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Original Articles

Immune checkpoint Ab enhances the antigen-specific anti-tumor effects by modulating both dendritic cells and regulatory T lymphocytes

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ABSTRACT

We determined the anti-tumor effects and possible mechanisms of an antigen-specific DNA vaccine combined with PD-1 or CTLA-4 blockade. Using the HPV16 E6/E7$^+$ syngeneic mouse tumor model, we investigated whether anti-CTLA-4 antibody (Ab) or anti-PD-1 Ab increases the antigen-specific anti-tumor effects and immune response induced by CTGF/E7 chimeric DNA vaccine and the possible mechanisms. Anti-PD-1 Ab or anti-CTLA-4 Ab combined with E7-specific DNA vaccine generated more potent antigen-specific immunity, including anti-E7 Abs and the number and cytotoxic activity of E7-specific cytotoxic CD8$^+$ T lymphocytes, and anti-tumor effects than E7-specific DNA vaccine alone. In addition, the number of systemic and intratumoral Tregs was lower with the anti-PD-1 or anti-CTLA-4 Ab and E7-specific DNA vaccine. Furthermore, anti-PD-1 and anti-CTLA-4 Abs could enhance the maturation and abilities of intratumoral DCs to activate E7-specific cytotoxic CD8$^+$ T cells. Immune checkpoint blockade overcomes the immunosuppressive status of the tumor-microenvironment to enhance the antigen-specific immunity and anti-tumor effects generated by an antigen-specific DNA vaccine. Antigen-specific immunotherapy combined with immune checkpoint blockade can be a novel strategy in clinical cancer therapy.

1. Introduction

An ideal antigen-specific cancer immunotherapy is a treatment modality that not only eradicates systemic tumors in multiple sites, but also discriminates between neoplastic and non-neoplastic cells through antigen-specific anti-tumor immune responses. Anti-tumor T cell activation is counteracted by immunosuppression mediated by tumors through stimulatory and inhibitory ligand-receptor interactions between T cells, dendritic cells, tumor cells, and macrophages in the tumor microenvironment [1]. One of these ligand-receptor interactions for inhibiting immune responses is involved with the appearance of various immune checkpoints, including cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1) during the process of tumorigenesis [2]. Therefore, targeting the immune checkpoints may have an impact on the caner immunotherapy.

Blockade of inhibitory immune checkpoint molecules such as CTLA-4 or PD-1 has been shown to be promising modalities in the clinic for many cancer types [3–5]. The anti-CTLA-4 monoclonal Ab, ipilimumab or anti-PD-1 monoclonal Ab, pembrolizumab has been demonstrated overall survival benefits in metastatic melanoma [4] or non-small cell lung cancer [6], respectively. However, the response rates of advanced melanoma patients treated with ipilimumab or pembrolizumab alone would be poor [7]. The possibility could be the lack of pre-existing anti-tumor T cell responses [8,9]. Therefore, immune checkpoint blockades combined with other treatment modalities having the abilities to prime the tumor-associated T cell responses may improve the therapeutic outcome.

The antigen-specific DNA vaccine can provoke antigen-specific anti-tumor immunity through antigen processing and presentation by antigen presenting cells (APCs) [10–12]. Unlike peptide vaccines

Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen-4; PD-1, programmed cell death protein 1; CTGF, connective tissue growth factor; IFN-γ, interferon-gamma; Tregs, regulatory T cells; Luc, luciferase; BMM, bone marrow monocyte; DC, dendritic cell; MHC, major histocompatibility complex

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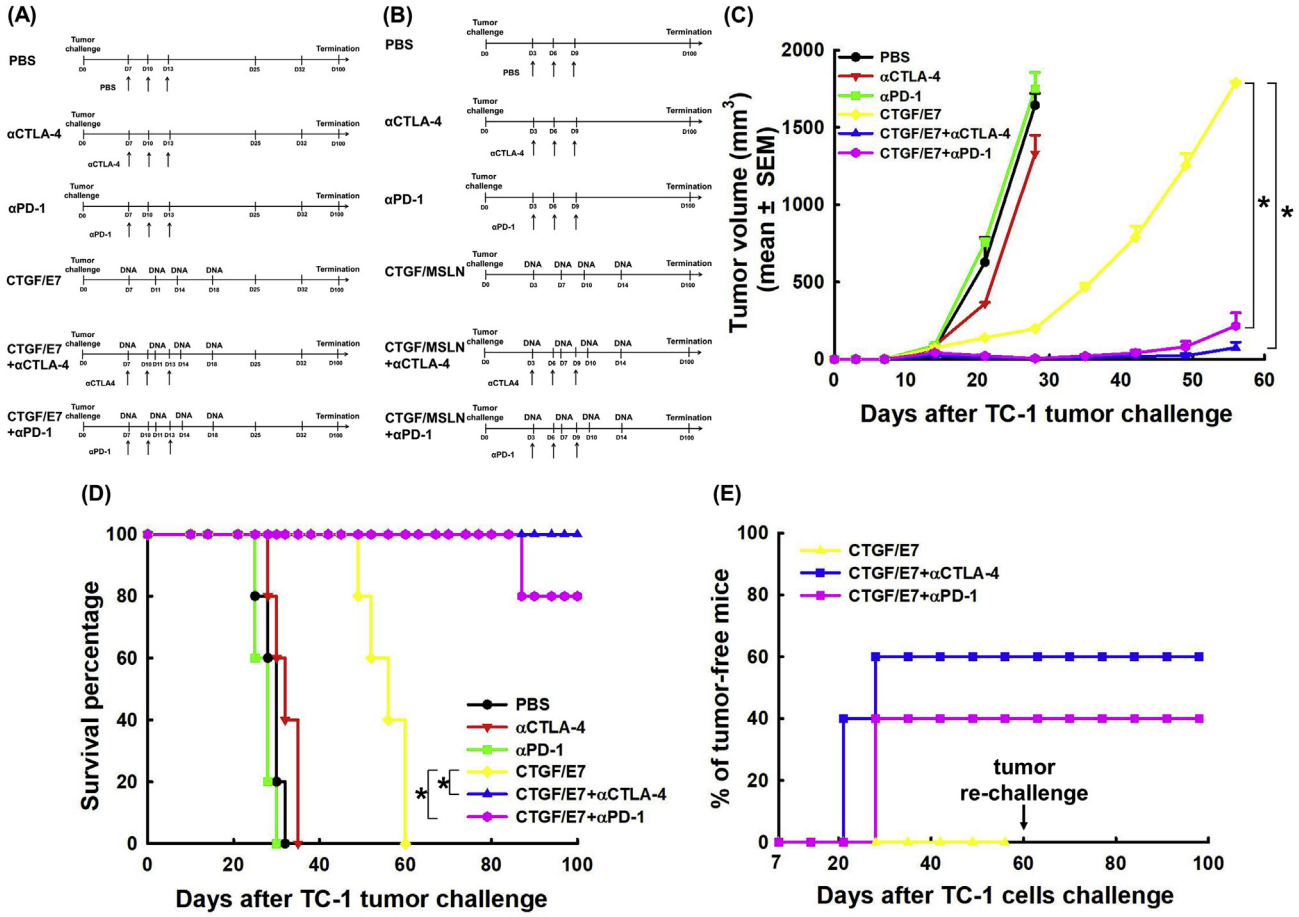

Fig. 1. Anti-tumor effects of CTGF/E7 DNA vaccine alone or with immune checkpoint inhibitor. (A) Diagram of the different treatment regimens for CTGF/E7 DNA vaccine with or without anti-CTLA-4 or PD-1 Ab for TC-1 tumor model. **(B)** Diagram of the different treatment regimens for CTGF/MSLN DNA vaccine with or without anti-CTLA-4 or PD-1 Ab for WF-3 tumor model. **(C)** Anti-tumor effects in mice treated with anti-CTLA-4 Ab alone, anti-PD-1 Ab alone, or CTGF/E7 DNA vaccine with or without the Abs. Anti-CTLA-4 or anti-PD-1 Ab alone had no anti-tumor effects in tumor-bearing mice (n = 5 per group, mean ± SEM). DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab enhanced the anti-tumor effects of DNA vaccine in tumor-bearing mice (p = 0.01, Kruskal-Wallis test). **(D)** Overall survival of mice treated with anti-CTLA-4 Ab alone, anti-PD-1 Ab alone, or CTGF/E7 DNA vaccine with or without the Abs. Mice were euthanized when the tumor diameter exceeded 1.5 cm or when they appeared sick (n = 5 per group). One hundred percent or eighty percent of mice that received CTGF/E7 DNA vaccination combined with anti-CTLA-4 Ab or anti-PD-1 Ab were alive 100 days after TC-1 tumor challenge respectively and none of the mice in CTGF/E7 DNA vaccine alone group could survive more than 60 days (p = 0.003, log-rank test). **(E)** Percentages of tumor-free mice immunized with CTGF/E7 DNA vaccine with or without the Abs. The percentages of tumor-free mice 60 days after treatment with CTGF/E7 DNA vaccine and anti-CTLA-4 Ab or anti-PD-1 Ab were 60% and 40%, respectively. Tumor-free mice were subsequently re-challenged with TC-1 tumor cells. All of the mice remained tumor-free 40 days after tumor re-challenge. All experiments were performed independently in triplicate.

restricted by histocompatibility leukocyte antigen (HLA)-matched concerns, DNA vaccines have the potential to induce both major histocompatibility complex (MHC)-I and II binding antigens for most patients. However, most DNA vaccine could not generate sufficiently vigorous anti-tumor immune responses in patients [13]. Several strategies, including metronomic chemotherapy [12], toll-like receptor (TLR) agonist, or recombinant vaccinia virus have been integrated to elevate the anti-tumor effects of DNA vaccines [14,15].

We previously demonstrated that the connective tissue growth factor (CTGF) linked to the E7 tumor antigen DNA vaccine significantly enhances the E7-specific anti-tumor effects by prolonging the lifespan of DCs [16]. However, this CTGF/E7 DNA vaccine alone is not potent enough to control established tumors [16]. CTGF linked to the mesothelin (MSLN) (CTGF/MSLN) DNA vaccine alone does not generate potent MSLN-specific immune responses to control MSLN-expressing tumor model, either [17]. In consideration of improving the anti-tumor efficacy of the CTGF/E7 or CTGF/MSLN DNA vaccine, inhibition of immune checkpoint with CTLA-4 or PD-1 was combined to treat the tumors. In the study, anti-tumor effects of this combinational treatment modality were investigated in both of the E7-expressing and MSLN-expressing tumor models. The roles of immune checkpoint blockades in the therapeutic strategy were also explored. Blockade of CTLA-4 or PD-1 with the antigen-specific DNA vaccine could generate more potent anti-tumor activity and immunity compared to checkpoint or vaccine alone. Consequently, we showed that antigen-specific DNA vaccine combined with immune checkpoint blockades is a potentially innovative approach for immunotherapy of antigen-expressing cancers in the survey.

2. Materials and methods

2.1. Cell lines

The TC-1 tumor cells [18], WF-3 tumor cells [19], and E7-specific $CD8^+$ T cells [20] were produced and maintained as described previously. Luciferase/GFP-expressing TC-1 (TC-1/LG) or WF-3 (WF-3/LG) cells were generated by transducing TC-1 or WF-3 cells with lentiviral vector, respectively [21,22]. Briefly, 293T cells generated lentiviral particles through transfection of pCMVΔR8.91, pMDG (Academia Sinica, Taipei, Taiwan), and pLKO/luciferase/AS3.1.EGFP3, in which the

luciferase gene was subcloned from pGL2-basic (Promega, Madison, WI) into pLKO/AS3.1.EGFP3 (Academia Sinica, Taipei, Taiwan). TC-1 or WF-3 cells were then infected with lentivirus using 8 μg/ml polybrene (Sigma, St Louis, MO). The TC-1/LG and WF-3/LG cells were sorted by flow cytometry, cultured, and then used in subsequent experiments.

2.2. Preparation of DNA vaccine

pcDNA3-CTGF/E7 and pcDNA3-CTGF/MSLN were generated in our laboratory as described previously [16,17]. The DNA construct was verified by DNA sequencing. The DNA bullet was also prepared as described previously [11]. The gold particle-coated DNA was delivered by a low pressure-accelerated gene gun (BioWare Technologies, Taipei, Taiwan) as described previously [11].

2.3. Mice

Six to 8-week-old female C57BL/6J mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and bred in the animal facility of the School of Medicine of National Taiwan University. All animal procedures were carried out according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. In all of the following experiments, the mice were divided into groups of five.

2.4. Administration of immune checkpoint inhibitors

Rat anti-mouse PD-1 (RMP1-14) and anti-CTLA-4 (9D9) Abs were purchased from BioXCell (West Lebanon, NH). The Ab was diluted with PBS and 200 (anti-mouse PD-1 Ab) or 100 (anti-CTLA-4 Ab) μg intraperitoneally injected into each mouse at indicated intervals (Fig. 1A and B).

2.5. In vivo tumor treatment

The treatment protocols for the DNA vaccine and/or immune checkpoint Ab are presented in Fig. 1A for TC-1 tumor model and Fig. 1B for WF-3 tumor model, respectively. First, each mouse was challenged with 1×10^5 TC-1 cells subcutaneously in the right leg or 1×10^5 WF-3/LG cells intraperitoneally. Anti-mouse PD-1 or anti-CTLA-4 Ab was administered 7, 10, and 13 days or 3, 6, 9 days after TC-1 or WF-3/LG tumor challenge with or without the DNA vaccine, respectively. For the CTGF/E7 DNA vaccine groups, mice were vaccinated with 16 μg of DNA 7, 11, 14, and 18 days after TC-1 tumor challenge. For the CTGF/MSLN DNA vaccine groups, mice were vaccinated with 16 μg of DNA 3, 7, 10, and 14 days after WF-3/LG tumor challenge. The diameter of TC-1 tumor was measured using calipers twice a week starting 7 days after tumor challenge and the tumor volume defined by $4\pi R^3/3$, where R is the radius of the tumor. Electronic balance was used to measure mice body weight twice a week from 10 to 25 days after TC-1 tumor challenge. And the bioluminescences of WF-3/LG tumor images were used to detect the tumor growth using the IVIS Imaging System Series 200 (Xenogen, Alameda, CA) twice a week until they died, or 100 days after tumor challenge. Mice were euthanized when TC-1 tumor reached 1.5 cm in diameter or they appeared sick, recorded as death for the survival curve.

2.6. Cell staining and flow cytometric analysis

To determine if antigen-specific $CD8^+$ T cells express CTLA-4 and/or PD-1 *in vitro* or *ex vivo*, the E7-specific $CD8^+$ T cells from splenocytes or TILs were stained with PE-conjugated anti-CD8a Ab (BD Pharmingen, San Jose, CA), APC-conjugated H-$2D^b$/$E7_{49-57}$ tetramers (MBL International Corporation), FITC-conjugated anti-PD-1 Ab (eBioscience, San Diego, CA), or FITC-conjugated anti-CTLA-4 Ab (Invitrogen Life Technologies, Carlsbad, CA) respectively. To evaluate the expression of various surface markers of Tregs from splenocytes or TILs, mice were challenged with TC-1 and immunized with DNA vaccine and/or the respective immune checkpoint Ab as described earlier. The mice were sacrificed on 25 days after tumor challenge.

Splenocytes or TILs were acquired from various groups on day 25 after tumor challenge (Fig. 1A) and prepared as described previously [12]. First, the Tregs were stained with PE-Cy5.5-conjugated anti-CD4 Ab (BD Pharmingen) and PE-conjugated anti-CD25 Ab (BD Pharmingen). Next, the cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen). Finally, the cells were stained with FITC-conjugated Foxp3 Ab (BioLegend). For the expression of surface PD-1, Tregs were stained with APC-conjugated anti-PD-1 Ab (BioLegend) before fixation and permeabilization. For the expression of CTLA-4, Tregs were stained with APC-conjugated anti-CTLA-4 Ab (eBioscience, San Diego, CA) after fixation and permeabilization.

To determine the expression of PD-1, PD-L1, and/or CTLA-4 by mature or immature DCs, mature or immature bone marrow monocyte (BMM)-derived DCs from mice were obtained, cultured, and harvested as described previously [23]. We also determine the expression of PD-1 and/or PD-L1 of mature or immature tumor-infiltrating DCs from mice treated with DNA vaccine as described earlier (Fig. 1A). For the PD-1/PD-L1 surface expression of TC-1 tumor cells, cells were stained with FITC-conjugated anti-PD-1 Ab (BioLegend) or PE-conjugated anti-PD-L1 Ab (BioLegend). For the expression of surface PD-1, the BMM-derived DCs or tumor-infiltrating DCs were stained with FITC-conjugated anti-CD11c Ab (BioLegend), APC-conjugated anti-PD-1 Ab (BioLegend), and PE-conjugated anti-CD80 Ab (BioLegend) or PE-conjugated anti-CD86 Ab (BioLegend) respectively. For the surface expression of PD-L1, BMM-derived DCs or tumor-infiltrating DCs were stained with PE-conjugated anti-CD11c Ab (BioLegend), FITC-conjugated anti-PD-L1 Ab (BioLegend), and PE-Cy5-conjugated anti-CD80 Ab (BioLegend) or PE-Cy5-conjugated anti-CD86 Ab (BioLegend) respectively. For the expression of CTLA-4, the BMM-derived DCs were stained with FITC-conjugated anti-CD11c Ab (BioLegend), APC-conjugated anti-CTLA-4 Ab (BioLegend). Flow cytometric analysis was performed using a BD Biosciences FACSCalibur flow cytometer (Becton Dickinson) with CELLQuest software.

2.7. Intracellular IFN-γ staining and MHC I-restricted E7 peptide H-$2D^b$ tetramer staining analyzed by flow cytometry

To determine antigen-specific antitumor immunities, mice were challenged with TC-1 tumor cells and immunized with CTGF/E7 DNA vaccine and/or immune checkpoint Ab (Fig. 1A). Splenocytes were harvested 7 days after the last DNA immunization [12] (=on day 25 after tumor challenge) and incubated with 1 μg/ml of MHC I-restricted E7 peptide (aa49–57) or 10 μg/ml of MHC II-restricted E7 peptide (aa30–67) overnight. Splenocytes cultured without E7 peptides were as negative controls. Golgistop (BD Pharmingen) was added 6 h before harvesting. The harvested splenocytes were stained with PE-conjugated anti-CD4 or anti-CD8a Ab (BD Pharmingen), and then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen). The splenocytes were then stained with FITC-conjugated IFN-γ Ab (BD Pharmingen). Splenocytes were also stained with FITC-conjugated anti-CD8a Ab (Abcam) and APC-conjugated H-$2D^b$/$E7_{49-57}$ tetramers (MBL International Corporation). The cells were analyzed using a BD Biosciences FACSCalibur flow cytometer.

2.8. Isolation of tumor-infiltrating lymphocytes (TILs)

TILs were prepared as described previously, with some modifications [11]. Briefly, mice were challenged with TC-1 tumor cells and immunized with CTGF/E7 DNA vaccine and/or immune checkpoint Ab. The mice were sacrificed and tumors excised 7 days after the last DNA immunization (=on day 25 after tumor challenge) as shown in Fig. 1A.

The tumors were dissected into small fragments and digested in 0.1 mg/ml collagenase in CTL medium at 37 °C overnight. After filtering through a 40-μm cell strainer (BD Falcon, San Jose, CA), the cell suspension was incubated for 30 min at 37 °C. After washing with CTL medium, mixing cell suspensions of CTL medium and balanced salt medium were layered on Ficoll-Paque medium (GE Healthcare, Pittsburgh, PA) before centrifugation. TILs from the white interface layer were collected and washed with PBS.

2.9. Detection of $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, IFN-γ-secreting antigen-specific $CD8^+$ cytotoxic T lymphocytes, antigen-specific $CD8^+$ cytotoxic T lymphocytes, and Tregs from TILs

$CD4^+$ T cells and $CD8^+$ T cells from the prepared TILs were stained with FITC-conjugated anti-CD3 Ab (BD Pharmingen), PE-conjugated anti-CD4 Ab, and PE-conjugated anti-CD8 Ab (BD Pharmingen). To determine the number of IFN-γ-secreting antigen-specific $CD8^+$ cytotoxic T TILs, the cells were incubated with or without MHC I-restricted E7 peptide (aa49–57), stained with PE-conjugated anti-CD8a Ab, fixed, and permeabilized using the Cytofix/Cytoperm kit and then stained with FITC-conjugated IFN-γ (BD Pharmingen) as described above. To determine the number of antigen-specific $CD8^+$ cytotoxic T lymphocytes of TILs, the TILs were stained with FITC-conjugated anti-CD8a Ab, and APC-conjugated-H-$2D^b$/$E7_{49\text{-}57}$ tetramers as described above. Tregs from the prepared TILs were stained with PE-Cy5-conjugated anti-CD4 Ab (BD Pharmingen), PE-conjugated anti-CD25 Ab (BD Pharmingen), and FITC-conjugated anti-Foxp3 Ab (BioLegend) as described previously [12]. The cells were analyzed by flow cytometry as described above.

2.10. In vivo activation markers of tumor-infiltrating $CD4^+$ or $CD8^+$ T cells

To further examine whether the blockade of PD-1 or CTLA-4 could enhance the expressions of T cell activation markers such as Ki-67 and CD69 of tumor-infiltrating $CD4^+$ or $CD8^+$ T cells *in vivo*, tumors were harvested from mice treated with CTGF/E7 DNA vaccine with or without Abs 7 days after the last DNA vaccination (=on day 25 after tumor challenge). TILs were first stained with APC-conjugated anti-CD4 Ab (eBioscience, San Diego, CA), PerCP-Cy5.5-conjugated anti-CD8a Ab (BioLegend), and PE-conjugated anti-CD69 Ab (BioLegend). TILs were fixed, and permeabilized using the Cytofix/Cytoperm kit and then stained with FITC-conjugated anti-Ki-67 Ab (BioLegend). The cells were analyzed by flow cytometry as described above.

2.11. Enzyme-linked immune-absorbent assay (ELISA) for anti-E7 Ab

Mice were challenged with TC-1 tumor cells and immunized with CTGF/E7 DNA vaccine and/or immune checkpoint Ab (Fig. 1A). Sera were collected 14 days after the last DNA vaccination (=on day 32 after tumor challenge). To detect E7-specific Abs in the sera, direct ELISA was used as described previously [24]. Briefly, a 96-microwell plate was coated with 100 μl of bacteria-derived HPV-16 E7 proteins (0.5 μg/ml) and incubated at 4 °C overnight before blocking with PBS containing 20% fetal bovine serum (FBS). Sera were serially diluted in PBS, added to the ELISA wells, and incubated at 37 °C for 2 h. After washing with PBS containing 0.05% Tween 20, the plate was incubated with a 1:2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG Ab (Zymed, San Francisco, CA) at room temperature for 1 h. The plate was then washed, developed with 1-Step Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1 M H_2SO_4. The ELISA plate was read at 450 nm using a standard ELISA reader.

2.12. In vitro tumor killing activities

In vitro and *ex vivo* tumor killing assays were performed to evaluate whether the blockade of PD-1 or CTLA-4 on E7-specific $CD8^+$ T cells or splenocytes from mice treated with DNA vaccine and/or immune checkpoint Ab can enhance the anti-tumor effects. E7-specific $CD8^+$ T cells were cultured with irradiated TC-1/LG tumor cells (1:8 ratio) in a 96-well plate (1×10^4 cells/well) for 24 h in the presence of anti-PD-1 Ab (10 μg/ml), anti-CTLA-4 Ab (10 μg/ml), or isotype Ab. Luciferin (Promega, Madison, WI) was added and the total flux (p/s) from each well measured using IVIS Imaging Systems.

Splenocytes were harvested from various groups on day 25 after tumor challenge as described earlier (Fig. 1A), and then co-cultured with irradiated TC-1/LG cells (10:1 ratio) in a 96-well plate (2×10^4 cells/well) for 24 h, and luciferin was added to detect the total flux (p/s) from each well using IVIS Imaging Systems as described earlier.

2.13. In vivo maturation status of DCs

To further evaluate whether the blockade of PD-1 or CTLA-4 enhances the maturation of DCs *in vivo*, inguinal lymph nodes (LNs) and tumors were harvested from mice treated with CTGF/E7 DNA vaccine and/or Abs 7 days after the last DNA vaccination (=on day 25 after tumor challenge). Single cell suspensions of LNs were prepared as described previously [11]. To detect the maturation status of DCs, the cells from LNs and TILs were stained with FITC-conjugated anti-CD11c Ab, PE-conjugated anti-CD80 Ab, and PE-Cy5-conjugated anti-CD86 Ab (BioLegend). The cells were analyzed by flow cytometry as described above.

2.14. Activation of antigen-specific $CD8^+$ cytotoxic T cells by DCs from TILs

To evaluate whether the DCs from TILs in mice treated with CTGF/E7 DNA vaccine and Ab could be more mature to stimulate the secretion of IFN-γ by cytotoxic T lymphocytes, $CD11c^+$-enriched DCs were isolated and sorted from TILs from mice in various groups as described previously, with some modifications [11]. Briefly, the $CD11c^+$ cells were enriched from TILs using magnetic CD11c MicroBeads UltraPure (Miltenyi Biotec, Auburn, CA) via positive selection using autoMACS Pro Separator. The $CD11c^+$ cells (2×10^4) were co-cultured with E7-specific $CD8^+$ T cells (2×10^6) (E:T ratio 1:100) and MHC I-restricted E7 peptide (aa49–57) for 16 h [25]. Next, the cells were stained with PE-conjugated anti-CD8a Ab (BD Pharmingen), fixed, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), stained with FITC-conjugated IFN-γ Ab, and analyzed using a BD Biosciences FACSCalibur flow cytometer as described above.

2.15. Immunofluorescent staining of TILs within the tumors

The mice were challenged with TC-1 tumor cells and immunized with CTGF/E7 DNA vaccine and/or immune checkpoint Ab as described above. Tumors were excised at the indicated days for the various groups. The cancerous tissues were prepared as frozen specimens [26]. Tumors were embedded in optimal cutting temperature (OCT) compound and cut into 10-μm sections using cryostats. The sections were mounted on slides. After fixation with cold methanol (−20 °C) for 20 min, the slides were incubated with 5% FBS for 10 min. To stain Tregs, rat anti-mouse CD4 (Abcam) and rabbit anti-mouse Foxp3 Abs (Abcam) were used. The maturation status of DCs was assessed by staining with rat anti-mouse CD80 Ab (Abcam) and FITC-conjugated anti-mouse CD11c Ab (Abcam).

After incubating with the primary Ab at 4 °C overnight, the sections were incubated with appropriate donkey anti-rat IgG H^+L (Alexa Fluor 594; Abcam) secondary Ab or anti-rabbit IgG H^+L (Alexa Fluor 488; Abcam) secondary Ab at room temperature for 2 h, followed by counter-staining with DAPI (Sigma-Aldrich, St. Louis, MO). After washing with PBS, the sections were cover-slipped using anti-fade

mounting medium (Invitrogen, Carlsbad, CA) and analyzed using a fluorescence microscope (Olympus BX51, Tokyo).

2.16. Maturation status of BMM-derived DCs treated with anti-PD-1 or anti-CTLA-4 Ab

Bone marrow cells from mice were prepared as described previously, with some modifications [23]. Briefly, bone marrow cells were collected from the femurs and tibias by flushing and the cells filtered through a 70-μm cell strainer (BD Falcon, San Jose, CA). Red blood cells were removed using RBC lysis buffer (eBioscience, San Diego, CA). Bone marrow cells (1×10^6 cells/well) were cultured in 24-well plates in culture medium supplemented with 10^3 U/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ) in a 5% CO_2 atmosphere at 37 °C for 6 days. Fresh GM-CSF-containing medium was replaced every 2 days.

To determine whether anti-PD-1 Ab or anti-CTLA-4 Ab could enhance the maturation of DCs, BMM-derived DCs treated with 50 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich Chemie GmbH) were used as a positive control. DCs were treated with or without LPS and 50 μg/ml of anti-PD-1 or anti-CTLA-4 Ab in a 5% CO_2 atmosphere at 37 °C overnight. The cells were stained with FITC-conjugated anti-CD11c Ab (BioLegend), PE-conjugated anti-CD80 Ab (BioLegend), anti-MHC class I (Biolegend), PE-Cy5-conjugated anti-CD86 Ab (BioLegend) or anti-MHC class II (Biolegend). The cells were analyzed by flow cytometry as described above.

2.17. Antigen presenting and processing abilities of the BMM-derived DCs treated with anti-CTLA-4 Ab by flow cytometric analysis

To analyze the influence of anti-CTLA-4 Ab on antigen processing ability, the BMM-derived DCs were treated with anti-CTLA-4 Ab (50 μg/ml) or isotype Ab, and 1 μg/ml FITC-conjugated OVA long peptide ($OVA_{323-339}$ [ISQAVHAAHAEINEAGR]) (Invitrogen) on day 6, with some modifications [21]. On day 7, the cells were then washed and stained with PE-conjugated anti-CD11c Ab (BioLegend), and assessed by flow cytometry.

To further analyze the influence of anti-CTLA-4 Ab on antigen presentation abilities, the BMM-de-rived DCs were treated with anti-CTLA-4 Ab (50 μg/ml) or isotype Ab, and 50 μg/ml FITC-conjugated OVA short peptide ($OVA_{257-264}$ [SIINFEKL]) (Invitrogen) on day 6 [21,23], and the cells were stained with PE-conjugated anti-CD11c Ab and assessed by flow cytometry next day as described earlier.

2.18. Histology

To access the toxicity of tumor-bearing mice immunized with CTGF/E7 DNA vaccine with or without immune checkpoint Ab, mice were sacrificed 7 days after the last DNA immunization (=on day 25 after tumor challenge) and the organs including colon, liver, kidney, and skin of the ear were collected from varous groups. These organs were fixed in 10% neutral-buffered formalin, and prepared as paraffin specimens and cut into 5-μm thick sections and stained with hematoxylin and eosin (H&E). H&E-stained tissue sections were imaged using optical microscope (Olympus BX51, Tokyo).

2.19. Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Mann-Whitney *U* test for comparisons of two groups and Kruskal Wallis test for comparisons of multiple groups were used to evaluate the data from surface marker staining in flow cytometric analysis, ELISA, and tumor treatment experiments. The log-rank test was used to evaluate the data from survival experiments. A $p < 0.05$ was considered significant.

3. Results

3.1. Immune checkpoint blockades enhanced the anti-tumor effects of E7-specific chimeric DNA vaccine

We first evaluated whether immune checkpoint Abs could enhance the anti-tumor effects of E7 antigen-specific DNA vaccine in E7-expressing tumor model. The protocols for *in vivo* therapeutic experiments were shown in Fig. 1A. Treatment was started when TC-1 tumors palpated 7 days after tumor challenge. TC-1 tumor volumes on day 28 were not different among mice treated with PBS (1643.2 ± 77.0 mm^3), anti-CTLA-4 Ab (1329.4 ± 119.7 mm^3), or anti-PD-1 Ab alone (1748.2 ± 107.0 mm^3, $p = 0.093$, Kruskal-Wallis test; Fig. 1C). However, mice immunized with CTGF/E7 DNA vaccine with or without Abs had smaller tumor volumes than mice treated with PBS ($p = 0.003$, Kruskal-Wallis test). Mice immunized with CTGF/E7 DNA vaccine and anti-CTLA-4 Ab (74.7 ± 35.5 mm^3) or anti-PD-1 Ab (215.8 ± 84.3 mm^3) had significantly smaller tumor volumes on day 56 than mice immunized with CTGF/E7 DNA vaccine alone (1788.5 ± 10.9 mm^3, $p = 0.01$, Kruskal-Wallis test; Fig. 1C). Tumor volume was not different in mice immunized with CTGF/E7 DNA and anti-CTLA-4 or anti-PD-1 Ab ($p = 0.18$, Mann-Whitney *U* test). Anti-CTLA-4 Ab or anti-PD-1 Ab monotherapy had no impact on mouse survival. Mice immunized with CTGF/E7 DNA vaccine and anti-CTLA-4 Ab or anti-PD-1 Ab had significantly longer survival than mice immunized with CTGF/E7 DNA vaccine alone ($p = 0.003$, log-rank test; Fig. 1D). One hundred percent or eighty percent of the mice that received the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab or anti-PD-1 Ab were alive 100 days after challenge with TC-1 tumor cells respectively (Fig. 1D). The percentage of tumor-free mice 60 days after treatment with CTGF/E7 DNA vaccine and anti-CTLA-4 Ab or anti-PD-1 Ab was 60% and 40%, respectively. To evaluate the memory of anti-tumor immunity, tumor-free mice were subsequently re-challenged with TC-1 tumor cells. All of the mice remained tumor-free 40 days after re-challenge (Fig. 1E).

Our results indicated that combination of E7 antigen-specific chimeric DNA vaccine and immune checkpoint inhibitors could generate more potent anti-tumor effects than DNA vaccine alone in E7-expressing tumor model.

3.2. Immune checkpoint blockades enhanced the antigen-specific $CD8^+$ T cell systemic and local immune responses generated by antigen-specific chimeric DNA vaccine

Then, we investigated whether immune checkpoint Ab regulates the antigen-specific immunological profiles induced by the antigen-specific DNA vaccine in systemic lymphoid organs, the spleen, and at the local tumor site. The protocols for *in vivo* immune response experiments on day 25 after tumor challenge were shown in Fig. 1A. In the systemic lymphoid organ, the spleen, the number of E7-specific IFN-γ-secreting $CD4^+$ helper T cells from splenocytes were significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (190.3 ± 12.4) or anti-PD-1 Ab (197.0 ± 20.0) groups compared to the CTGF/E7 DNA vaccine alone group (71.7 ± 8.3, $p = 0.003$, Kruskal-Wallis test; Fig. 2A). The number of E7-specific IFN-γ-secreting $CD4^+$ helper T cells was not different between the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and vaccine with anti-PD-1 Ab groups ($p = 0.39$, Mann-Whitney *U* test).

Representative results of flow cytometric analysis of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T cells from splenocytes are shown in Fig. 2B. The number of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T cells was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (1472.3 ± 65.7) or anti-PD-1 Ab (1206.3 ± 116.9) groups compared to the CTGF/E7 DNA vaccine alone group (665.0 ± 44.3, $p = 0.01$, Kruskal-Wallis test; Fig. 2C). The number of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T cells was not different between the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and vaccine with anti-PD-1 Ab

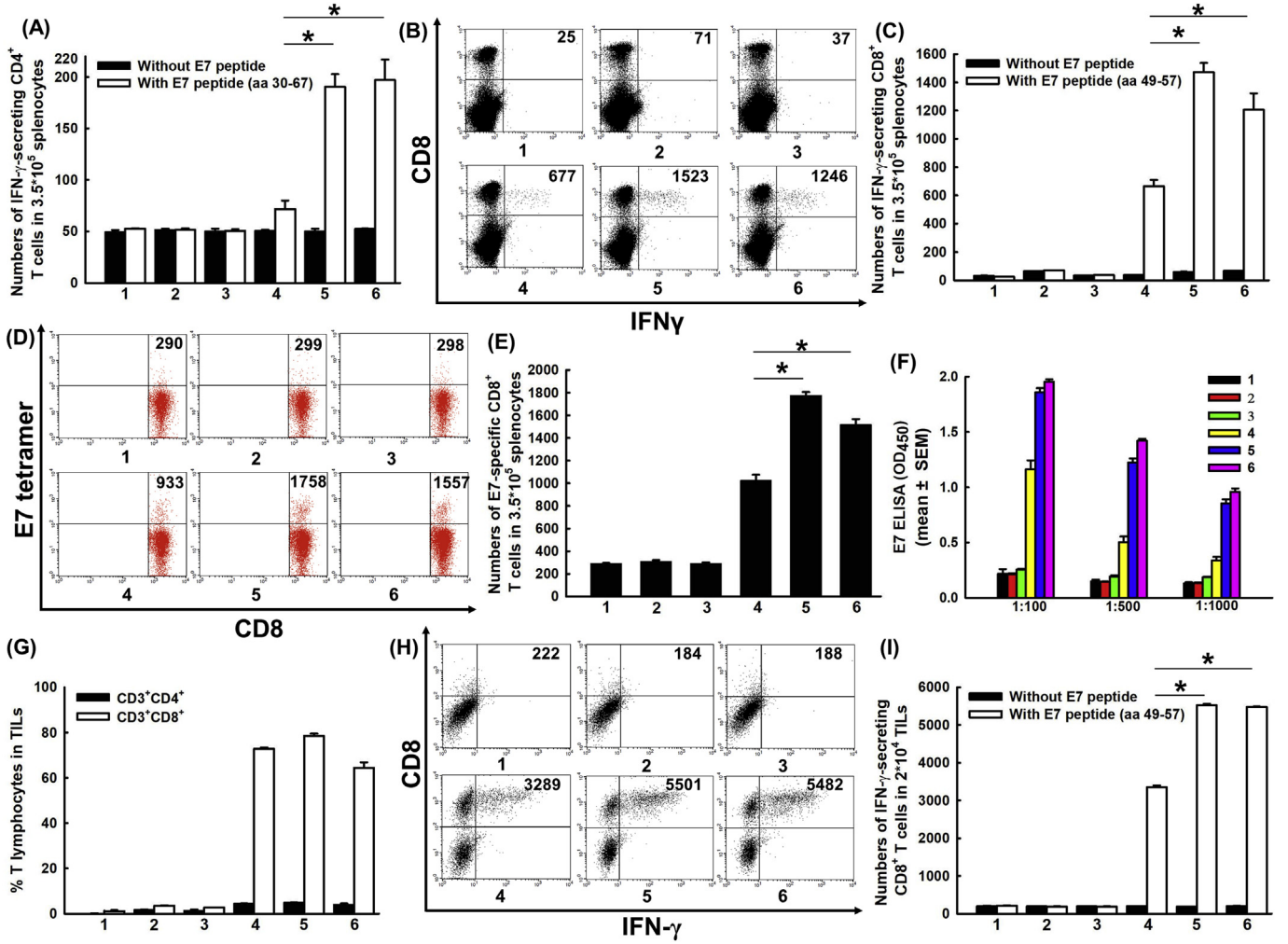

Fig. 2. Antigen-specific immunoprofiles of mice treated with CTGF/E7 DNA vaccine alone or with immune checkpoint inhibitor. (A) Numbers of E7-specific IFN-γ-secreting $CD4^+$ helper T-cell precursors/3.5×10^5 splenocytes with (open columns) or without (filled columns) the corresponding MHC II-restricted E7 peptide (aa 30–67) on day 25 after tumor challenge in various vaccinated groups as determined by flow cytometry (n = 5 per group, mean ± SEM). DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab significantly augmented antigen-specific $CD4^+$ T cell immune responses compared to DNA vaccine alone ($p = 0.003$, Kruskal-Wallis test). **(B)** Representative figures of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T-cell precursors/3.5×10^5 splenocytes in various groups (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). **(C)** Numbers of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T-cell precursors/3.5×10^5 splenocytes with (open columns) or without (filled columns) the corresponding MHC I-restricted E7 peptide (aa 49–57) in various groups as determined by flow cytometry (n = 5 per group, mean ± SEM). DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab significantly augmented antigen-specific $CD8^+$ T cell immune responses compared to DNA vaccine alone ($p = 0.01$, Kruskal-Wallis test). **(D)** Representative figures of E7-specific $CD8^+$ cytotoxic T precursors/3.5×10^5 splenocytes by HPV-16 E7 tetramer staining in various groups (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). **(E)** Numbers of E7-specific $CD8^+$ cytotoxic T precursors/3.5×10^5 splenocytes in various groups as determined by HPV-16 E7 tetramer staining (n = 5 per group, mean ± SEM). DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab significantly augmented the numbers of antigen-specific $CD8^+$ T cells compared to DNA vaccine alone ($p < 0.001$, Kruskal-Wallis test). **(F)** Titers of E7-specific antibodies on day 32 after tumor challenge in various groups as detected by ELISA (n = 5 per group, mean ± SEM). DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab significantly augmented antigen-specific Abs in sera compared to DNA vaccine alone ($p < 0.001$, Kruskal-Wallis test). **(G)** Percentages of $CD4^+$ and $CD8^+$ T lymphocytes from TILs in various groups (n = 5 per group, mean ± SEM). The percentages of $CD4^+$ ($p = 0.33$) and $CD8^+$ ($p = 0.07$) T lymphocytes from TILs of mice treated with CTGF/E7 DNA vaccine combined with anti-CTLA-4 or anti-PD-1 were similar to the DNA vaccine alone group (both by Kruskal-Wallis test). **(H)** Representative figures of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T-cell precursors/2×10^4 TILs in various groups. **(I)** Numbers of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T-cell precursors/2×10^4 TILs with (open columns) or without (filled columns) the corresponding MHC I-restricted E7 peptide (aa 49–57) in various groups (n = 5 per group, mean ± SEM). DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab significantly augmented the numbers of E7-specific $CD8^+$ T cells in TILs compared to DNA vaccine alone ($p = 0.001$, Kruskal-Wallis test) (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). All experiments were performed independently in triplicate.

groups ($p = 0.20$, Mann-Whitney *U* test). Furthermore, the representative figures of tetramer staining of E7-specific $CD8^+$ cytotoxic T cells from splenocytes are shown in Fig. 2D. The numbers of E7-specific $CD8^+$ cytotoxic T cells also dramatically increased in mice treated with CTGF/E7 DNA vaccine with anti-CTLA-4 (1777.3 ± 28.9) and anti-PD-1 Ab (1519.2 ± 48.7) groups compared with those treated with DNA vaccine alone group (1026.5 ± 48.5, $p < 0.001$, Kruskal-Wallis test; Fig. 2E).

The antigen-specific humoral immunity of various groups was further analyzed by detecting the anti-E7 Abs. The titers of E7-specific Abs were significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (1.858 ± 0.038) or anti-PD-1 Ab (1.955 ± 0.021) groups than the

CTGF/E7 DNA vaccine alone group (1.164 ± 0.079, $p < 0.001$, Kruskal-Wallis test, in 1:100 dilution; Fig. 2F). The titers of E7-specific Abs were not different among the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and vaccine with anti-PD-1 Ab groups ($p = 0.27$, Mann-Whitney *U* test).

In the local tumor site, the percentage of $CD4^+$ T TILs was not significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (4.74 ± 0.17%) or anti-PD-1 Ab (3.93 ± 0.60%) groups compared to the CTGF/E7 DNA vaccine alone group (4.35 ± 0.20%, $p = 0.33$, Kruskal-Wallis test; Fig. 2G). The percentages of $CD8^+$ T TILs were also similar in all groups (CTGF/E7 DNA vaccine with anti-CTLA-4 (78.52 ± 0.99%) or anti-PD-1 Ab (64.42 ± 2.41%), CTGF/E7 DNA vaccine alone (72.82 ± 0.59%); $p = 0.07$, Kruskal-Wallis test; Fig. 2G). However, representative numbers of flow cytometric analysis of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T cells from TILs are shown in Fig. 2H. The number of antigen-specific IFN-γ-secreting $CD8^+$ cytotoxic T TILs was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (5519.3 ± 37.2) or anti-PD-1 Ab (5475.7 ± 11.1) groups than the CTGF/E7 DNA vaccine alone group (3351.8 ± 47.3, $p = 0.001$, Kruskal-Wallis test; Fig. 2I). The number of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T cells was not different between the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and vaccine with anti-PD-1 Ab groups ($p = 0.40$, Mann-Whitney *U* test).

Thus, our results indicate that immune checkpoint inhibitor enhances the number of antigen-specific $CD8^+$ cytotoxic T cells generated by antigen-specific chimeric DNA vaccine in the spleen and local tumors.

3.3. Combination of DNA vaccine with immune checkpoint blockades decreases the frequencies of Tregs in splenocytes and tumors in mice by reducing CTLA-4 expression of splenocytes and TILs

We further investigated whether immune checkpoint Abs influence the Tregs from splenocytes and tumors from mice vaccinated with an antigen-specific DNA vaccine. In the systemic lymphoid organ, the spleen, representative flow cytometry results of Tregs from splenocytes are shown in Fig. 3A. The percentage of Tregs was significantly reduced in the CTGF/E7 DNA vaccine with anti-CTLA-4 (8.75 ± 0.07%) or anti-PD-1 Ab (9.01 ± 0.07%) groups compared to the CTGF/E7 DNA vaccine alone group (11.48 ± 0.18%, $p = 0.004$, Kruskal-Wallis test; Fig. 3B). The percentage of Tregs was not different between the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and vaccine with anti-PD-1 Ab groups ($p = 0.14$, Mann-Whitney *U* test). The percentage of CTLA-4^+ $CD4^+CD25^+FoxP3^+$ Tregs was significantly lower in the CTGF/E7 DNA vaccine with anti-CTLA-4 (3.47 ± 0.05%) or anti-PD-1 Ab (3.64 ± 0.06%) groups compared to the CTGF/E7 DNA vaccine alone group (6.35 ± 0.17%, $p = 0.01$, Kruskal-Wallis test; Fig. 3C). The percentage of CTLA-4^+ $CD4^+CD25^+FoxP3^+$ Tregs was not different between the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and vaccine with anti-PD-1 Ab groups ($p = 0.20$, Mann-Whitney *U* test). However, the percentage of PD-1^+ $CD4^+CD25^+FoxP3^+$ Tregs was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (25.07 ± 0.41%) or anti-PD-1 Ab (25.79 ± 0.11%) groups than in the CTGF/E7 DNA vaccine alone group (22.92 ± 0.11%, $p = 0.01$, Kruskal-Wallis test; Fig. 3D).

We also investigated whether anti-CTLA-4 or anti-PD-1 Ab combined with the CTGF/E7 DNA vaccine decreases the number of Tregs in tumors. Representative flow cytometry results and immunofluorescent staining of tumor-infiltrating Tregs are shown in Fig. 3E and Supplementary Fig. 1A, respectively. The CTGF/E7 DNA vaccine with anti-CTLA-4 (6.37 ± 0.21%) or anti-PD-1 Ab (12.66 ± 0.71%) groups had significantly lower percentages of tumor-infiltrating Tregs than the CTGF/E7 DNA vaccine alone group (22.52 ± 0.33%, $p = 0.03$, Kruskal-Wallis test) and the other groups ($p = 0.04$, Kruskal-Wallis test; Fig. 3F). In addition, the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab group had significantly lower percentages of tumor-infiltrating Tregs than the CTGF/E7 DNA vaccine with anti-PD-1 Ab group ($p = 0.002$, Mann-Whitney *U* test). The ratio of tumor-infiltrating $CD4^+$ cells/Tregs in different groups was shown in Fig. 3G. The CTGF/E7 DNA vaccine with anti-CTLA-4 (0.75 ± 0.02) or anti-PD-1 Ab groups (0.41 ± 0.07) had higher $CD4^+$/Treg ratios than the CTGF/E7 DNA vaccine alone group (0.19 ± 0.01, $p = 0.03$, Kruskal-Wallis test; Fig. 3G). The CTGF/E7 DNA vaccine with anti-CTLA-4 Ab group also had higher tumor-infiltrating total $CD8^+$ cells/Tregs ratios (10.28 ± 0.78) than the CTGF/E7 DNA vaccine with anti-PD-1 Ab group (4.67 ± 0.51) or DNA vaccine alone group (3.32 ± 0.19, $p = 0.01$, Kruskal-Wallis test; Fig. 3H). Moreover, the CTGF/E7 DNA vaccine with anti-CTLA-4 Ab group had higher tumor-infiltrating antigen-specific cytotoxic $CD8^+$ T cells/Tregs ratios (4.29 ± 0.12) than the CTGF/E7 DNA vaccine with anti-PD-1 Ab group (2.21 ± 0.08) or DNA vaccine alone group (0.73 ± 0.03, $p = 0.001$,Kruskal-Wallis test; Fig. 3I).

Thus, our results indicate that immune checkpoint inhibitor combined with the DNA vaccine decreases Tregs in both the spleen and tumors, and reduces their expression of CTLA-4 compared to the DNA vaccine alone.

3.4. In vitro and ex vivo antigen-specific $CD8^+$ cytotoxic T cells treated with anti-CTLA-4 or anti-PD-1 Ab have enhanced tumor killing activities

In vitro tumor killing assays were performed to evaluate whether the tumor cell killing activities of $CD8^+$ cytotoxic T cells can be enhanced by blocking the CTLA-4 or PD-1 molecule. The representative luminescence results for TC-1/LG tumor cells co-cultured with E7-specific $CD8^+$ cytotoxic T cells or splenocytes are shown in Fig. 4A and C, respectively. The E7-specific $CD8^+$ cytotoxic T cells pretreated with anti-CTLA-4 ($8.1 \pm 0.5 \times 10^6$ p/s) or anti-PD-1 Ab ($7.1 \pm 0.4 \times 10^6$ p/s) had significantly less luminescence than those pretreated with isotype Ab ($1.1 \times 10^7 \pm 0.9 \times 10^6$ p/s, $p = 0.03$, Kruskal-Wallis test; Fig. 4B).

The *ex vivo* tumor killing effects of splenocytes were also evaluated. The representative luminescence activities of TC-1/LG cells co-cultured with splenocytes from mice treated with CTGF/E7 DNA vaccine with or without anti-CTLA-4 or anti-PD-1 Ab are shown in Fig. 4C. The CTGF/E7 DNA vaccine with anti-CTLA-4 ($1.4 \pm 0.5 \times 10^8$ p/s) or anti-PD-1 Ab ($1.4 \pm 0.9 \times 10^8$ p/s) resulted in significantly lower luminescence activities than CTGF/E7 DNA vaccine alone ($2.0 \pm 0.4 \times 10^8$ p/s, $p = 0.04$, Kruskal-Wallis test; Fig. 4D). The luminescence activities were not different between mice treated with CTGF/E7 DNA vaccine with anti-CTLA-4 Ab and those treated with vaccine and anti-PD-1 Ab ($p = 0.70$, Mann-Whitney *U* test).

Our results indicate that the tumor killing activities of E7-specific $CD8^+$ cytotoxic T lymphocyte can be enhanced by immune checkpoint blockades.

3.5. Both PD-1 and CTLA-4 are highly expressed on antigen-specific $CD8^+$ T cells and Tregs of splenocytes

The inhibition of CTLA-4 or PD-1 combined with CTGF/E7 DNA vaccine generated similar anti-tumor effects and immune responses (Figs. 1 and 2); therefore, we further evaluated regulatory mechanisms of the inhibitory surface receptors, CTLA-4 and PD-1. As shown in Fig. 5A and B, both CTLA-4 and PD-1 were highly expressed on E7-specific cytotoxic $CD8^+$ cytotoxic T cells. 9.6% (Fig. 5C2) and 19.3% (Fig. 5C3) of Tregs from splenocytes expressed CTLA-4 and PD-1, respectively. Consequently, our results indicate that antigen-specific $CD8^+$ cytotoxic T cells and Tregs expressed both PD-1 and CTLA-4.

3.6. Higher percentage of immature BMM-derived DCs express PD-1 and PD-L1 than mature BMM-derived DCs

CTLA-4 is expressed exclusively on T cells, and PD-1 is broadly expressed on activated T cells, B cells, and myeloid cells [27]. In addition to lymphocytes, we investigated whether the inhibitory surface

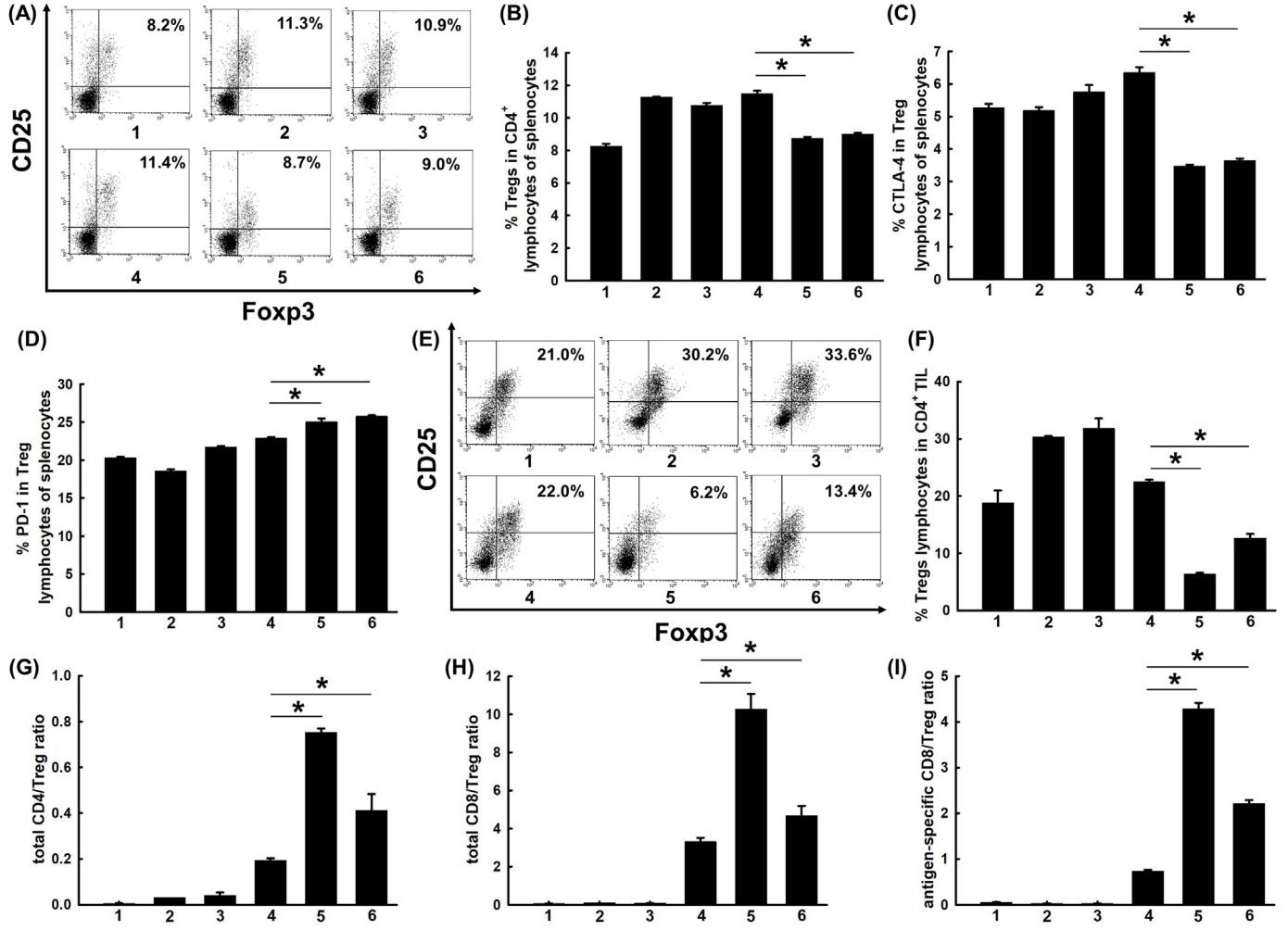

Fig. 3. Alteration of Tregs from the splenocytes and TILs of mice treated with CTGF/E7 DNA vaccine with or without anti-CTLA-4 or anti-PD-1 Ab. (A) Representative numbers of Tregs from the splenocytes on day 25 after tumor challenge of various groups by flowcytometric analysis (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). **(B)** Percentages of Tregs among the $CD4^+$ T lymphocytes from splenocytes from various groups (n = 5 per group, mean ± SEM). The percentage of Tregs was significantly lower in the CTGF/E7 DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups than the CTGF/E7 DNA vaccine alone group (p = 0.004, Kruskal-Wallis test). **(C)** The percentages of CTLA-4^+ Tregs from various groups (n = 5 per group, mean ± SEM). The percentage of CTLA-4^+ Tregs was lower in the DNA vaccine with anti-CTLA-4 Ab or anti-PD-1 Ab groups than the DNA vaccine alone group (p = 0.01, Kruskal-Wallis test). **(D)** The percentages of PD-1^+ Tregs from various groups (n = 5 per group, mean ± SEM). The percentage of PD-1^+ Tregs was higher in the DNA vaccine with anti-CTLA-4 Ab or anti-PD-1 Ab groups than the DNA vaccine alone group (p = 0.01, Kruskal-Wallis test). **(E)** Representative numbers of Tregs from TILs in various groups by flowcytometric analysis (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). **(F)** Percentages of Tregs from TILs in various groups (n = 5 per group, mean ± SEM). The percentage of tumor-infiltrating Tregs was significantly decreased in the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups compared to the DNA vaccine or Ab alone groups (p = 0.04, Kruskal-Wallis test). **(G)** The ratios of total $CD4^+$ T cells to Tregs in TILs of various groups. The ratio of total $CD4^+$ T cells to Tregs from TILs was significantly higher in the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups than the DNA vaccine alone group (p = 0.03, Kruskal-Wallis test). **(H)** The ratios of total $CD8^+$ T cells to Tregs in TILs of various groups. The ratio of total $CD8^+$ T cells to Tregs from TILs was significantly higher in the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups than the DNA vaccine alone group (p = 0.01, Kruskal-Wallis test). **(I)** The ratios of antigen-specific cytotoxic $CD8^+$ T cells to Tregs in TILs of various groups. The ratio of antigen-specific cytotoxic $CD8^+$ T cells to Tregs from TILs was significantly higher in the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups than the DNA vaccine alone groups (p = 0.001, Kruskal-Wallis test) (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). All experiments were performed independently in triplicate.

receptor and its ligand are expressed on other cells, including DCs and tumor cells. The representative of flow cytometric analysis of CD80, CD86, PD-1 or PD-L1 from BMM-derived $CD11c^+$ DCs are shown in Fig. 5D and F. A greater proportion of $CD80^-CD11c^+$ DCs (7.52 ± 0.16%) and $CD86^-CD11c^+$ DCs (10.2 ± 0.12%) expressed PD-1 than $CD80^+CD11c^+$ DCs (1.45 ± 0.03%) and $CD86^+CD11c^+$ DCs (1.67 ± 0.04%) ($CD80^-CD11c^+PD\text{-}1^+$ vs. $CD80^+CD11c^+PD\text{-}1^+$, p = 0.002; $CD86^-CD11c^+PD\text{-}1^+$ vs. $CD86^+CD11c^+PD\text{-}1^+$, p = 0.002, Mann-Whitney *U* test; Fig. 5E). In addition, a greater proportion of $CD80^-CD11c^+$ DCs (11.68 ± 0.1%) and $CD86^-CD11c^+$ DCs (9.03 ± 0.17%) expressed PD-L1 than $CD80^+CD11c^+$ DCs (4.08 ± 0.11%) and $CD86^+CD11c^+$ DCs (3.41 ± 0.04%) ($CD80^-CD11c^+PD\text{-}L1^+$ vs. $CD80^+CD11c^+PD\text{-}L1^+$, p = 0.002; $CD86^-CD11c^+PD\text{-}L1^+$ vs. $CD86^+CD11c^+PD\text{-}L1^+$, p = 0.002, Mann-Whitney *U* test; Fig. 5G). As shown in Fig. 5H, the PD-1 was not expressed on the TC-1 tumor cells. Whereas, the PD-L1, was highly expressed on the TC-1 tumor cells (Fig. 5I).

Our results revealed that immature DCs could express higher PD-1 and PD-L1 molecules than mature DCs in BMM-derived DCs. The PD-L1 could also be noted to express on the tumor cells.

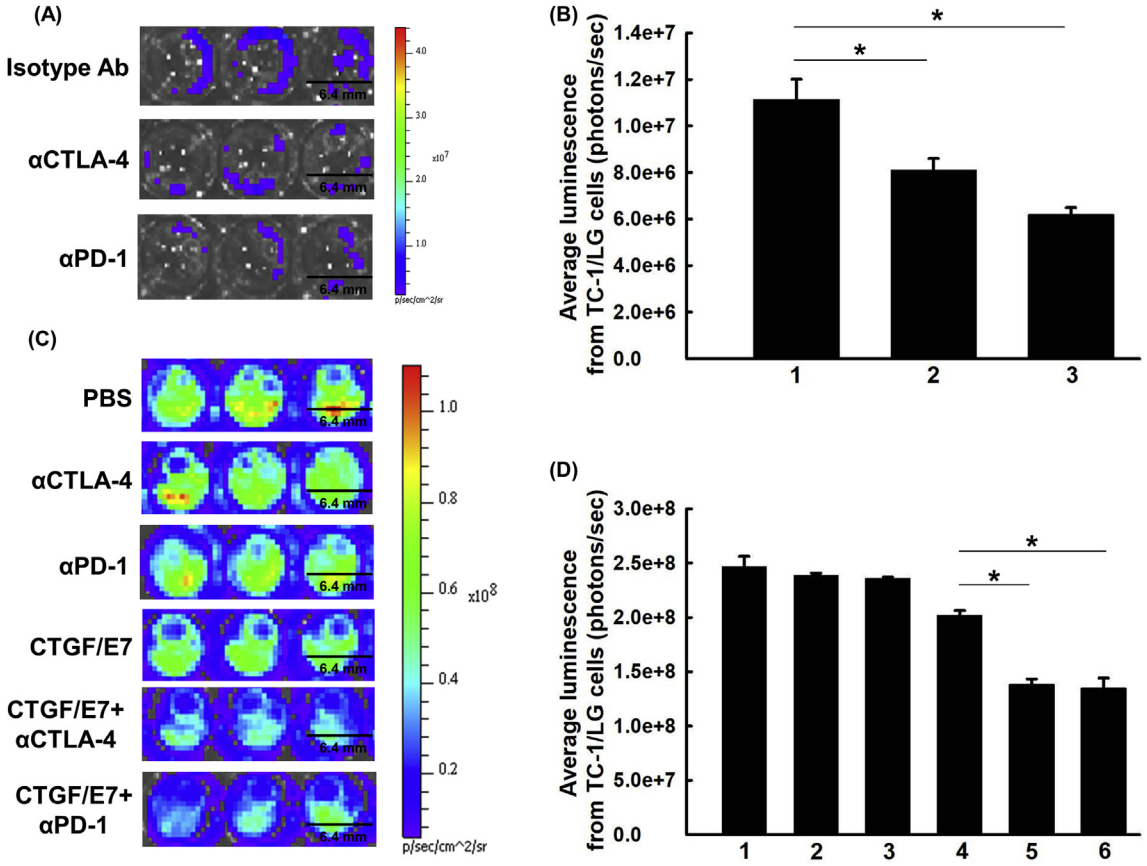

Fig. 4. ***In vitro*** **tumor specific killing activity of antigen-specific CD8$^+$ cytotoxic T cells or splenocytes from various immunized groups. (A)** Representative luminescence images of the *in vitro* tumor-specific killing activity of E7-specific CD8$^+$ cytotoxic T cells treated with or without anti-CTLA-4 or anti-PD-1 immune checkpoint Ab. **(B)** Quantification of the average luminescence of TC-1/LG cells in various groups (1: isotype Ab, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab). Anti-CTLA-4 or anti-PD-1 Ab significantly enhanced tumor killing activities of E7-specific CD8$^+$ cytotoxic T cells ($p = 0.03$, Kruskal-Wallis test). **(C)** Representative luminescence images of the *ex vivo* tumor-specific killing activity of splenocytes from mice vaccinated with CTGF/E7 DNA vaccine and treated with or without anti-CTLA-4 or anti-PD-1 Ab. **(D)** Quantification of the average luminescence of TC-1/LG cells co-cultured with splenocytes from various groups ($n = 5$ per group, mean ± SEM) (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). Anti-CTLA-4 or anti-PD-1 Ab significantly enhanced the tumor killing activities of E7-specific CD8$^+$ cytotoxic T lymphocytes from CTGF/E7 DNA vaccinated mice ($p = 0.04$, Kruskal-Wallis test). All experiments were performed independently in triplicate.

3.7. Anti-CTLA-4 and anti-PD-1 Abs enhance the maturation of DCs in LNs and tumors in vivo

To examine whether anti-CTLA-4 or anti-PD-1 Ab enhances the maturation of DCs *in vivo*, DCs were obtained from the inguinal LNs and tumors of mice in various groups. The representative results of flow cytometric analysis of CD80$^+$CD11c$^+$ and CD86$^+$CD11c$^+$ DCs from inguinal LNs are shown in Fig. 6A. The percentage of CD80$^+$CD11c$^+$ DCs was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (3.00 ± 0.10%) or anti-PD-1 Ab (4.71 ± 0.11%) groups than in the CTGF/E7 DNA vaccine alone group (2.32 ± 0.02%, $p < 0.001$, Kruskal-Wallis test; Fig. 6B). In addition, the percentage of CD86$^+$CD11c$^+$ DCs was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (3.63 ± 0.06%) or anti-PD-1 Ab (5.32 ± 0.02%) groups than in the CTGF/E7 DNA vaccine alone group (2.85 ± 0.01%, $p < 0.001$, Kruskal-Wallis test; Fig. 6C).

In the local tumor site, the representative results of flow cytometric analysis and immunofluorescent staining of tumor-infiltrating CD80$^+$CD11c$^+$ DCs are shown in Fig. 6D and Supplementary Fig. 1B, respectively. The percentage of tumor-infiltrating CD80$^+$CD11c$^+$ DCs was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (9.27 ± 0.08%) or anti-PD-1 Ab (6.23 ± 0.09%) groups than in the CTGF/E7 DNA vaccine alone group (3.59 ± 0.25%, $p < 0.001$, Kruskal-Wallis test; Fig. 6E). In addition, the percentage of tumor-infiltrating CD80$^+$CD11c$^-$ cells (other possible APCs) was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (27.4 ± 0.13%) or anti-PD-1 Ab (14.7 ± 0.08%) groups than in the CTGF/E7 DNA vaccine alone group (6.37 ± 0.41%, $p < 0.001$, Kruskal-Wallis test; Fig. 6F). The percentage of tumor-infiltrating CD86$^+$CD11c$^+$ DCs was significantly higher in the CTGF/E7 DNA vaccine with anti-PD-1 (8.35 ± 0.15%) or anti-CTLA-4 Ab (7.73 ± 0.08%) groups than in the DNA vaccine alone group (6.79 ± 0.15%, $p < 0.001$, Kruskal-Wallis test; Fig. 6G).

Thus, our results revealed that the immune checkpoint blockades could enhance the maturation of DCs and other possible APCs in tumor sites.

3.8. Anti-CTLA-4 or anti-PD-1 Ab enhances the activation of antigen-specific cytotoxic CD8$^+$ T cells from tumor-infiltrating DCs

Next, we evaluated whether the immune checkpoint blockades enhance the activation of antigen-specific cytotoxic CD8$^+$ T cells from tumor-infiltrating DCs. CD11c$^+$-enriched DCs were isolated using magnetic CD11c beads from TILs of mice with various treatment modalities and then tumor-infiltrating CD11c$^+$ DCs were co-cultured with the E7-specific CD8$^+$ cytotoxic T cells. To account for the variations of IFN-γ-secreting E7-specific CD8$^+$ cytotoxic T cells pulsed with tumor-infiltrating DCs of different groups, the fold change of E7-specific

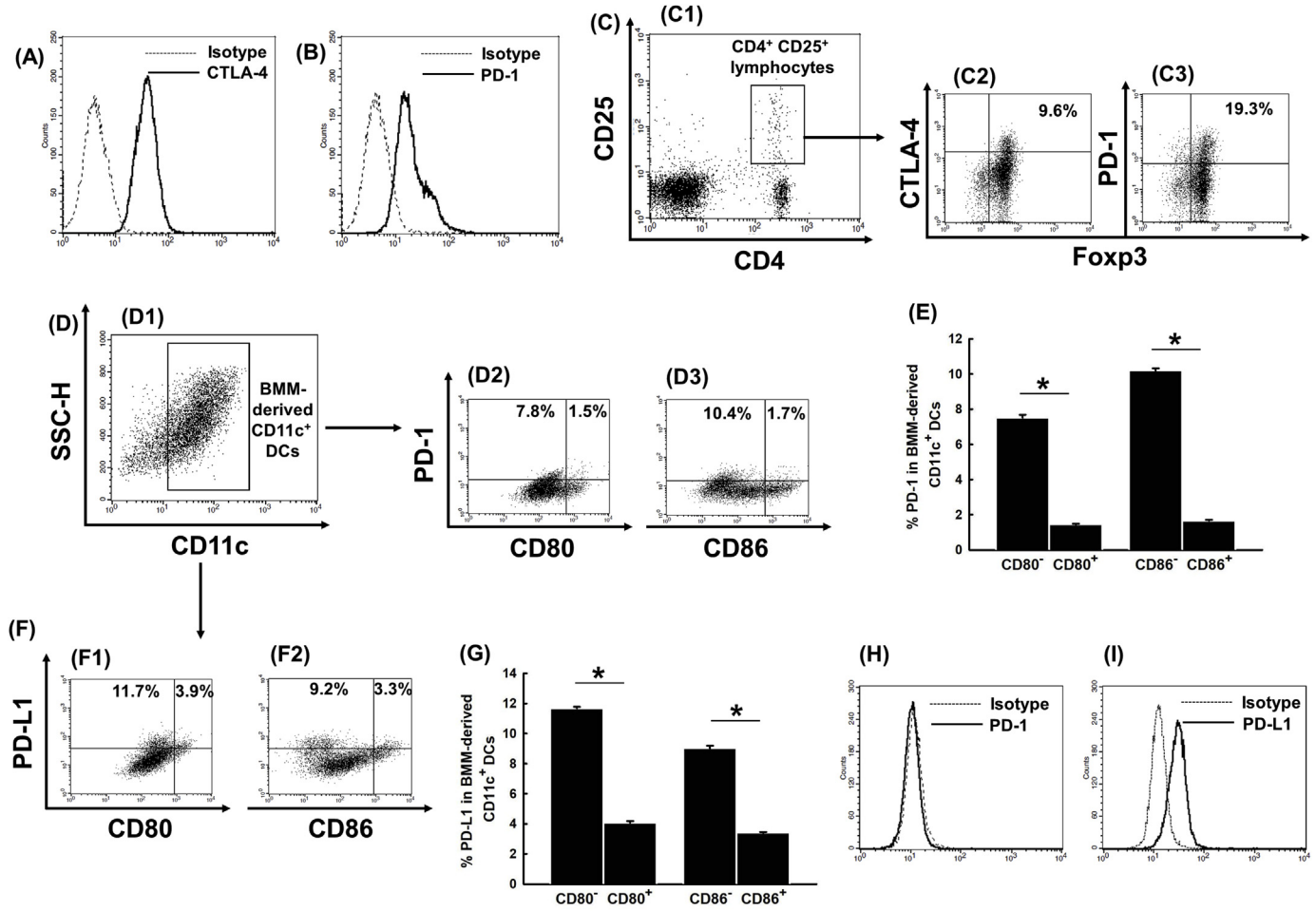

Fig. 5. Expression of CTLA-4, PD-1, and PD-L1 in E7-specific CD8$^+$ T cells, Tregs, BMM-derived DCs, and tumor cells. (A–B) Flow cytometric analysis of the expression of CTLA-4 and PD-1 in E7-specific T cells. CTLA-4 and PD-1 were both highly expressed on E7-specific T cells. **(C)** Expression of CTLA-4 and PD-1 in Tregs from splenocytes. Both CTLA-4 and PD-1 were expressed on Foxp3$^+$ Tregs. **(D–G)** Expression of PD-1/PD-L1 on BMM-derived DCs. Higher percentages of CD80$^-$CD11c$^+$ and CD86$^-$CD11c$^+$ DCs (immature DCs) expressed PD-1/PD-L1 than mature DCs ($p = 0.002$, Mann-Whitney *U* test). **(H–I)** The expression of PD-1/PD-L1 in TC-1 tumor cells. The TC-1 tumor cells expressed PD-L1 but not PD-1. All experiments were performed independently in triplicate.

IFN-γ-secreting CD8$^+$ cytotoxic T cells was further calculated. The average number of IFN-γ-secreting E7-specific CD8$^+$ cytotoxic T cells of PBS group was used as baseline. The fold changes of E7-specific IFN-γ-secreting CD8$^+$ cytotoxic T lymphocytes were significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (33.4 ± 0.52) and anti-PD-1 Ab (32.06 ± 0.42) groups than those in the CTGF/E7 DNA vaccine alone group (8.77 ± 0.06) and the other groups ($p = 0.004$, Kruskal-Wallis test; Fig. 6H).

Our results revealed that immune checkpoint blockades could augment the number of antigen-specific CD8$^+$ T cells by modulating tumor-infiltrating DCs.

3.9. Immune checkpoint blockades could also enhance the anti-tumor effects of MSLN-specific chimeric DNA vaccine

Finally, we evaluated whether immune checkpoint Abs could also enhance the anti-tumor effects of MSLN-specific DNA vaccine in MSLN-expressing tumor model. The protocols for *in vivo* therapeutic experiments were shown in Fig. 1B. Treatment was started when WF-3/LG tumors injected 3 days after tumor challenge. The luciferase activities of WF-3/LG tumor-bearing mice in various groups as detected by the IVIS system were shown in Fig. 7A. The CTGF/MSLN DNA vaccine combined with anti-CTLA-4 Ab ($4.6 \pm 0.4 \times 10^6$) or anti-PD-1 Ab ($4.7 \pm 0.4 \times 10^6$) exhibited the least luminescence of all the groups after 28 days of WF-3/LG tumor cell injection (PBS: $1.5 \pm 0.1 \times 10^7$, anti-CTLA-4 Ab: $1.4 \pm 0.1 \times 10^7$, anti-PD-1 Ab: $1.4 \pm 0.1 \times 10^7$, CTGF/MSLN alone: $1.2 \pm 0.1 \times 10^7$; $p < 0.001$, Kruskal-Wallis test, Fig. 7B). Sixty percent of the mice that received the CTGF/MSLN DNA vaccine with anti-CTLA-4 Ab or anti-PD-1 Ab were alive 100 days after WF-3/LG tumor challenge. Whereas, all of the mice in the other groups died less than 60 days after tumor challenge ($p = 0.005$, log-rank test, Fig. 7C).

Our results indicated that combination of MSLN antigen-specific chimeric DNA vaccine and immune checkpoint inhibitors could also generate more potent anti-tumor effects than DNA vaccine alone in MSLN-expressing tumor model.

3.10. Anti-PD-1 Ab enhances the maturation of BMM-derived DCs by upregulating CD80/86 and MHC I molecules

As both PD-1 and PD-L1 were expressed on mature and immature DCs (Fig. 5E and G), the maturation status of DCs may be inhibited through the interaction of PD-1 and PD-L1. We determined whether anti-PD-1 Ab enhances the maturation of BMM-derived DCs. The CD80/86 expression of DCs treated with anti-PD-1 Ab are shown in Supplementary Fig. 2A. The anti-PD-1 Ab-treated DCs had significantly higher percentages of CD11c$^+$ CD80$^+$ cells than the isotype Ab-treated group (25.88 ± 0.93% vs. 17.57 ± 0.16%, $p < 0.001$, Mann-Whitney *U* test; Supplementary Fig. 2B). In addition, the percentage of CD11c$^+$ CD86$^+$ cells was significantly higher in the anti-PD-1 Ab-

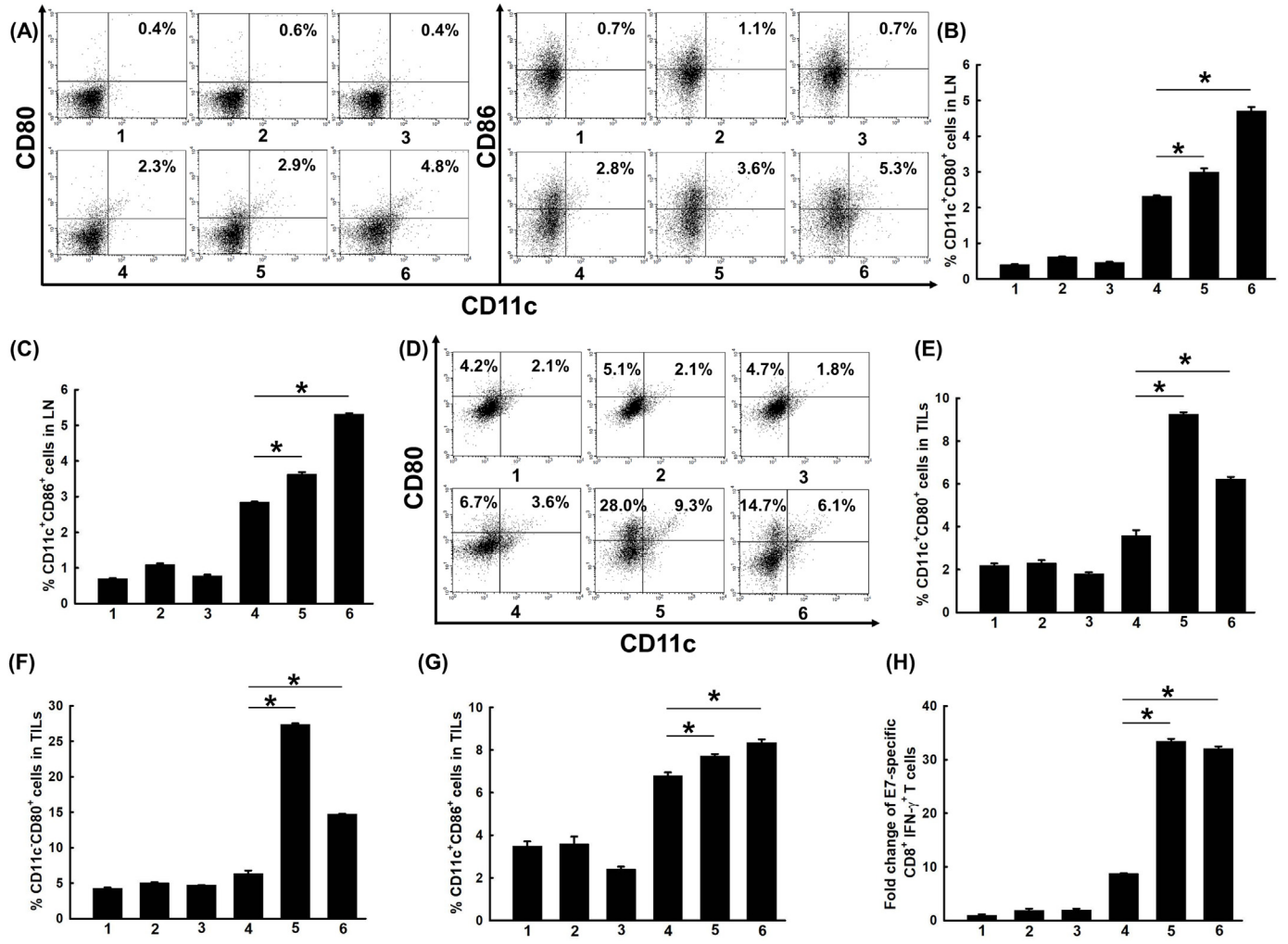

Fig. 6. Maturation status and function of dendritic cells (DCs) in lymph nodes and tumors of mice treated with CTGF/E7 DNA vaccine and/or immune checkpoint Ab. (A) Flow cytometric analysis of the proportion of $CD80^+CD11c^+$ or $CD86^+CD11c^+$ DCs in lymph nodes on day 25 after tumor challenge from various groups (1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab). **(B)** Percentages of $CD80^+CD11c^+$ DCs in lymph nodes from various groups (n = 5 per group, mean ± SEM). The CD80 maturation marker of $CD11c^+$ DCs significantly increased in lymph nodes from the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups compared to the DNA vaccine alone group ($p < 0.001$, Kruskal-Wallis test). **(C)** Percentages of $CD86^+$ $CD11c^+$ DCs in lymph nodes from various groups (n = 5 per group, mean ± SEM). The CD86 maturation marker of $CD11c^+$ DCs significantly increased in lymph nodes from the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups compared to the DNA vaccine alone group ($p < 0.001$, Kruskal-Wallis test). **(D)** Flow cytometric analysis of the proportions of $CD80^+CD11c^+$ DCs and $CD80^+CD11c^-$ antigen-presenting cells in tumors from various groups. **(E)** Percentages of $CD80^+CD11c^+$ DCs in tumors from various groups (n = 5 per group, mean ± SEM). The percentage of CD80-expressing $CD11c^+$ DCs was significant higher in tumors from the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups compared to the DNA vaccine alone group ($p < 0.001$, Kruskal-Wallis test). **(F)** Percentages of $CD80^+$ $CD11c^-$ cells in tumors from various groups (n = 5 per group, mean ± SEM). The percentage of CD80-expressing $CD11c^-$ cells was significantly higher in tumors from the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups compared to the DNA vaccine alone group ($p < 0.001$, Kruskal-Wallis test). **(G)** Percentages of $CD86^+CD11c^+$ DCs in tumors from various groups (n = 5 per group, mean ± SEM). The percentage of CD86-expressing $CD11c^+$ DCs was significantly higher in tumors from the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups compared to the DNA vaccine alone group ($p < 0.001$, Kruskal-Wallis test). **(H)** Fold changes in IFN-γ-secreting E7-specific $CD8^+$ cytotoxic T-cells co-cultured with $CD11c^+$-enriched cells isolated from the TILs of various vaccinated groups (n = 5 per group, mean ± SEM). The numbers of IFN-γ-secreting E7-specific $CD8^+$ cytotoxic T cells pulsed by tumor-infiltrating DCs of PBS group was defined as baseline. DCs from the DNA vaccine with anti-CTLA-4 or anti-PD-1 Ab groups stimulated significant fold changes of E7-specific $CD8^+$ IFN-γ secreting T cells than those from the DNA vaccine alone group ($p = 0.004$, Kruskal-Wallis test). Column1: PBS, 2: anti-CTLA-4 Ab, 3: anti-PD-1 Ab, 4: CTGF/E7 DNA vaccine, 5: CTGF/E7 DNA vaccine with anti-CTLA-4 Ab, 6: CTGF/E7 DNA vaccine with anti-PD-1 Ab. All experiments were performed independently in triplicate.

treated group than in the isotype Ab-treated group (24.83 ± 1.17% vs. 14.90 ± 0.17%, $p < 0.001$, Mann-Whitney *U* test; Supplementary Fig. 2C).

MHC class I and II expression in DCs treated with anti-PD-1 Ab is shown in Supplementary Fig. 2D. The anti-PD-1 Ab-treated DCs had significantly higher percentages of $CD11c^+$ MHC I^+ cells than the isotype Ab-treated group (21.63 ± 1.37% vs. 14.31 ± 0.69%, $p = 0.004$, Mann-Whitney *U* test; Supplementary Fig. 2E). However, no difference in the percentage of $CD11c^+$ MHC II^+ cells was found between the anti-PD-1 Ab and isotype Ab-treated groups (39.49 ± 0.52% vs. 40.80 ± 0.14%, $p = 0.32$, Mann-Whitney *U* test; Supplementary Fig. 2F).

Thus, our results indicated that anti-PD-1 Ab enhances the expression of surface markers of mature DCs.

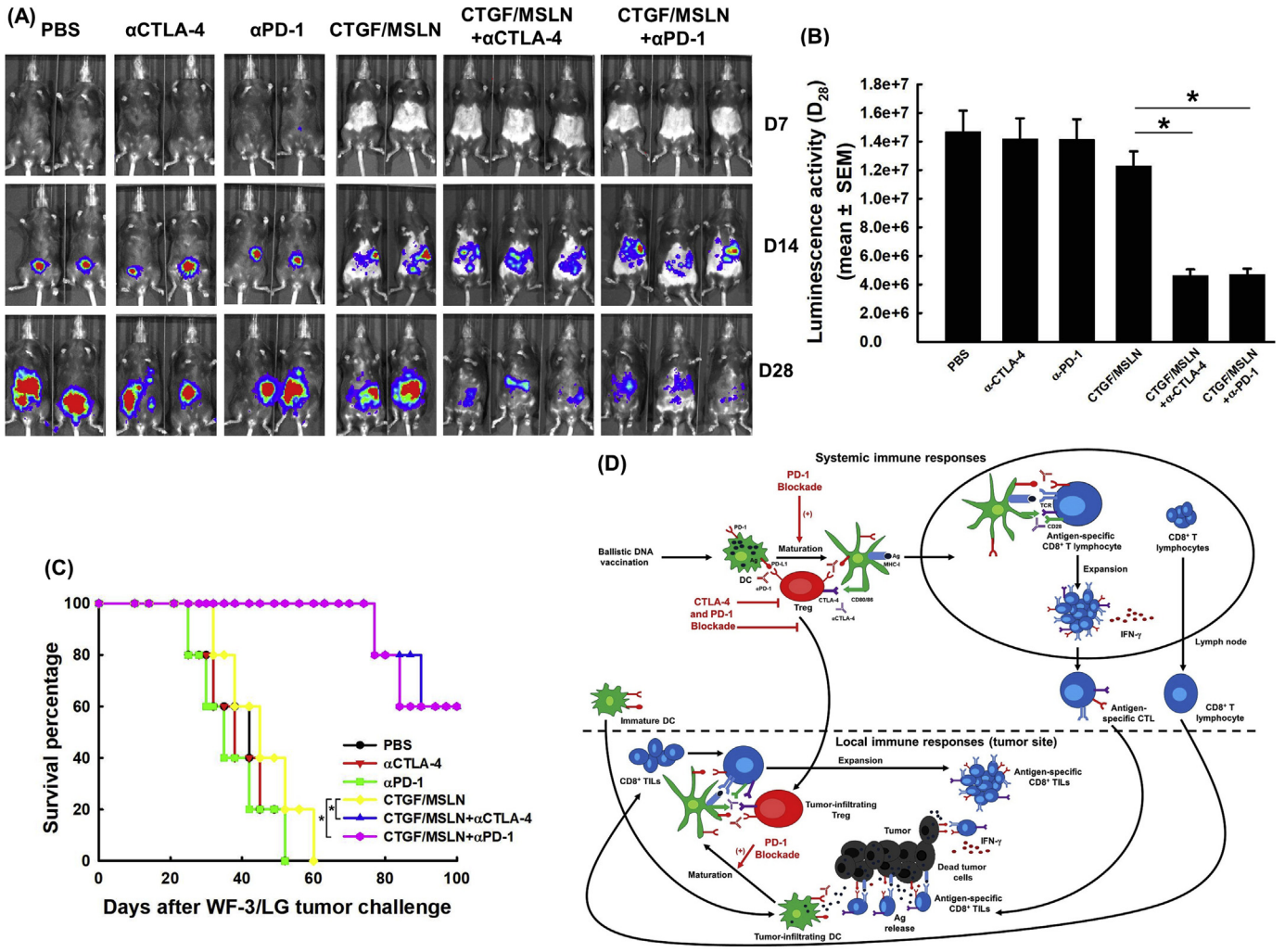

Fig. 7. Anti-tumor effects of CTGF/MSLN DNA vaccine with or without immune checkpoint inhibitor. (A) Representative luminescence images of WF-3/LG tumor-bearing mice in various groups using the IVIS system at indicated intervals. **(B)** Luminescences of WF-3/LG tumor-bearing mice in various groups (mean ± SEM). Mice immunized with the CTGF/MSLN DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Ab exhibited the least luminescence ($p < 0.001$, Kruskal Wallis test). **(C)** Overall survival of mice treated with CTGF/MSLN DNA vaccine with or without respective Ab. Sixty percent of mice that received CTGF/MSLN DNA vaccine combined with anti-CTLA-4 Ab or anti-PD-1 Ab were alive 100 days after WF-3/LG tumor challenge. None of the mice in CTGF/MSLN DNA vaccine alone group could survive more than 60 days after tumor challenge ($p = 0.005$, log-rank test). All experiments were performed independently in duplicate. **(D)** Schematic diagram shows more potent anti-tumor effects of antigen-specific DNA vaccine combined with PD-1 or CTLA-4 blockade. Anti-CTLA-4 and PD-1 Abs enhanced the antigen-specific $CD8^+$ T cell responses through targeting antigen-specific $CD8^+$ T cells and Tregs. In addition, PD-1 blockade could promote the maturation of DCs through targeting PD-1/PD-L1 pathway between DCs and tumor cells.

3.11. Combination of DNA vaccine with immune checkpoint blockades does not induce significant changes of histopathology and body weight in mice

To further evaluate whether CTGF/E7 DNA vaccine combined with immune checkpoint blockades induced the toxicity of the mice. Various organs of the mice in various groups were examined. No significant or specific tissue damages or immune responses noted in these organs of the mice in different groups (Supplementary Fig. 3A). Besides, there was no significant changes of the body weight of mice in various groups (Supplementary Fig. 3B).

Thus, our results revealed that the strategy of DNA vaccine combine with immune checkpoint blockades was well tolerated without definite serious toxicities to the mice.

3.12. DNA vaccine combined with anti-CTLA-4 or anti-PD-1 Abs could enhance the expression of Ki-67 activation marker on the T lymphocytes of TILs

We further examined where the frequencies of activated $CD4^+$ and $CD8^+$ T cells in the TILs of DNA vaccine combined with immune checkpoint blockades were higher than those of DNA vaccine. So the T cell activation markers such as Ki-67 and CD69 were stained. The percentage of tumor-infiltrating Ki-67^+CD4^+ T cells was significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (5.92 ± 0.13%) or anti-PD-1 Ab (5.87 ± 0.11%) groups than in the DNA vaccine alone group (4.19 ± 0.08%, $p = 0.003$, Kruskal-Wallis test; Supplementary Fig. 4A). However, the percentage of CD69 (DNA: 6.95 ± 0.04%, DNA with anti-CTLA-4Ab: 7.02 ± 0.03%, DNA with anti-PD-1 Ab: 6.96 ± 0.04%, $p = 0.38$, Kruskal-Wallis test; Supplementary Fig. 4B) of tumor-infiltrating $CD4^+$ T cells was no difference in the three groups. In addition, the percentage of tumor-infiltrating Ki-67^+CD8^+ T cells was also significantly higher in the CTGF/E7 DNA vaccine with anti-CTLA-4 (39.65 ± 1.3%) or anti-PD-1 Ab (39.67 ± 0.97%) groups than in the DNA vaccine alone group (30.75 ± 0.88%, $p = 0.003$, Kruskal-Wallis test; Supplementary Fig. 4C). The percentage of CD69 (DNA: 56.83 ± 0.54%, DNA with anti-CTLA-4 Ab: 58.15 ± 0.12%, DNA with anti-PD-1 Ab: 58.04 ± 0.08%, $p = 0.11$, Kruskal-Wallis test; Supplementary Fig. 4D) of tumor-infiltrating $CD8^+$ T cells was no

difference in these three groups, either.

Our results indicated that immune checkpoint blockades could enhance the activation of tumor-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes of the TILs by increasing Ki-67 expressions.

3.13. Both of $CD8^+$ T cells and Tregs could highly express PD-1 and CTLA-4 molecules in TILs

Both of the CTLA-4 and PD-1 molecules were highly expressed on E7-specific $CD8^+$ cytotoxic T cells and Tregs in the splenocytes of mice treated with CTGF/E7 DNA vaccine alone (Fig. 5A–C). So we further examined whether E7-specific cytotoxic $CD8^+$ T cells and Tregs in tumors of mice treated with DNA vaccine could also express CTLA-4 and PD-1 molecules. As shown in Supplementary Fig. 5A, there were 8.3% and 12.7% of E7-specific cytotoxic $CD8^+$ T cells of TILs expressed CTLA-4 and PD-1 molecules, respectively. There were 9.9% and 34.7% of Tregs of TILs expressed CTLA-4 and PD-1, respectively (Supplementary Fig. 5B).

Consequently, our results indicated that antigen-specific cytotoxic $CD8^+$ T cells and Tregs in tumors could express both of PD-1 and CTLA-4 molecules.

3.14. Higher percentages of immature DCs express PD-1 and PD-L1 molecules than mature DCs in TILs

Because higher percentages of immature BMM-derived DCs could express PD-1 and PD-L1 molecules than mature BMM-derived DCs (Fig. 5D–G), we further investigated whether the similar phenomena could be observed in TILs of tumors in mice treated with CTGF/E7 DNA vaccine. A greater proportion of $CD80^-CD11c^+$ DCs (52.65 ± 0.17%) and $CD86^-CD11c^+$ DCs (53.24 ± 0.05%) expressed PD-1 than $CD80^+CD11c^+$ DCs (5.44 ± 0.13%) and $CD86^+CD11c^+$ DCs (4.85 ± 0.02%) in tumors ($p = 0.03$, Mann-Whitney *U* test; Supplementary Fig. 5C). A greater proportion of $CD80^-CD11c^+$ DCs (23.41 ± 0.52%) and $CD86^-CD11c^+$ DCs (35.24 ± 0.60%) also expressed PD-L1 than $CD80^+CD11c^+$ DCs (7.67 ± 0.20%) and $CD86^+CD11c^+$ DCs (5.94 ± 0.19%) in tumors ($p = 0.002$, Mann-Whitney *U* test; Supplementary Fig. 5D).

Our results revealed that higher percentages of immature DCs could express both of PD-1 and PD-L1 molecules than mature DCs in tumors.

3.15. Immune checkpoint blockades could enhance the numbers of antigen-specific $CD8^+$ TILs generated by antigen-specific chimeric DNA vaccine

Because immune checkpoint inhibitor enhanced the numbers of E7-specific IFN-γ-secreting $CD8^+$ cytotoxic T cells in tumors (Fig. 2I), we further examined whether similar phenomena could be observed in tumors by staining HPV-16 E7 tetramer. Representative figures of flow cytometric analysis of tetramer staining E7-specific $CD8^+$ T cells from TILs are shown in Supplementary Fig. 5E. The numbers of E7-specific $CD8^+$ cytotoxic T cells from TILs significantly increased in mice treated with CTGF/E7 DNA vaccine with anti-CTLA-4 (5064.0 ± 56.9) and anti-PD-1 Ab (4834.7 ± 37.4) groups compared with those treated with DNA vaccine alone group (3256.0 ± 11.5) ($p = 0.004$, Kruskal-Wallis test; Supplementary Fig. 5F).

Our results revealed that anti-CTLA-4 Ab or anti-PD-1 Ab could enhance the numbers of antigen-specific $CD8^+$ T cells in the tumors of mice treated with antigen-specific DNA vaccine.

3.16. Anti-CTLA-4 Ab does not induce the maturation, antigen-processing, and antigen-presenting activities of BMM-derived DCs

Because DNA vaccine combined with anti-CTLA-4 Ab could enhanced the DC maturation compared to the DNA vaccine alone *in vivo* (Fig. 6). We further evaluated whether the blockade of CTLA-4 could directly enhance the maturation status, antigen-processing, and antigen-presenting activities of BMM-derived DCs *in vitro*. Only 1.3% of BMM-derived $CD11c^+$ DCs expressed CTLA-4 molecule at baseline (Supplementary Fig. 6A). The percentages of $CD11c^+CD80^+$ or $CD11c^+CD86^+$ cells were no difference between the anti-CTLA-4 Ab and isotype Ab-treated groups ($CD11c^+CD80^+$: 25.39 ± 1.41% vs. 25.14 ± 0.66%, $p = 0.94$; $CD11c^+CD86^+$: 27.56 ± 1.50% vs. 27.21 ± 0.74%, $p = 0.94$, both by Mann-Whitney *U* test; Supplementary Figs. 6B–C).

The percentages of MHC class I and II in DCs treated with anti-CTLA-4 Ab are shown in Supplementary Figs. 6D and 6E. The percentages of $CD11c^+$MHC I^+ or $CD11c^+$MHC II^+ cells were no difference between the anti-CTLA-4 Ab and isotype Ab-treated groups ($CD11c^+$MHC I^+: 19.22 ± 0.13% vs. 20.41 ± 0.11%, $p = 0.14$; $CD11c^+$MHC II^+: 48.66 ± 0.05% vs. 47.59 ± 2.03%, $p = 0.79$, both by Mann-Whitney *U* test).

The percentages of FITC-$OVA_{257–264}$ short peptide pulsed-DCs treated with anti-CTLA-4 Ab were no difference as compared with those treated with isotype Ab ($p = 0.14$, Mann-Whitney *U* test; Supplementary Fig. 6F). The percentages of FITC-conjugated $OVA_{323-339}$ long peptide pulsed-DCs treated with anti-CTLA-4 Ab were no difference compared with those treated with isotype Ab, either ($p = 0.25$, Mann-Whitney *U* test; Supplementary Fig. 6G).

Our results revealed that anti-CTLA-4 Ab does not induce the maturation and function of BMM-derived DCs. DNA vaccine with blockade of CTLA-4 enhancing the maturation and function of DCs may be mediated by suppression of Tregs.

4. Discussion

Our results reveal that immune checkpoint blockade, such as anti-CTLA-4 Ab or anti-PD-1 Ab, enhances the antigen-specific anti-tumor effects, and cell-mediated and humoral immunities, of an antigen-specific DNA vaccine than the DNA vaccine alone. The immune checkpoint inhibitors decreased the number of Tregs in both splenocytes and TILs. In addition, anti-CTLA-4 Ab and anti-PD-1 Ab enhanced the maturation and antigen-presenting abilities of DCs *in vivo,* promoting the anti-tumor activities of the antigen-specific DNA vaccine.

Antigen-specific chimeric DNA vaccines, including CTGF/E7 DNA vaccine could generate potent antigen-specific immunity and anti-tumor effects to control small tumors [16]. However, the CTGF/E7 DNA vaccine alone is not potent enough to control established tumors in a subcutaneous tumor model (Fig. 1C and D). Therefore, a new combinational strategy is needed to enhance the anti-tumor effects of the antigen-specific chimeric DNA vaccine.

Tumor cells could downregulate the amplitude of T cell activation and suppress anti-tumor immunity through the CTLA-4 and PD-1 pathways [27]. Like the previous report [27], the antigen-specific $CD8^+$ T cells and Tregs expressed both CTLA-4 and PD-1 (Fig. 5A–C). Anti-CTLA-4 Ab or anti-PD-1 Ab enhanced the antigen-specific tumor killing effects *in vitro* and *ex vivo* in this study (Fig. 4). PD-L1 can be upregulated by oncogenes, such as activated AKT and STAT3 [28], or IFN-γ secreted by activated anti-tumor T cells [29,30]. It is expressed mainly on the surface of tumor cells in many different cancer types and inhibits anti-tumor T cell immunity [31]. PD-L1 was also detected on TC-1 tumor cells in our survey (Fig. 5I). Therefore, inhibition of immune checkpoint molecules, such as CTLA-4, PD-1, and PD-L1 blockades could promote anti-tumor effects by inhibiting the immune-suppressive effects.

The poorly immunogenic cancers, such as highly progressive mammary carcinoma, SM1 [32], and highly tumorigenic murine melanoma, B16-BL6 [33], could respond to anti-CTLA-4 Ab combined with cell-based vaccine. Peptide vaccines targeting E7 antigen combined with modulation of immune checkpoint molecules such as anti-PD-1 Ab have been shown more potent anti-tumor effects than E7 peptide vaccine alone in HPV-associated cancer model [34,35]. Duperret et al. also reported that combination therapy of DNA vaccine with anti-CTLA-4 Ab

or anti-PD-1 Ab enhanced more robust anti-tumor effects than DNA vaccine alone [8]. Therefore, an antigen-specific DNA vaccine combined with immune checkpoint inhibitor may have potential to produce potent antigen-specific anti-tumor immunities and effects to control larger tumors. As demonstrated in this current study, the antigen-specific DNA vaccine combined with anti-PD-1 Ab or anti-CTLA-4 Ab could generate more potent antigen-specific anti-tumor immunities and effects than DNA vaccine alone for treating established tumors (Figs. 1–2).

Immune checkpoint inhibitor could suppress immuno-suppressive lymphocytes, such as Tregs, which are crucial in suppression of both autoimmune and anti-tumor immune responses [36,37]. Our previous study demonstrated that depletion of Tregs corrects the imbalance of immunologic profiles between pro- and anti-tumor immunities and generates more potent anti-tumor effects *in vivo* [26]. In addition, previous studies showed that targeting Tregs with an immune checkpoint Ab, such as anti-CTLA-4 Ab [38,39] or anti-PD-1 Ab [40], enhances anti-tumor immunities. Recently, Kurose et al. showed that depletion of Tregs by infusion of anti-CCR4 Ab, KW-0761, was safe and well tolerated in a phase I clinical trial in solid cancer patients [41]. In the present study, the anti-CTLA-4 Ab and anti-PD-1 Ab reduced the number of Tregs systemically (i.e., splenocytes) and locally (i.e., TILs), and enhanced the potency of the antigen-specific DNA vaccine (Fig. 3).

Because the $CD8^+$ cytotoxic T cell tested in Fig. 4B was E7 antigen-specific $CD8^+$ cytotoxic T cell line, so immune checkpoint blockade could be more effective to enhance the killing effects to the E7-expression TC-1/LG tumor cells than isotype Ab treated group. However, the splenocytes of mice treated with PBS, anti-CTLA-4 Ab alone, or anti-PD-1 Ab alone groups in Fig. 4D had fewer E7 antigen-specific $CD8^+$ cytotoxic T cells (Fig. 2E), so the tumor-killing effect might be abolished in the *ex vivo* experiments.

Immune checkpoint inhibitors could enhance the maturation and function of DCs. The CTLA-4 molecule expressed on T cells, including Tregs, has a higher affinity for the counterreceptor of the DC maturation molecules CD80 and CD86, and outcompetes CD28 costimulation for the activation of T lymphocytes [1,42,43]. In the present study, CTLA-4 blockade rescued the maturation and function of DCs in LNs and tumors (Fig. 6). In addition, PD-L1 was expressed on DCs (Fig. 5G and supplementary Fig. 5D). Thus, tumor cells and DCs could interact with antigen-specific $CD8^+$ T cells to down-regulate the anti-tumor immune responses through the PD-1 and PD-L1 interaction. Tumor cells could also induce the expression of PD-1 on tumor-infiltrating DCs to suppress DC-mediated anti-tumor immunities [44,45]. As shown in the present study, PD-1 blockade could enhance the maturation of BMM-derived DCs and the maturation and function of DCs in LNs and the tumor microenvironment. In addition, no difference between the maturation status of BMM-derived DCs treated with or without isotype Ab (data not shown), which may exclude the activation of DCs via FcR pathway. Thus, the blockade of CTLA-4 or PD-1 could enhance anti-tumor immunity through the modulation of DCs.

As shown in Fig. 7D, CTLA-4 and PD-1 blockades enhanced the antigen-specific $CD8^+$ T cell responses through targeting antigen-specific $CD8^+$ T and Tregs. PD-1 blockade had additional role to promote DCs maturation through targeting PD-1/PD-L1 pathway of DCs and tumor cells. Therefore, immune checkpoint blockade of CTLA-4 or PD-1 combined with an antigen-specific DNA vaccine generates more potent antigen-specific effector T cell responses by enhancing the maturation and functions of DCs and decreasing immuno-suppressive Tregs. Our results provide a novel strategy for developing antigen-specific cancer immunotherapies and treatment.

Ethics approval

Animal handling and procedures were approved by the animal ethic committee of College of Medicine, National Taiwan University.

Conflicts of interest

No potential conflicts of interest was disclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.039.

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