution to remove peroxidase at room temperature for 10min. Then, the sections were washed by PBS for 3times (10min/time) and blocked by normal goat serum or 5% BSA for 1h at 37°C. The sections were then incubated with rat anti-mouse Foxp3 monoclonal antibody (1:200) at 4°C overnight. On the second day, the tissue sections were treated with instant SABC kit according to provided protocol. Finally, the sections were stained with hematoxylin and sealed for observation under microscope.

***In vivo* antibody treatment**

3 x 10⁶ U14 cells were subcutaneously injected as described above. Seven days post tumor cell injection, anti-PD-L1 antibody [*In vivo* mab anti-mouse PD-L1 (B7-H1), BioX Cell, BE0101]

and anti-CD25 (IL-2R α , *In vivo* plus anti-mouse CD25, BioX Cell, BP0012) was intraperitoneally injected (200 μ g per dose per mouse) as indicated schedule. Mice were euthanized and tumors were harvested after 5–6 antibody injections. The resected tumors were photographed and measured.

Cell and tissue FACS analysis

The peripheral blood, tumor, draining lymph node and spleen were isolated from mice. Then, the single cell suspension for these sample were prepared. The single cell suspension was stained with the following antibodies: anti-mouse CD3, anti-mouse CD4, rabbit anti-mouse CD25 at 4 °C for 30 minutes in dark. For intracellular cytokine staining (FOXP3 and Ki67), the cells were resuspended in Fixation/ Permeabilization solution (Cytofix/Cytoperm Kit; BD Biosciences) and incubate at 4°C for 40min. The samples were centrifuged for 500g for 5min. Then, anti-mouse FOXP3 and anti-Ki67 were stained for 30min at 4°C and washed for two times by cold PBS. For the sorting of Tregs, the staining panel is the same as mentioned above. The isolated cells were resuspended with 1ml Trizol reagent and the total RNA was extract for RT-PCR.

Results.

1 . PD-L1 shows high expression of PD-L1 in cervical tumor cell lines and tumor tissue.

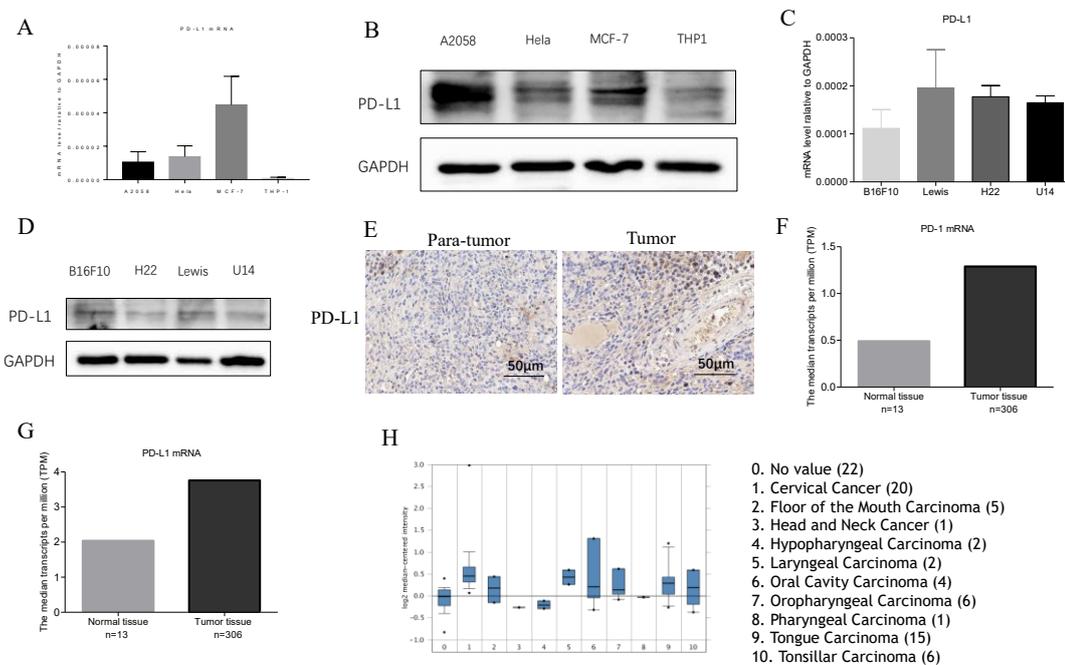


Figure1. PD-L1 shows high expression of PD-L1 in tumor cell lines and tumor tissue.

A. The mRNA level of PD-L1 in HeLa cells and other three human tumor cell lines.

B. The protein level of PD-L1 in HeLa cells and other three human tumor cell lines. The uncropped blots provided in supplementary figure 1a.

C. The mRNA level of PD-L1 in U14 cells and other three mouse tumor cell lines.

D. The protein level of PD-L1 in U14 cells and other three mouse tumor cell lines. The uncropped blots provided in supplementary figure 1b.

E. The detection of expression of PD-L1 by IHC in human cervical tumor tissue. The pictures were magnified for 400 times. Scale bar: 50 μ m.

(F-G). The mRNA level of PD-1 (F) and PD-L1(G) in cervical squamous cell carcinoma patients. The original data of the two graphs was analysed by the GEPIA database for cervical squamous cell

carcinoma.

H. PD-L1 expression in different types of cancer. The original data of this graph comes from the Oncomine database.

The expression of PD-1 and PD-L1 are the important predicative biomarker for anti-PD-1/L1 therapy. Therefore, we first investigated the expression of PD-L1 expression in human and mouse cervical cell lines. Our results showed that Hela and U14 cell line has relatively high level of PD-L1 expression compared with other tumor types in mRNA (Fig. 1A and 1C) and protein level (Fig. 1B and 1D), indicating the potential therapy of anti-PD-L1 therapy in cervical cancer. To investigate the expression of PD-1 and PD-L1 expression in cervical patient samples, we conduct immunohistochemistry for PD-1/L1 in the tumor tissue and corresponding normal tissue of cervical patients. We found that the expression of PD-1 and PD-L1 significantly increase in tumor tissue compared to the corresponding normal tissue of patients (Fig.1E). In addition, we analyzed the mRNA level of PD-1 and PD-L1 in human cervical squamous cell carcinoma by GEPIA database and Oncomine database. We observed that PD-1 (Fig.1F) and PD-L1 (Fig. 1G) showed higher expression in cancer patients than the health control, and cervical cancer had a relative high PD-L1 expression compared with other common cancer types (Fig. 1H). Taken together, the PD-1/L1 signaling pathway may be active in cervical cancer.

2 . The therapeutic efficiency of anti-PD-L1 therapy is partially compromised by the upregulating immunosuppression in tumor.

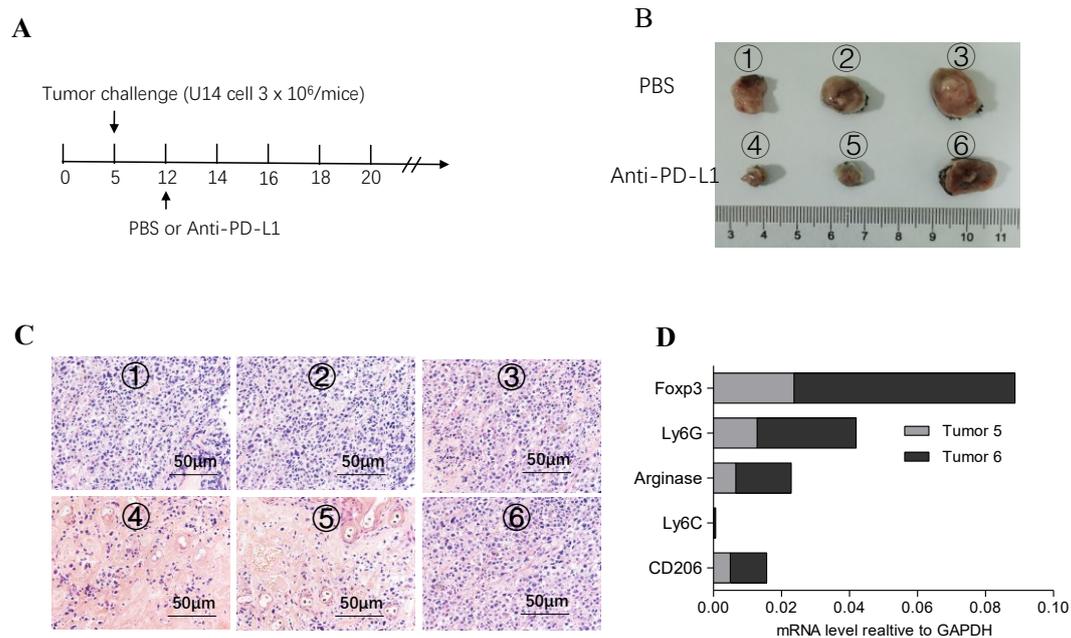


Figure2. The therapeutic efficiency of anti-PD-L1 therapy is partially compromised by the upregulating immunosuppression in tumor.

A. The anti-PD-L1 treatment schedule in syngeneic tumor mouse model. 3×10^6 U14 cervical cells was subcutaneously injected on the flank of 7-weeks C57BL/6 mouse. Anti-PD-L1 was intraperitoneally injected in mouse seven days post tumor challenge.

B. The representative picture of tumors after five times anti-PD-L1 therapy. The tumor was numbered with Arabic numerals.

C. The HE dying for the tumor tissue in (B). The pictures were magnified for 400 times. Scale bar:

50 μ m.

D. The mRNA level of immunosuppressive molecular, including CD206, Ly6C, Ly6G, Foxp3, and Arginase in tumor ⑤ and tumor ⑥.

To investigate the effect of anti-PD-L1 on tumorigenesis of cervical cancer, we constructed the syngeneic tumor model in immune competent C57BL/6 mouse. Anti-PD-L1 or PBS was administrated according the treatment schedule (Fig. 2A). We found that U14 cell line had 100% tumor formation rate in C57BL/6 mice. Anti-PD-L1 treatment significantly depressed the growth of xenografted tumor in most of mice (Fig.2B). Interestingly, tumors were not response to anti-PD-L1 treatment in about 30% mice. To further investigated the tumor microenvironment situation, we conduct HE dye for the tumors. We found that the tumors responding to anti-PD-L1 treatment showed the higher levels tumor necrosis and immune cell infiltration than the unresponsive ones (Fig.2C), indicating the strong immunosuppressive activity. Therefore, we extract mRNA from responsive and unresponsive tumors to anti-PD-L1 therapy and detected the immunosuppressive activity by several vital molecules, including Foxp3, CD206, Arginase, Ly6c and Ly6G. Our results showed that the unresponsive tumors showed higher immunosuppressive activity than the responsive one (Fig.2D). Collectively, the excessive upregulation of Tregs level after anti-PD-L1 treatment may undermine the therapeutic efficiency.

3 . The excessive upregulating Tregs in tumors after anti-PD-L1 treatment is associated with the compromised therapeutic efficiency.

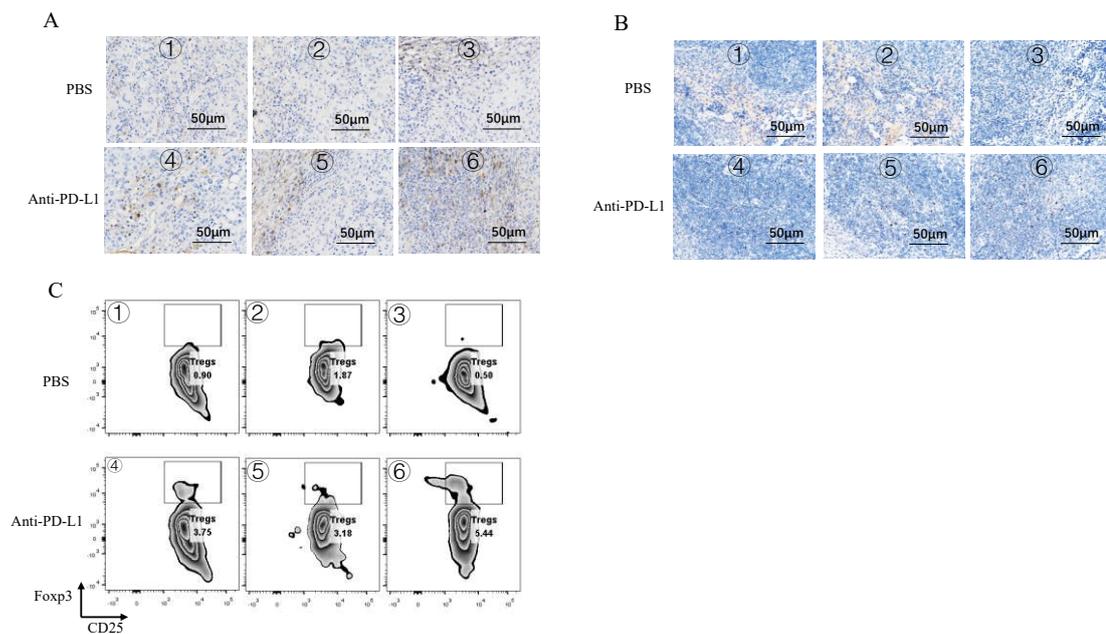


Figure3. The excessive upregulating Tregs in tumors after anti-PD-L1 treatment is associated with the compromised therapeutic efficiency.

A: The Foxp3 IHC assay for the tumor tissue in (B). The pictures were magnified for 400 times. Scale bar: 50 μ m.

B: The Foxp3 IHC assay for the spleen in the corresponding host of tumors in (B). The pictures were magnified for 400 times. Scale bar: 50 μ m.

C: The detection of Tregs in the tumors of (B) with flow cytometry. The Tregs was defined as CD45⁺CD3⁺CD4⁺CD25⁺Foxp3⁺.

As shown in the figure 2D, the unresponsive tumor showed very high level of Foxp3 compared to the responsive tumor and other immunosuppressive marker. Therefore, we hypothesized that the upregulated Tregs level may account for the compromised anti-tumor effect in unresponsive ones. The IHC results showed that Tregs had a relatively high level in unresponsive tumors (Fig. 3A) and corresponding spleens (Fig.3B) compared to responsive tumors. To further identified our finding, we conducted the flow cytometry to detect the frequency of Tregs in tumors. Consistently, we indeed observed the highest Tregs frequency in unresponsive tumor (Fig.3C). Of note, we also found that anti-PD-L1 promoted the frequency of Tregs in both responsive and unresponsive tumors at different degree (Fig.3C).

4 . Anti-PD-L1 treatment upregulates Tregs level and Tregs depletion strengthens the therapeutic efficiency. in multiple immune organs.

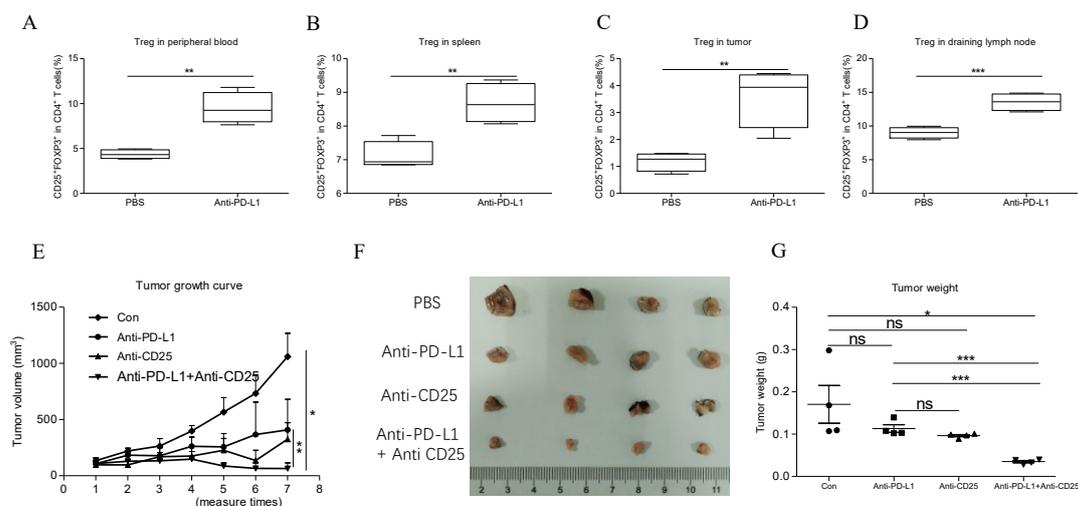


Figure4. Anti-PD-L1 treatment upregulates Tregs level and Tregs depletion strengthen the therapeutic efficiency.

(A-D). The change of Tregs level in peripheral blood (A), spleen (B), tumor (C) and DLN (D) in the syngeneic tumor model. The above samples were collected at the endpoint. N=4. Two-tailed unpaired T-test was performed.

(E): The tumor growth curve under different treatment. The tumor was measured with a caliper, and the tumor volumes were calculated with the formula: $\frac{1}{2} \times \text{length} \times \text{width} \times \text{width}$. Two-tailed unpaired T-test was performed.

(F): the tumor pictures after different treatment. N=4.

(G): The tumor weight in different groups. n=4. The tumors were weight with a analytical balance. Two-tailed unpaired T-test was performed.

ns: no significant difference, * $p < 0.05$, ** $p < 0.01$ compared to the control groups. A P value less than 0.05 was considered to be statistically significant.

Although anti-PD-L1 could effectively depressed the growth rate of tumor in the mouse model, only 20% reduction of tumor weight was achieved. Therefore, we hypothesized that Tregs depletion could enhance anti-PD-L1 efficacy. Therefore, we used PBS, anti-PD-L1, anti-CD25 or anti-PD-L1 plus anti-CD25 to treated cervical tumor mouse model (Fig. S2A). Then, we performed C-flow cytometry of Tregs population in the various immune organs after several five times immunotherapy (Fig. S2B). Our results showed that anti-PD-L1 significantly increased the percentage of Tregs in peripheral blood (Fig. 4A), spleen (Fig. 4B), tumors (Fig. 4C) and lymph node (Fig. 4D).

Importantly, anti-PD-L1 plus anti-CD25 treatment significantly inhibited the growth of syngeneic tumor compared to PBS or anti-PD-L1 or anti-CD25 alone (Fig 4E). The tumors were harvest at the endpoint, and the tumor weight in anti-PD-L1 plus anti-CD25 treatment group was significantly smaller than the control group or anti-PD-L1 or anti-CD25 group alone (Fig 4F and 4G). Taken together, Tregs depletion could strengthen the therapeutic effect of anti-PD-L1 treatment by decreasing upregulating immunosuppression after immunotherapy.

5 . The increased Tregs proliferation depresses the level of effector CD8⁺ T cells.

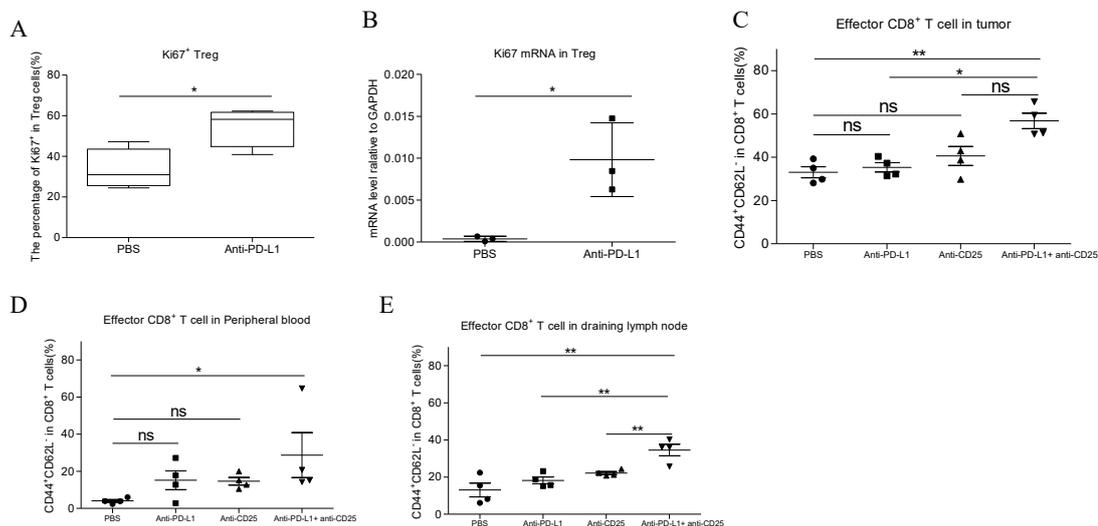


Figure5. The increased Tregs proliferation depresses the level of effector CD8⁺ T cells.

(A). The change of Ki67⁺ Tregs in the peripheral blood of mice after anti-PD-L1 treatment. The blood was collected at the endpoint. N=4. Two-tailed unpaired T-test was performed.

(B). The mRNA level of Ki67 in sorted Tregs cells from the spleen of mouse. N=3. Two-tailed unpaired T-test was performed.

(C-E). The change of the frequency of effector CD8⁺ T cells in tumor (C), the peripheral blood (D) and DLN (E) at the endpoint. N=4. The effector CD8⁺T cell was defined as CD3⁺CD8⁺CD62L⁻CD44⁺. Two-tailed unpaired T-test was performed.

ns: no significant difference, * p < 0.05, ** p < 0.01 compared to the control groups. A P value less than 0.05 was considered to be statistically significant.

To figure out the reason for the increase of Tregs, we analyzed the signature of Tregs after anti-PD-L1 treatment. We found that anti-PD-L1 treatment significantly upregulated the percentage of Ki67⁺ Tregs, indicating the increasing Tregs proliferation (Fig. 5A). Additionally, we also sorted Tregs for PBS and PD-L1 treated group to detect the mRNA level of Ki67 transcription. Consistently, Ki67 showed the higher mRNA level in anti-PD-L1 group compared with the PBS group (Fig.5B). Therefore, the increased Tregs after anti-PD-L1 therapy may associated with increasing proliferation of Tregs.

Considering of the important role of effector CD8⁺ T cells (defined by CD3⁺CD8⁺CD62L⁻CD44⁺) in anti-tumor response. We respectively analyzed the frequency of effector T cells after PBS, or anti-PD-L1 or anti-CD25 or anti-PD-L1 plus anti-CD25 treatment. Our results that anti-PD-L1 plus anti-CD25 treatment group had a significantly higher level of effector CD8⁺ T cells than PBS and PD-L1 group (Fig.5C and 5D). In the draining lymph node, we observed the more distinct

increase of effector CD8⁺ T cells in the combination group compared to any of the other three groups (Fig. 5E). In conclusion, the increased effector CD8⁺ T cells may be associated with the better therapeutic effect after Tregs depletion in cervical tumor model.

Discussion

Currently, immunotherapy had aroused the widely concern of researcher focusing on various tumor types [21]. Although several clinical trials had verified the effect of anti-PD-L1 on advanced tumors, most of patients had great difficulty in maintaining the long-lasting response to immune checkpoint mediated immunotherapy and barely eliminate tumor cells [8, 11]. However, the underlying mechanism is not very clear in cervical cancer. Here, we found that anti-PD-L1 partially inhibit tumor growth in the syngeneic mouse model, which could be depressed the autonomously increased Tregs proliferation and frequency in multiple immune organs and tumor tissue. We also found that Tregs depletion significantly enhanced the tumor depression effect of anti-PD-L1 treatment *in vivo*. Therefore, our research provides a novel insight for the limited anti-tumor efficacy in cervical cancer.

The increased Tregs may be one of the important mechanisms of cervical tumors to resist immunotherapy efficacy. Under tumor conditions, various cytokines, such as GM-CSF, IL6, TNF- α , and other chemokines [22, 23]. Previous studies showed that IL6 and Tumor necrosis factor α (TNF α) could promote Tregs proliferation in tumor sites. The recent study reported that anti-PD-L1 treatment or the Rhein and combination therapy groups upregulated the IL6 level established 4T1 breast cancer xenografts [24]. Consistently, we also observed the slightly increase of IL6 in the tumor tissue after anti-PD-L1 therapy (data not show). TNF is a potent pro-inflammatory cytokine, which played a vital role in the balance of tumor microenvironment. Benoît L Salomon et al reported that TNF is able to increase expansion, stability, and possibly function of Tregs via TNFR2 [23]. In addition, Lack of interleukin-6 in the tumor microenvironment augments type-1 immunity and increases the efficacy of anti-PD-L1 therapy in CT26 cells mouse model [25]. Collectively, we supposed that the increased Tregs in our mouse model probably caused by immunotherapy induced IL6 expression.

Although we observed the enhanced anti-tumor effect after Tregs depletion during anti-PD-L1 treatment in mouse model, we also should be careful for the quickly use of this strategy in cervical cancer patients. A few studies reported that Tregs depleted mice suffered serious autoimmune disease [26]. Furthermore, anti-PD-L1 also may lead to huge immune storm in the host. Therefore, much more attention should be paid on the treatment related adverse event in cervical cancer patients during Tregs depletion combined anti-PD-L1 treatment in cervical cancer patients in the future.

In conclusion, we found that anti-PD-L1 treatment upregulated Tregs levels in cervical cancer mouse model, and Tregs depletion maybe a promising adjuvant treatment of anti-PD-L1 therapy for cervical cancer treatment.

Ethics approval and consent to participate

All methods were carried out in accordance with relevant guidelines and regulations at Shanghai JiaoTong University. All animal protocols were approved by Shanghai JiaoTong University Institutional Animal Care and Use Committee.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Author contribution

Fengying Xu and Fengying Zhang wrote the manuscript. Fengying Xu, Fengying Zhang, Qian Wang and Ying Xu construct the mouse model and collected all related data in figure 2-5. Fengying Xu, Fengyi Zhang, Shuifeng Xu and Caihong Zhang performed the flow cytometry in figure 2-4. Lihua Wang designed and supervised the project.

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Reference.

1. Suh DH, Kim JW, Aziz MF, Devi UK, Ngan HY, Nam JH, Kim SC, Kato T, Ryu HS, Fujii S *et al*: **Asian society of gynecologic oncology workshop 2010**. *J Gynecol Oncol* 2010, **21**(3):137-150.
2. Benard VB, Thomas CC, King J, Massetti GM, Doria-Rose VP, Saraiya M, Centers for Disease C, Prevention: **Vital signs: cervical cancer incidence, mortality, and screening - United States, 2007-2012**. *MMWR Morb Mortal Wkly Rep* 2014, **63**(44):1004-1009.
3. Gustafsson L, Ponten J, Zack M, Adami HO: **International incidence rates of invasive cervical cancer after introduction of cytological screening**. *Cancer Causes Control* 1997, **8**(5):755-763.
4. Tsikouras P, Zervoudis S, Manav B, Tomara E, Iatrakis G, Romanidis C, Bothou A, Galazios G: **Cervical cancer: screening, diagnosis and staging**. *J BUON* 2016, **21**(2):320-325.
5. Kim JY, Byun SJ, Kim YS, Nam JH: **Disease courses in patients with residual tumor following concurrent chemoradiotherapy for locally advanced cervical cancer**. *Gynecol Oncol* 2017, **144**(1):34-39.
6. Hertlein L, Lenhard M, Kirschenhofer A, Kahlert S, Mayr D, Burges A, Friese K: **Cetuximab monotherapy in advanced cervical cancer: a retrospective study with five patients**. *Arch Gynecol Obstet* 2011, **283**(1):109-113.
7. Herrera FG, Chan P, Doll C, Milosevic M, Oza A, Syed A, Pintilie M, Levin W, Manchul L, Fyles A: **A prospective phase I-II trial of the cyclooxygenase-2 inhibitor celecoxib in patients with carcinoma of the cervix with biomarker assessment of the tumor**

- microenvironment.** *Int J Radiat Oncol Biol Phys* 2007, **67**(1):97-103.
8. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, Yokoi T, Chiappori A, Lee KH, de Wit M *et al*: **Durvalumab after Chemoradiotherapy in Stage III Non-Small-Cell Lung Cancer.** *N Engl J Med* 2017, **377**(20):1919-1929.
 9. Mezache L, Paniccia B, Nyinawabera A, Nuovo GJ: **Enhanced expression of PD L1 in cervical intraepithelial neoplasia and cervical cancers.** *Mod Pathol* 2015, **28**(12):1594-1602.
 10. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC *et al*: **Improved survival with ipilimumab in patients with metastatic melanoma.** *N Engl J Med* 2010, **363**(8):711-723.
 11. Lheureux S, Butler MO, Clarke B, Cristea MC, Martin LP, Tonkin K, Fleming GF, Tinker AV, Hirte HW, Tsoref D *et al*: **Association of Ipilimumab With Safety and Antitumor Activity in Women With Metastatic or Recurrent Human Papillomavirus-Related Cervical Carcinoma.** *JAMA Oncol* 2018, **4**(7):e173776.
 12. Chinn Z, Stoler MH, Mills AM: **PD-L1 and IDO expression in cervical and vulvar invasive and intraepithelial squamous neoplasias: implications for combination immunotherapy.** *Histopathology* 2019, **74**(2):256-268.
 13. Meng Y, Liang H, Hu J, Liu S, Hao X, Wong MSK, Li X, Hu L: **PD-L1 Expression Correlates With Tumor Infiltrating Lymphocytes And Response To Neoadjuvant Chemotherapy In Cervical Cancer.** *J Cancer* 2018, **9**(16):2938-2945.
 14. Frenel JS, Le Tourneau C, O'Neil B, Ott PA, Piha-Paul SA, Gomez-Roca C, van Brummelen EMJ, Rugo HS, Thomas S, Saraf S *et al*: **Safety and Efficacy of Pembrolizumab in Advanced, Programmed Death Ligand 1-Positive Cervical Cancer: Results From the Phase Ib KEYNOTE-028 Trial.** *J Clin Oncol* 2017, **35**(36):4035-4041.
 15. Borcoman E, Le Tourneau C: **Pembrolizumab in cervical cancer: latest evidence and clinical usefulness.** *Ther Adv Med Oncol* 2017, **9**(6):431-439.
 16. Chung HC, Ros W, Delord JP, Perets R, Italiano A, Shapira-Frommer R, Manzuk L, Piha-Paul SA, Xu L, Zeigenfuss S *et al*: **Efficacy and Safety of Pembrolizumab in Previously Treated Advanced Cervical Cancer: Results From the Phase II KEYNOTE-158 Study.** *J Clin Oncol* 2019, **37**(17):1470-1478.
 17. Adurthi S, Krishna S, Mukherjee G, Bafna UD, Devi U, Jayshree RS: **Regulatory T cells in a spectrum of HPV-induced cervical lesions: cervicitis, cervical intraepithelial neoplasia and squamous cell carcinoma.** *Am J Reprod Immunol* 2008, **60**(1):55-65.
 18. Chen R, Gong Y, Zou D, Wang L, Yuan L, Zhou Q: **Correlation between subsets of tumor-infiltrating immune cells and risk stratification in patients with cervical cancer.** *PeerJ* 2019, **7**:e7804.
 19. Zhang T, Jiao J, Jiao X, Zhao L, Tian X, Zhang Q, Ma D, Cui B: **Aberrant frequency of TNFR2(+) Treg and related cytokines in patients with CIN and cervical cancer.** *Oncotarget* 2018, **9**(4):5073-5083.
 20. Punt S, van Vliet ME, Spaans VM, de Kroon CD, Fleuren GJ, Gorter A, Jordanova ES: **FoxP3(+) and IL-17(+) cells are correlated with improved prognosis in cervical adenocarcinoma.** *Cancer Immunol Immunother* 2015, **64**(6):745-753.
 21. Boutros C, Tarhini A, Routier E, Lambotte O, Ladurie FL, Carbonnel F, Izzeddine H, Marabelle A, Champiat S, Berdelou A *et al*: **Safety profiles of anti-CTLA-4 and anti-PD-**

- 1 antibodies alone and in combination.** *Nat Rev Clin Oncol* 2016, **13**(8):473-486.
22. Johnson DE, O'Keefe RA, Grandis JR: **Targeting the IL-6/JAK/STAT3 signalling axis in cancer.** *Nat Rev Clin Oncol* 2018, **15**(4):234-248.
23. Salomon BL, Leclerc M, Tosello J, Ronin E, Piaggio E, Cohen JL: **Tumor Necrosis Factor alpha and Regulatory T Cells in Oncoimmunology.** *Front Immunol* 2018, **9**:444.
24. Shen Z, Zhu B, Li J, Qin L: **Rhein Augments Antiproliferative Effects of Atezolizumab Based on Breast Cancer (4T1) Regression.** *Planta Med* 2019, **85**(14-15):1143-1149.
25. Ohno Y, Toyoshima Y, Yurino H, Monma N, Xiang H, Sumida K, Kaneumi S, Terada S, Hashimoto S, Ikeo K *et al.* **Lack of interleukin-6 in the tumor microenvironment augments type-1 immunity and increases the efficacy of cancer immunotherapy.** *Cancer Sci* 2017, **108**(10):1959-1966.
26. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T: **Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease.** *Immunol Rev* 2006, **212**:8-27.

Figures

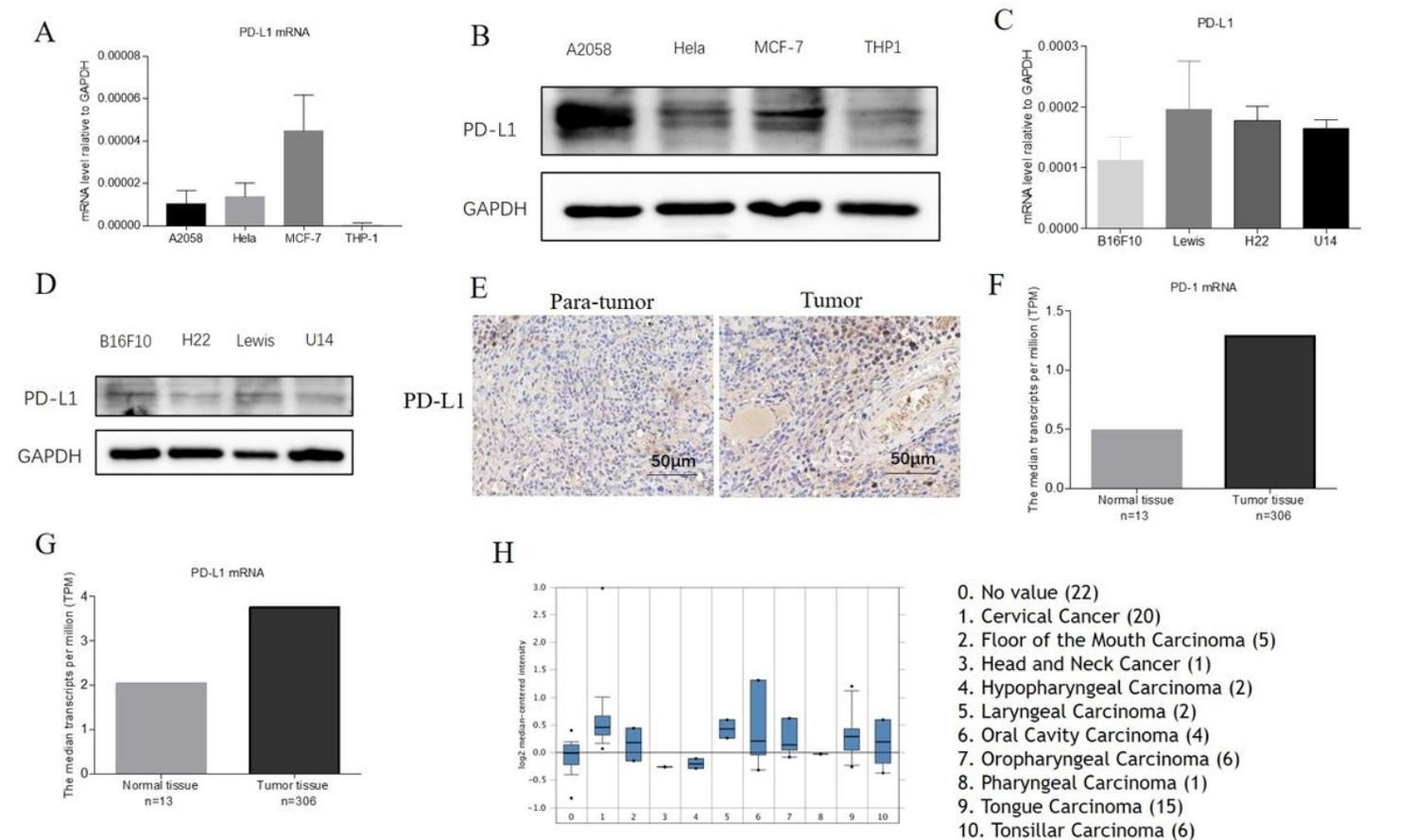


Figure 1

PD-L1 shows high expression of PD-L1 in tumor cell lines and tumor tissue. A. The mRNA level of PD-L1 in HeLa cells and other three human tumor cell lines. B. The protein level of PD-L1 in HeLa cells and other three human tumor cell lines. The uncropped blots provided in supplementary figure 1a. C. The mRNA level of PD-L1 in U14 cells and other three mouse tumor cell lines. D. The protein level of PD-L1 in U14 cells and other three mouse tumor cell lines. The uncropped blots provided in supplementary figure 1b. E. The detection of expression of PD-L1 by IHC in human cervical tumor tissue. The pictures were magnified for 400 times. Scale bar: 50µm. (F-G). The mRNA level of PD-1 (F) and PD-L1(G) in cervical squamous cell carcinoma patients. The original data of the two graphs was analysed by the GEPIA database for cervical squamous cell carcinoma patients. The original data of the two graphs was analysed by the GEPIA database for cervical squamous cell carcinoma patients. The original data of the two graphs was analysed by the GEPIA database for cervical squamous cell carcinoma patients.

0. No value (22)
 1. Cervical Cancer (20)
 2. Floor of the Mouth Carcinoma (5)
 3. Head and Neck Cancer (1)
 4. Hypopharyngeal Carcinoma (2)
 5. Laryngeal Carcinoma (2)
 6. Oral Cavity Carcinoma (4)
 7. Oropharyngeal Carcinoma (6)
 8. Pharyngeal Carcinoma (1)
 9. Tongue Carcinoma (15)
 10. Tonsillar Carcinoma (6)

PD-L1 mRNA level relative to GAPDH: A2058 0.000000, HeLa 0.000020, MCF-7 0.000040, THP-1 0.000060, THP-10.00008

PD-L1 mRNA Normal tissue 0.12, Tumor tissue 0.34, n=13, n=306

The median transcripts per million (TPM) PD-1 mRNA Normal tissue 0.51, Tumor tissue 1.5, n=13, n=306

The median transcripts per million (TPM) PD-L1 mRNA Normal tissue 2.0, Tumor tissue 3.8, n=13, n=306

log₂ median-centered intensity: 0.0, 0.5, 0.4, 0.0, 0.4, 0.5, 1.3, 0.6, 0.0, 0.4, 0.6

L1GAPDHB16F10H22LewisU14DEPara-tumorTumorPD-L150µm50µm carcinoma. H. PD-L1 expression in different types of cancer. The original data of this graph comes from the Oncomine database.

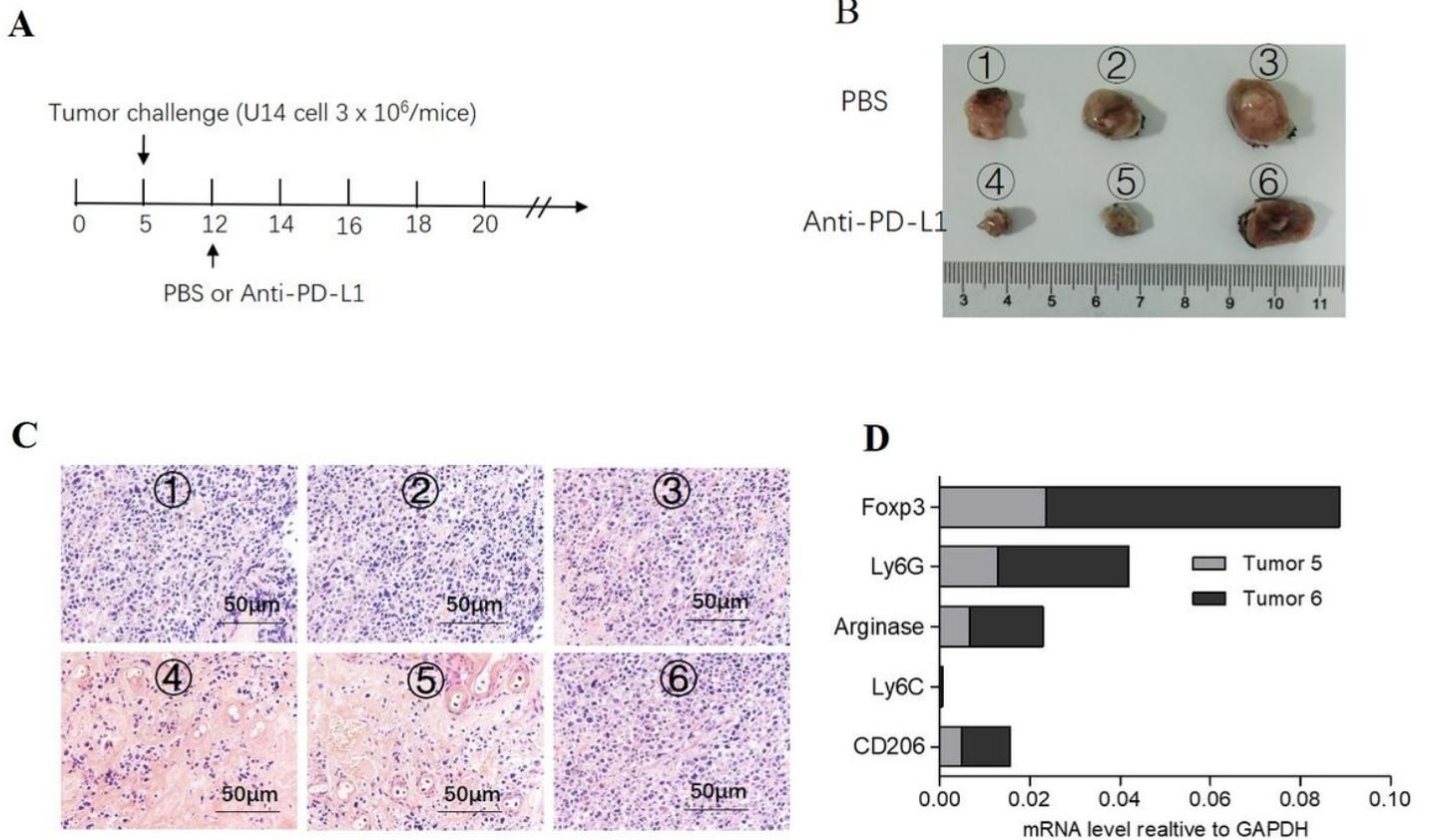


Figure 2

The therapeutic efficiency of anti-PD-L1 therapy is partially compromised by the upregulating immunosuppression in tumor. A. The anti-PD-L1 treatment schedule in syngeneic tumor mouse model. 3×10^6 U14 cervical cells was subcutaneously injected on the flank of 7-weeks C57BL/6 mouse. Anti-PD-L1 was intraperitoneally injected in mouse seven days post tumor challenge. B. The representative picture of tumors after five times anti-PD-L1 therapy. The tumor was numbered with Arabic numerals. C. The HE dyeing for the tumor tissue in (B). The pictures were magnified for 400 times. Scale bar: $50 \mu\text{m}$. D. The mRNA level of immunosuppressive molecular, including CD206, Ly6C, Ly6G, Foxp3, and Arginase in tumor ⑤ and tumor ⑥.

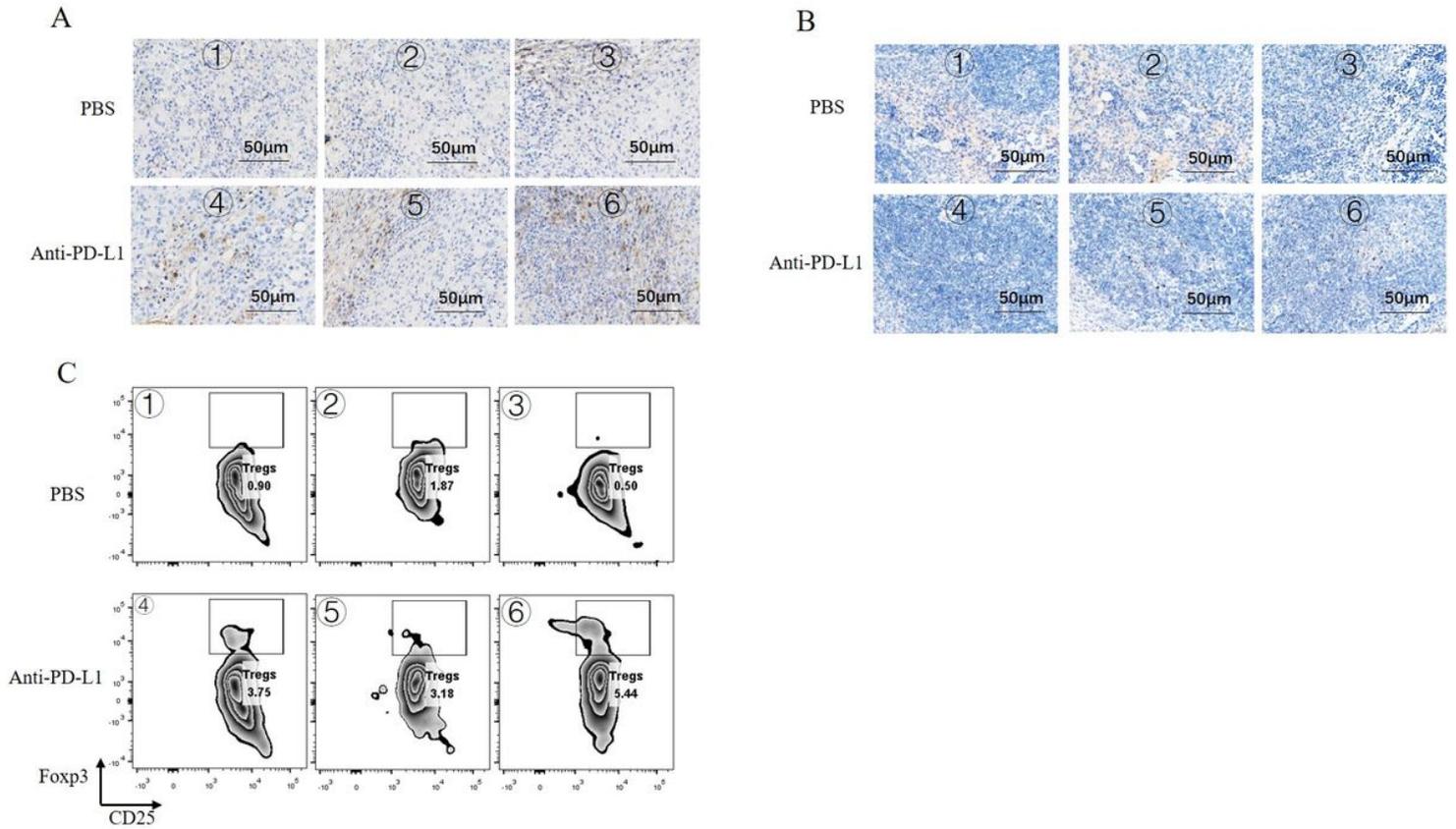


Figure 3

The excessive upregulating Tregs in tumors after anti-PD-L1 treatment is associated with the compromised therapeutic efficiency. A: The Foxp3 IHC assay for the tumor tissue in (B). The pictures were magnified for 400 times. Scale bar: 50μm. B: The Foxp3 IHC assay for the spleen in the corresponding host of tumors in (B). The pictures were magnified for 400 times. Scale bar: 50μm. C: The detection of Tregs in the tumors of (B) with flow cytometry. The Tregs was defined as CD45+CD3+CD4+CD25+Foxp3+.

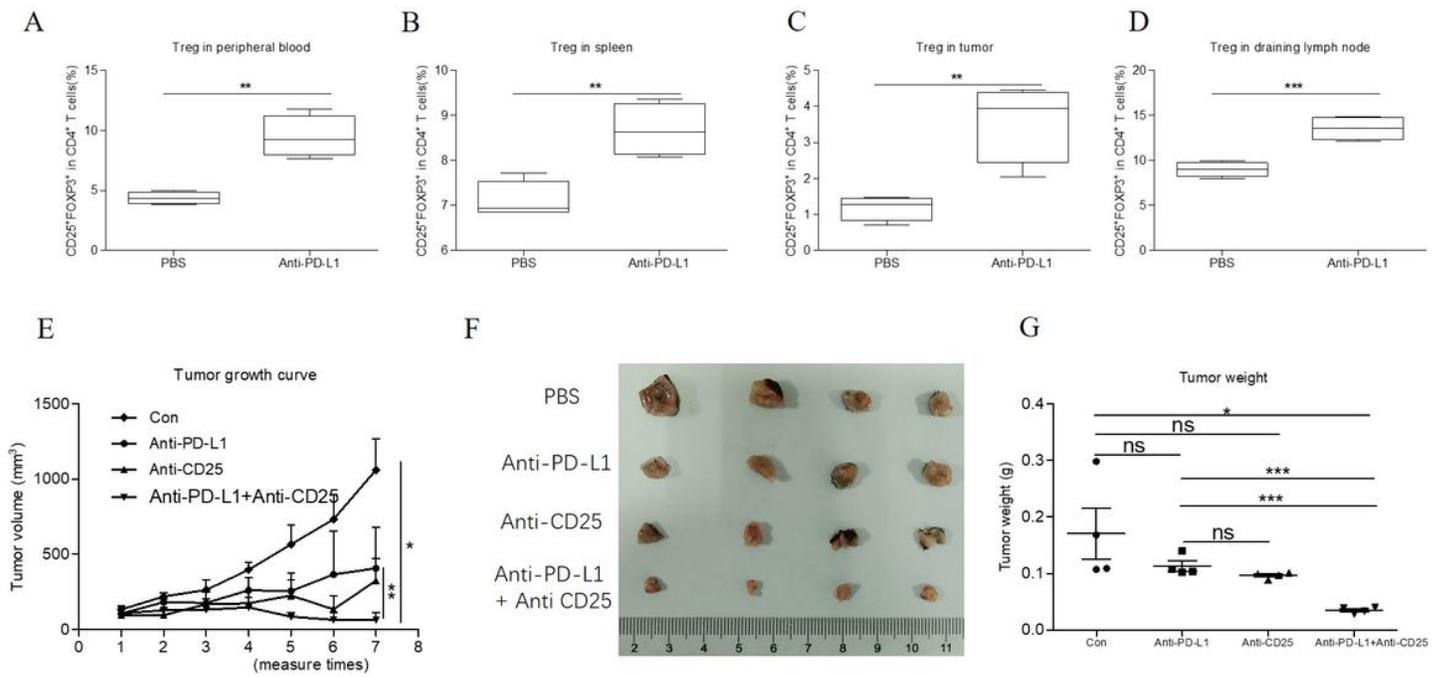


Figure 4

Anti-PD-L1 treatment upregulates Tregs level and Tregs depletion strengthen the therapeutic efficiency. (A-D). The change of Tregs level in peripheral blood (A), spleen (B), tumor (C) and DLN (D) in the syngeneic tumor model. The above samples were collected at the endpoint. N=4. Two-tailed unpaired T-test was performed. (E): The tumor growth curve under different treatment. The tumor was measured with a caliper, and the tumor volumes were calculated with the formula: $\frac{1}{2} \times \text{length} \times \text{width} \times \text{width}$. Two-tailed unpaired T-test was performed. (F): the tumor pictures after different treatment. N=4. (G): The tumor weight in different groups. n=4. The tumors were weight with a analytical balance. Two-tailed unpaired T-test was performed. ns: no significant difference, * $p < 0.05$, ** $p < 0.01$ compared to the control groups. A P value less than 0.05 was considered to be statistically significant.

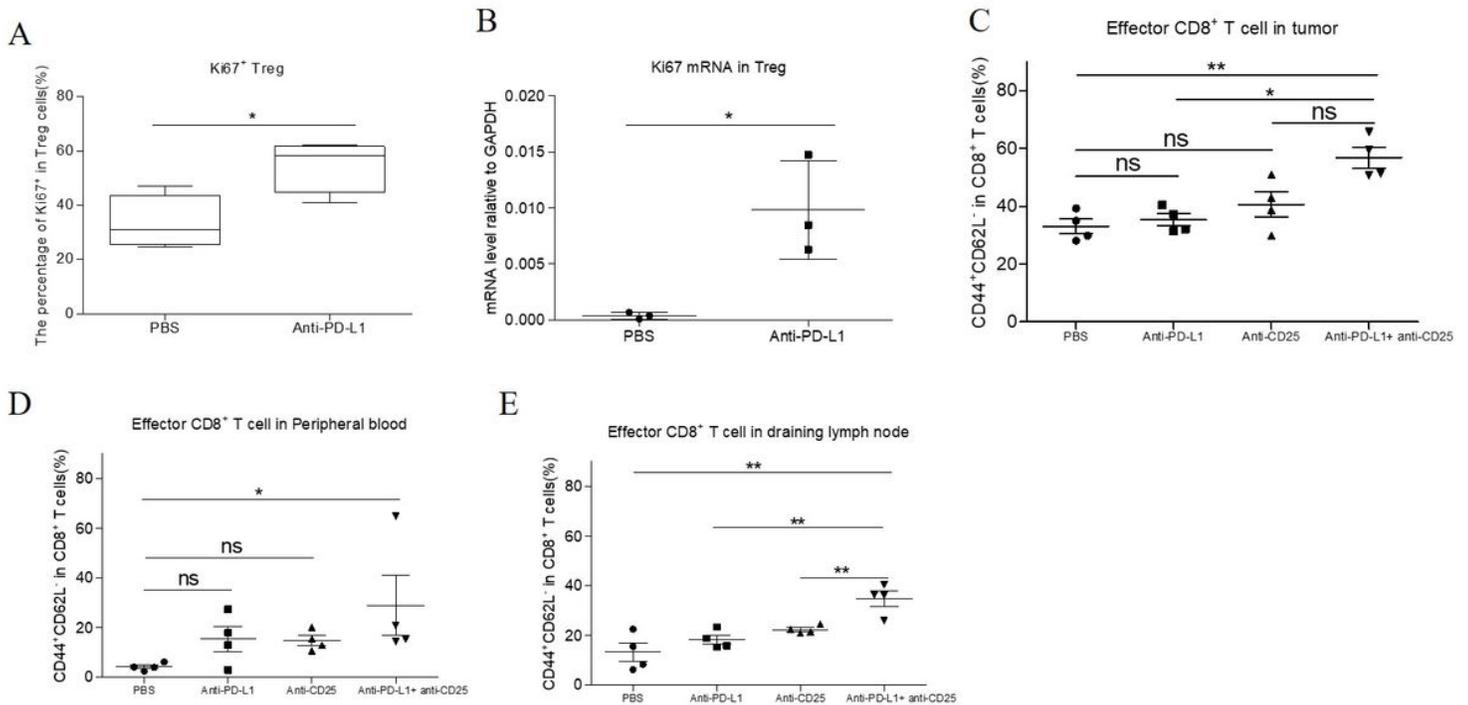


Figure 5

The increased Tregs proliferation depresses the level of effector CD8⁺ T cells. (A). The change of Ki67⁺ Tregs in the peripheral blood of mice after anti-PD-L1 treatment. The blood was collected at the endpoint. N=4. Two-tailed unpaired T-test was performed. (B). The mRNA level of Ki67 in sorted Tregs cells from the spleen of mouse. N=3. Two-tailed unpaired T-test was performed. (C-E). The change of the frequency of effector CD8⁺ T cells in tumor (C), the peripheral blood (D) and DLN (E) at the endpoint. N=4. The effector CD8⁺ T cell was defined as CD3⁺CD8⁺CD62L⁻CD44⁺. Two-tailed unpaired T-test was performed. ns: no significant difference, * p < 0.05, ** p < 0.01 compared to the control groups. A P value less than 0.05 was considered to be statistically significant.

Supplementary Files

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- [SupplementaryTheInterplayBetweenRegularTCellsandImmunotherapyinCervicalCancer.pptx](#)