ORIGINAL ARTICLE

Immunologic Significance of CD80/CD86 or Major Histocompatibility Complex-II Expression in Thymic Epithelial Tumors

Hideki Ikeda, MD,[a,b,c] Joji Nagasaki, MD, PhD,[a,d] Daiki Shimizu, MD,[e] Yuki Katsuya, MD, PhD,[f] Hidehito Horinouchi, MD, PhD,[c] Yukio Hosomi, MD, PhD,[g] Etsuko Tanji, MS,[a] Takekazu Iwata, MD, PhD,[e] Makiko Itami, MD, PhD,[h] Masahito Kawazu, MD, PhD,[a] Yuichiro Ohe, MD, PhD,[c] Takuji Suzuki, MD, PhD,[b] Yosuke Togashi, MD, PhD[a,d,*]

[a]*Chiba Cancer Center, Research Institute, Chiba, Japan*
[b]*Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, Japan*
[c]*Department of Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan*
[d]*Department of Tumor Microenvironment, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan*
[e]*Division of Thoracic Surgery, Chiba Cancer Center, Chiba, Japan*
[f]*Department of Experimental Therapeutics, National Cancer Center Hospital, Tokyo, Japan*
[g]*Department of Thoracic Oncology and Respiratory Medicine, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan*
[h]*Department of Surgical Pathology, Chiba Cancer Center, Chiba, Japan*

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ABSTRACT

Introduction: Unresectable or recurrent thymic epithelial tumors (TETs) have a poor prognosis, and treatment options are limited. This study aimed to investigate the immunologic significance of CD80/CD86 or major histocompatibility complex class II (MHC-II) expression in TETs, as potential predictive biomarkers for immune checkpoint inhibitors (ICIs).

Methods: We analyzed CD80, CD86, MHC class I (MHC-I), and MHC-II expression in TETs using immunohistochemistry and investigated their association with T-cell infiltration or ICI efficacy. In addition, we generated CD80- or MHC-II–expressing mouse tumors, evaluated the effects of ICIs, and analyzed tumor-infiltrating lymphocytes. We also performed tumor-rechallenge experiments in vivo.

Results: We found that approximately 50% and 30% of TETs had high expression of CD80/CD86 and MHC-II in tumor cells, respectively, and that this expression was related to T-cell infiltration in clinical samples. In mouse models, both CD80 and MHC-II increase the effects of ICIs. In addition, senescent T cells and long-lived memory precursor effector T cells were significantly decreased and increased, respectively, in tumor-infiltrating lymphocytes from CD80-expressing tumors, and rechallenged tumors were completely rejected after the initial eradication of

*Corresponding author.

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Address for correspondence: Yosuke Togashi, MD, PhD, Department of Tumor Microenvironment, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. E-mail: ytogashi1584@gmail.com

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CD80-expressing tumors by programmed cell death protein 1 blockade. Indeed, patients with CD80-high thymic carcinoma had longer progression-free survival with anti–programmed cell death protein 1 monoclonal antibody.

Conclusions: Half of the TETs had high expression of CD80/CD86 or MHC-II with high T-cell infiltration. These molecules could potentially increase the effects of ICIs, particularly inducing a durable response. CD80/CD86 and MHC-II can be predictive biomarkers of ICIs in TETs, promoting the development of drugs for such TETs.

Keywords: Thymic epithelial tumor; Cancer immunotherapy; CD80/CD86; MHC; Memory precursor effector T cell

Introduction

Thymic epithelial tumors (TETs), which are divided into thymomas and thymic carcinomas, are relatively rare thoracic malignancies. Although surgical resection of early stage TETs has a good prognosis, with a 10-year overall survival (OS) rate of 80%, unresectable or recurrent TETs have a poor prognosis despite combined systemic chemotherapy.[1–6] Although the efficacy of the anti-angiogenic drug lenvatinib in patients with advanced thymic carcinomas has been revealed by the results of the phase 2 REMORA trial,[7] treatment options are limited and more effective therapies are warranted.

Immune checkpoint inhibitors (ICIs) such as programmed cell death protein-1 (PD-1) or programmed death-ligand 1 (PD-L1) blockade therapies exhibit efficacy through reinvigoration of $CD8^+$ effector T cells in the tumor microenvironment (TME) and have improved OS in various types of cancer.[8–10] Efficacy, which has been found in lung cancer,[11–13] is also expected in TETs, and several clinical trials have been conducted.[14–17] Although noteworthy responses have been reported in a fraction of patients, not all patients responded, and severe immune-related adverse events (irAEs) have been observed in some patients.[14–17] Therefore, predictive biomarkers are urgently required to identify responders.

The thymus is the site of T-cell maturation and differentiation and is the center of adaptive immunity.[18,19] Normal thymic epithelial cells express CD80 and CD86, which regulate T-cell activation by binding to the co-stimulatory molecule CD28.[20] Normal thymic epithelial cells also express abundant major histocompatibility complex (MHC) class II (MHC-II) and are involved in T-cell maturation and differentiation.[21,22] Considering their origin, these molecules may be expressed in TETs, unlike other solid tumors.[23,24] As these molecules can activate antitumor T-cell responses, ICIs can be more effective in CD80/CD86- or MHC-II–expressing TETs. Thus, this study was conducted to elucidate immunologic significance of CD80/86 and MHC-II expressed in TETs. In addition, we investigated their roles as potential biomarkers for anti–PD-1 monoclonal antibody in patients with TETs given their immunologic roles.

Materials and Methods

Patients and Samples

A total of 86 patients with TET who underwent surgical resection at Chiba Cancer Center between 1999 and 2020 and seven patients with thymic carcinoma who received nivolumab (3 mg/kg) after platinum-based chemotherapy in the PRIMER study[16] were enrolled in this study (Table 1 and Supplementary Tables 1–3). Patients' clinical information was obtained from their medical records. The study design was approved by the appropriate ethics review board at Chiba Cancer Center (R04-011), National Cancer Center (2021-384). This study was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained by opting out on the website of our institution.

Immunohistochemistry

For immunohistochemistry (IHC), we used formalin-fixed, paraffin-embedded (FFPE) samples at the diagnosis. The FFPE sections (3 μm) were deparaffinized with xylene, rehydrated, and subjected to antigen retrieval in a microwave oven for 20 minutes. After inhibition of endogenous peroxidase activity, individual slides were incubated overnight at 4°C with anti-CD4 monoclonal antibody (SP35; Abcam, Cambridge, UK), anti-CD8 monoclonal antibody (SP16, Abcam), anti-CD80 monoclonal antibody (EPR1157(2); Abcam), anti-CD86 monoclonal antibody (PA5-88284; Invitrogen, Waltham, MA), anti–MHC-A, -B, -C monoclonal antibody (EMR8-5; HKD, Hokkaido, Japan), and anti–MHC-DP, MHC-DQ, MHC-DR monoclonal antibody (CR3/43, Dako, Santa Clara, CA). The slides were then incubated with EnVision reagent (Dako), and the color reaction was developed using 2% 3,3-diaminobenzidine (DAB) in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen peroxidase. High CD80, CD86, MHC-I, and MHC-II expression in tumor cells was defined as greater than 50% positivity in tumor cells, as previously described.[25,26] Intratumoral $CD4^+$ and $CD8^+$ T cells were counted in four fields (0.0625 mm^2) containing tumor cells that were randomly selected and counted on each slide. The average of the four area counts for each patient was used for statistical analysis.

Table 1. Patient Characteristics (N = 86)

Features	CD80/CD86			MHC-II		
	Low (n = 43)	High (n = 43)	P	Low (n = 54)	High (n = 32)	p
Age, y [median] (range)	64 (32-80)	65 (24-86)	0.43	65 (43-86)	63 (24-80)	0.099
Sex (male/female)	24/19	22/21	0.67	31/23	15/17	0.34
Performance status (0 or 1/2 or 3)	41/2	40/3	0.64	51/3	30/2	0.89
WHO classification (A/AB/B1/B2/B3/C)	3/14/6/10/0/10	5/10/5/11/11/1	0.064[a]	4/15/8/15/6/6	4/9/3/6/5/5	>0.99[a]
Masaoka stage (I or II/III or IV)	28/15	31/12	0.64	39/15	20/12	0.35
Double cancer (yes/no)	12/31	14/29	0.64	18/36	8/24	0.47
Myasthenia gravis (yes/no)	4/39	1/42	0.17	4/50	1/31	0.65
Previous treatment (yes/no)	4/39	5/38	>0.99	5/49	4/28	0.72
CD4 [median] (range)	197.8 (1.25-521.8)	258.8 (10.5-539.8)	0.033	192.8 (1.25-539.8)	318.1 (1.5-531)	0.037
CD8 [median] (range)	149.5 (3.75-462.8)	338.3 (7.5-564)	0.025	251.3 (3.75-482.5)	267.1 (7.5-564)	0.68
CD80/CD86 (low/high)	-	-	-	23/31	20/12	0.074
MHC-I (low/high)	2/41	2/41	>0.99	4/50	0/32	0.11
MHC-II (low/high)	23/20	31/12	0.074	-	-	-

Note: The relationships between groups were compared using Fisher's exact test or a *t* test.
[a]A/AB/B1 versus B2/B3.
MHC, major histocompatibility complex; performance status, Eastern Cooperative Oncology Group performance status.

Data Sets

The Cancer Cell Line Encyclopedia (CCLE) data sets were used to evaluate CD80, CD86, and MHC-II (HLA-DRA and HLA-DRB1) mRNA expression in various cancer cell lines.[27–29]

Cell Lines and Reagents

The E.G7 (mouse thymic T-cell lymphoma, RRID: CVCL_3505) cell line was purchased from ATCC (Manassas, VA). The MC-38 cell line (mouse colon cancer, RRID: CVCL_B288) was obtained from Kerafast (Boston, MA). These cell lines were maintained in RPMI medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal calf serum (Cytiva, Tokyo, Japan). All tumor cells were used after confirming that they were *Mycoplasma* (-) by *Mycoplasma* testing using the polymerase chain reaction Mycoplasma Detection Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The rat anti-mouse PD-1 monoclonal antibody (RMP1-14; Bio X Cell, Lebanon, NH), anti–CTLA-4 monoclonal antibody (UC10-4F10-11; Bio X Cell), control rat IgG2a monoclonal antibody (RTK2758), and control rat IgG1κ monoclonal antibody (RTK2071) were obtained from BioLegend (San Diego, CA).

Constructs, Virus Production, and Transfection

Mouse *Cd80* or *Ciita* cDNA was subcloned into pBABE-puro (Addgene, Watertown, MA), which was transfected into a packaging cell line using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, MA). After 48 hours, the supernatant was concentrated and transfected into cell lines (E.G7 and MC-38). The expression of CD80 and MHC-II was evaluated using flow cytometry.

In Vivo Animal Models

Female C57BL/6J mice (6–8 wk old) were purchased from SLC Japan (Shizuoka, Japan). C57BL/6J-Prkdc<scid>/Rbrc mice (B6 SCID; RBRC01346) were provided by RIKEN BRC (Tsukuba, Japan) through the National BioResource Project of MEXT/AMED, Japan. E.G7 cells (4×10^6) or MC-38 cells (1×10^6) were subcutaneously inoculated, and tumor volume was monitored every 3 days. The means of the long and short diameters were used to generate the tumor growth curves. Anti–PD-1 monoclonal antibody (200 μg/mouse), anti–CTLA-4 monoclonal antibody (100 μg/mouse), or control monoclonal antibody was administered intraperitoneally three times every 3 days thereafter. Tumors were harvested 14 days after tumor cell inoculation to collect tumor-infiltrating lymphocytes (TILs) for evaluation using flow

cytometry. All in vivo experiments were performed at least twice. Furthermore, we performed rechallenge mouse experiments. Briefly, mice that had completely eradicated the initial tumors after anti–PD-1 monoclonal antibody were secondarily challenged with parental tumor cells on day 32. All the mice were maintained under specific pathogen-free conditions at the animal facility of the Institute of Biophysics. Mouse experiments were approved by the Animal Committee for Animal Experimentation of the Chiba Cancer Center. All experiments met the National Research Council's Guide for the Care and Use of Laboratory Animals.

Flow Cytometry Analyses

Flow cytometry was performed as described.[30] Briefly, the cells were washed with phosphate-buffered saline containing 2% fetal calf serum and stained with surface antibodies. Intracellular staining was performed with specific antibodies and a FOXP3/transcription factor staining buffer set (Thermo Fisher Scientific) according to the manufacturer's instructions. For intracellular staining, the cells were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) and ionomycin (2 μg/mL) (Sigma Aldrich, St. Louis, MO). GolgiPlug reagent (1.3 μL/mL) (BD Biosciences, Franklin Lakes, NJ) was added during the last 4 hours of the culture. The samples were assessed using FACSVerse (BD Biosciences) and FlowJo software (BD Biosciences). Staining antibodies were diluted according to the manufacturer's instructions. The antibodies used in flow cytometry are summarized in Supplementary Table 4.

Statistical Analyses

GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used for statistical analyses. The relationships between the groups were compared using Fisher's exact test or a *t* test. The relationships of continuous variables between or among the groups were compared using the *t* test or one-way analysis of variance (ANOVA), respectively. The relationships between tumor volume curves were compared using two-way ANOVA. For multiple testing, Bonferroni corrections were used. In patients who received surgical resection in Chiba Cancer Center, recurrence-free survival and OS were defined as the time from surgery to the first observation of disease progression or death from any cause and the time from surgery to death from any cause, respectively. In the PRIMER study, progression-free survival (PFS) and OS were defined as the time from nivolumab administration to the first observation of disease progression or death from any cause and the time from nivolumab administration to death from any cause, respectively. Recurrence-free survival, PFS, and OS were analyzed using the Kaplan-Meier method and compared among the groups using the log-rank test. All tests were two tailed, and *p* values less than 0.05 were considered statistically significant.

Data Availability Statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Statement

The study design was approved by the appropriate ethics review board at Chiba Cancer Center (R04-011), National Cancer Center (2021-384). This study was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained by opting out on the website of our institution. Mouse experiments were approved by the Animal Committee for Animal Experimentation of the Chiba Cancer Center. All experiments met the National Research Council's Guide for the Care and Use of Laboratory Animals.

Results

Half of TETs Had High Expression of CD80/CD86 or MHC-II, Which Is Related to T-Cell Infiltration

We first stained the FFPE samples from 86 patients, who underwent surgical resection at Chiba Cancer Center, with TET. The patient characteristics are summarized in Table 1 and Supplementary Tables 1 and 2. A total of 43 patients (50%) had high expression of both CD80 and CD86, 32 patients (37.2%) had high expression of MHC-II, and most had high MHC-I expression in tumor cells (Fig. 1*A* and Table 1). In contrast, CCLE data sets have revealed that these molecules, especially CD80/CD86, are mainly expressed in hematological malignancies but not in other solid tumors (Supplementary Figs. 1 and 2). Patients with type B3 thymoma exhibited high CD80/CD86 expression, whereas patients with thymic carcinoma (type C) exhibited lower expression levels (Supplementary Fig. 3*A*). In contrast, MHC-I and MHC-II expression levels were comparable among the WHO types (Supplementary Fig. 3*A*). Patients with thymic carcinoma exhibited remarkably low T-cell infiltration, and those with type A or B3 thymoma also exhibited relatively low T-cell infiltration (Supplementary Fig. 3*B*). Patients with high expression of CD80/CD86 tended to, but not significantly, have low expression of MHC-II, suggesting the relationship between antitumor immunity and tumorigenesis in TETs (Table 1). Both $CD4^+$ and $CD8^+$ T cells highly infiltrated the TME of CD80/CD86-high TETs (Fig. 1*B*). $CD4^+$ T cells, but not $CD8^+$ T cells, highly infiltrated the TME of MHC-II–high TETs (Fig. 1*C*). It was difficult to evaluate the prognostic significance according

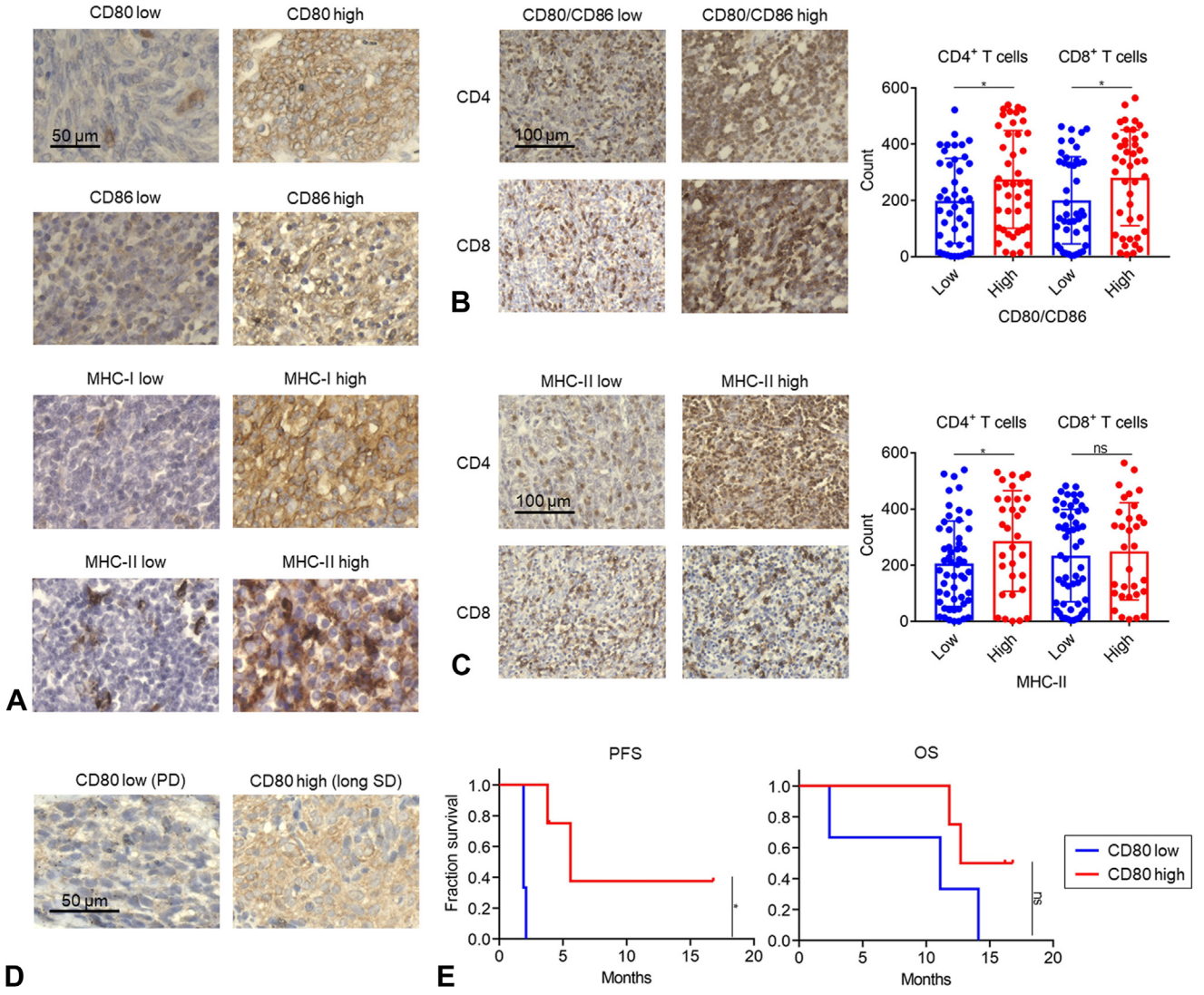

Figure 1. Expression of CD80/CD86 or MHC-II in TETs. For CD80, CD86, MHC-I, MHC-II, CD4, and CD8 staining, FFPE samples from 86 patients with TET who underwent surgical resection were used. (*A*) Representative staining for CD80, CD86, MHC-I, and MHC-II, representative staining for (*B*) CD4 and CD8 according to CD80/CD86 expression or (*C*) MHC-II (left), and the summaries (right) are illustrated. In addition, we used FFPE samples from patients with thymic carcinoma treated with the anti-PD-1 monoclonal antibody. PFS and OS were defined as the time from nivolumab administration to the first observation of disease progression or death from any cause and the time from nivolumab administration to death from any cause, respectively. Representative staining of (*D*) CD80 and (*E*) survival curves according to CD80 expression are illustrated. *t* tests were used in (*B*) and (*C*) for statistical analyses. The means and SDs are found in (*B*) and (*C*). OS and PFS were analyzed using the Kaplan-Meier method and compared among the groups using the log-rank test. * $p < 0.05$. FFPE, formalin-fixed, paraffin-embedded; MHC, major histocompatibility complex; ns, not significant; OS, overall survival; PD-1, programmed cell death protein 1; PFS, progression-free survival; TET, thymic epithelial tumor.

to the expression of these molecules because most patients did not experience any events (Supplementary Fig. 4).

CD80 Expressed in Tumor Cells Increases Antitumor Immunity and Efficacy of ICIs

To elucidate the role of CD80/CD86 in antitumor immunity, we created CD80-expressing mouse tumor cell lines (E.G7 and MC-38) (Fig. 2*A*). CD80-expressing E.G7 or MC-38 tumors exhibited slower growth in immunocompetent mice compared with the controls (Fig. 2*B*), whereas there was no considerable growth difference in tumor growth in immunodeficient mice (Supplementary Fig. 5). When we administered anti–PD-1 monoclonal antibody at the same time on day 4, CD80-expressing E.G7 or MC-38 tumors responded drastically, compared with the controls (Fig. 2*C*). Because the sizes between CD80-expressing tumors and the controls were

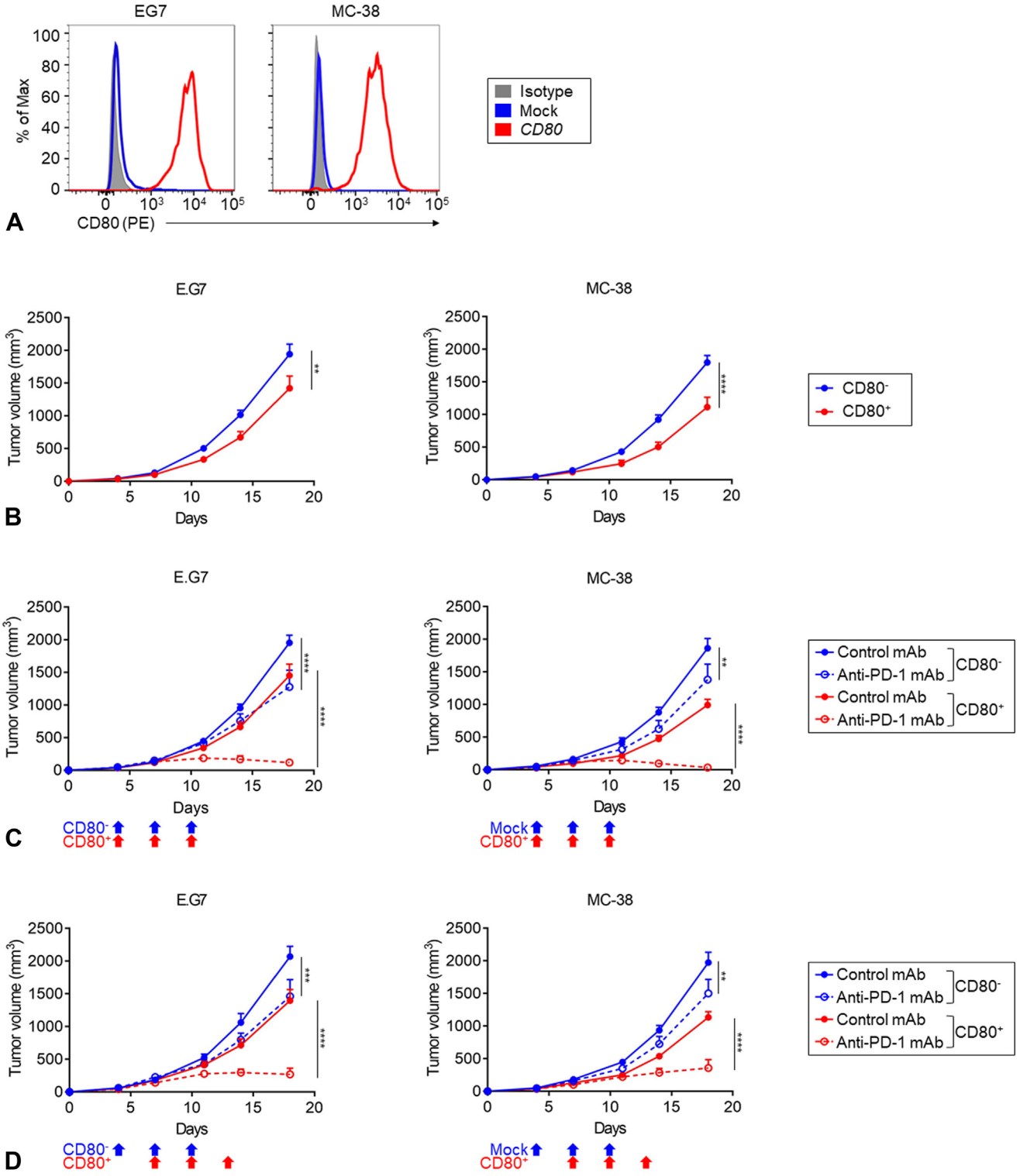

Figure 2. In vivo efficacy of PD-1 blockade against $CD80^{-}$ or $CD80^{+}$ tumors. (*A*) CD80 expression in mouse cancer cell lines. Mouse *Cd80* was subcloned into pBABE-puro and transfected into cell lines retrovirally (E.G7 and MC-38). Representative flow cytometry staining is found from triplicate experiments. (*B*) Tumor growth in immunocompetent mice. Cells (E.G7, 4×10^6; MC-38 cells, 1×10^6) were inoculated subcutaneously into immunocompetent wild-type mice (n = 8 per group), and tumor volume was monitored every three days. The means of the long and short diameters were used to generate tumor growth curves (left, E.G7; right, MC-38). (*C* and *D*) Efficacy of PD-1 blockade against E.G7 tumors in immunocompetent mice. We administered (*C*) anti-PD-1 monoclonal antibody at the same time on day 4 or (*D*) when tumor size reached same size of approximately 100 mm^3 ($CD80^{-}$ [mock], day 4; $CD80^{+}$, day 7) (n = 8 per group). Tumor volume was monitored every three days, and the means of the long and short diameters were used to generate tumor growth curves (left, E.G7; right, MC-38). All in vivo experiments were performed in duplicate, with similar results. Two-way ANOVA with or without Bonferroni correction was used. The mean and SEMs are illustrated. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. ANOVA, analysis of variance; mAbs, monoclonal antibody; PD-1, programmed cell death protein 1.

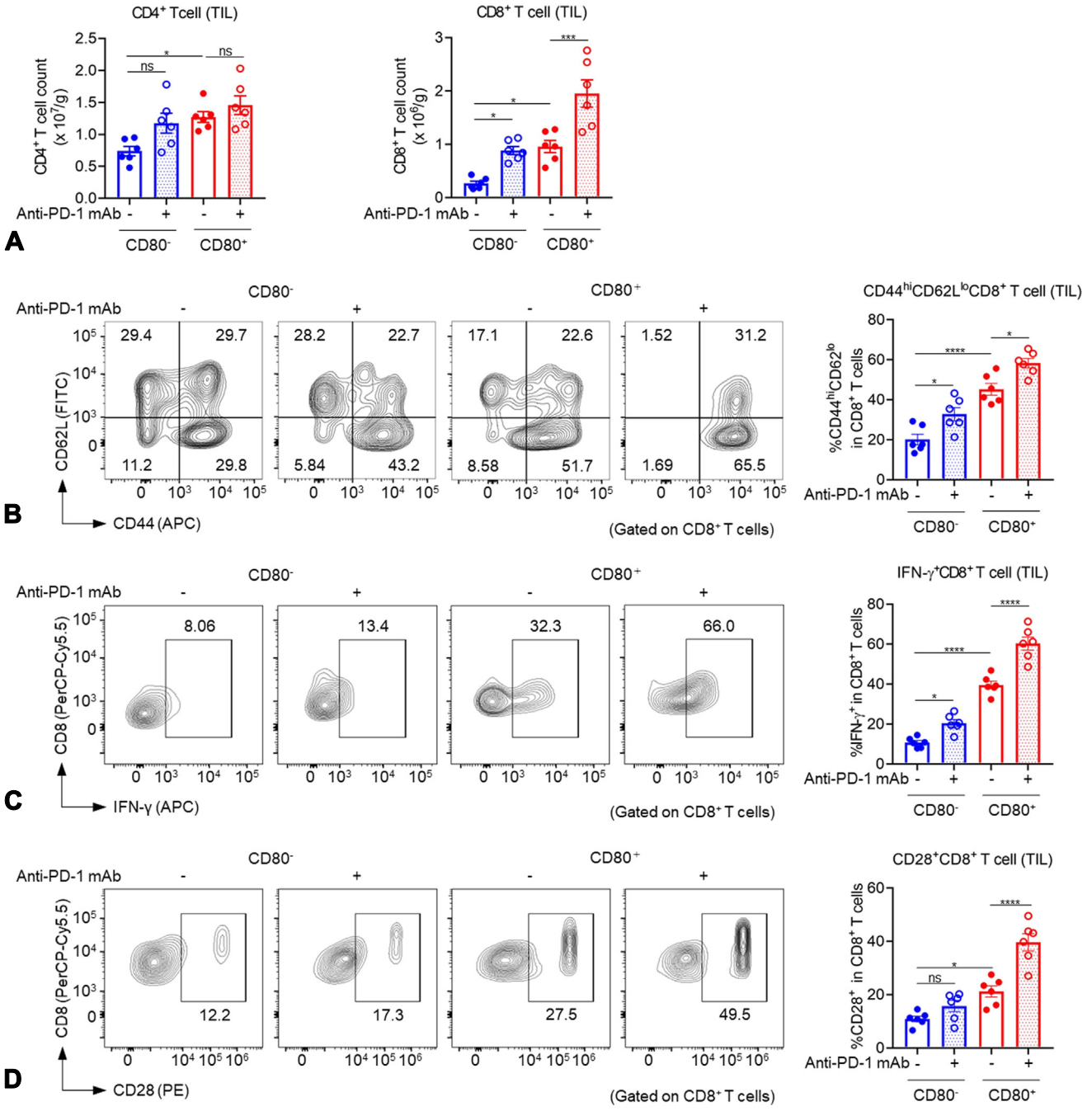

Figure 3. TIL analyses according to CD80 expression. Mouse experiments were performed as described in Figure 2*C*. Tumors were harvested 14 days after tumor cell inoculation to collect TILs for evaluation using flow cytometry (n = 6 per group). We evaluated TILs when anti-PD-1 monoclonal antibody was administered at the same time on day 4. (*A*) T-cell infiltration. Both $CD4^+$ and $CD8^+$ T cells were counted and compensated for the tumor weight. Summaries of T-cell counts/tumor weights are found. Proportions of (*B*) $CD44^{hi}CD62L^{lo}CD8^+$ T cells, (*C*) IFN-γ^+CD8^+ T cells, and (*D*) $CD28^+CD8^+$ T cells in TILs. Representative flow cytometry staining (left) and the summaries (right) are found. All in vivo experiments were performed in duplicate, with similar results. One-way ANOVA with Bonferroni correction was used for the statistical analyses. The mean and SEMs are found. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. ANOVA, analysis of variance; mAb, monoclonal antibody; ns, not significant; PD-1, programmed cell death protein 1; TIL, tumor-infiltrating lymphocyte.

different, we administered anti–PD-1 monoclonal antibody when the tumor size reached the same size of approximately 100 mm^3 ($CD80^-$ [mock], day 4; $CD80^+$, day 7). The anti–PD-1 monoclonal antibody also exhibited significant efficacy against CD80-expressing E.G7 or MC-38 tumors (Fig. 2*D*). Both $CD4^+$ and $CD8^+$ T cells highly infiltrated the TME of CD80-expressing tumors compared with the control (Fig. 3*A*). The

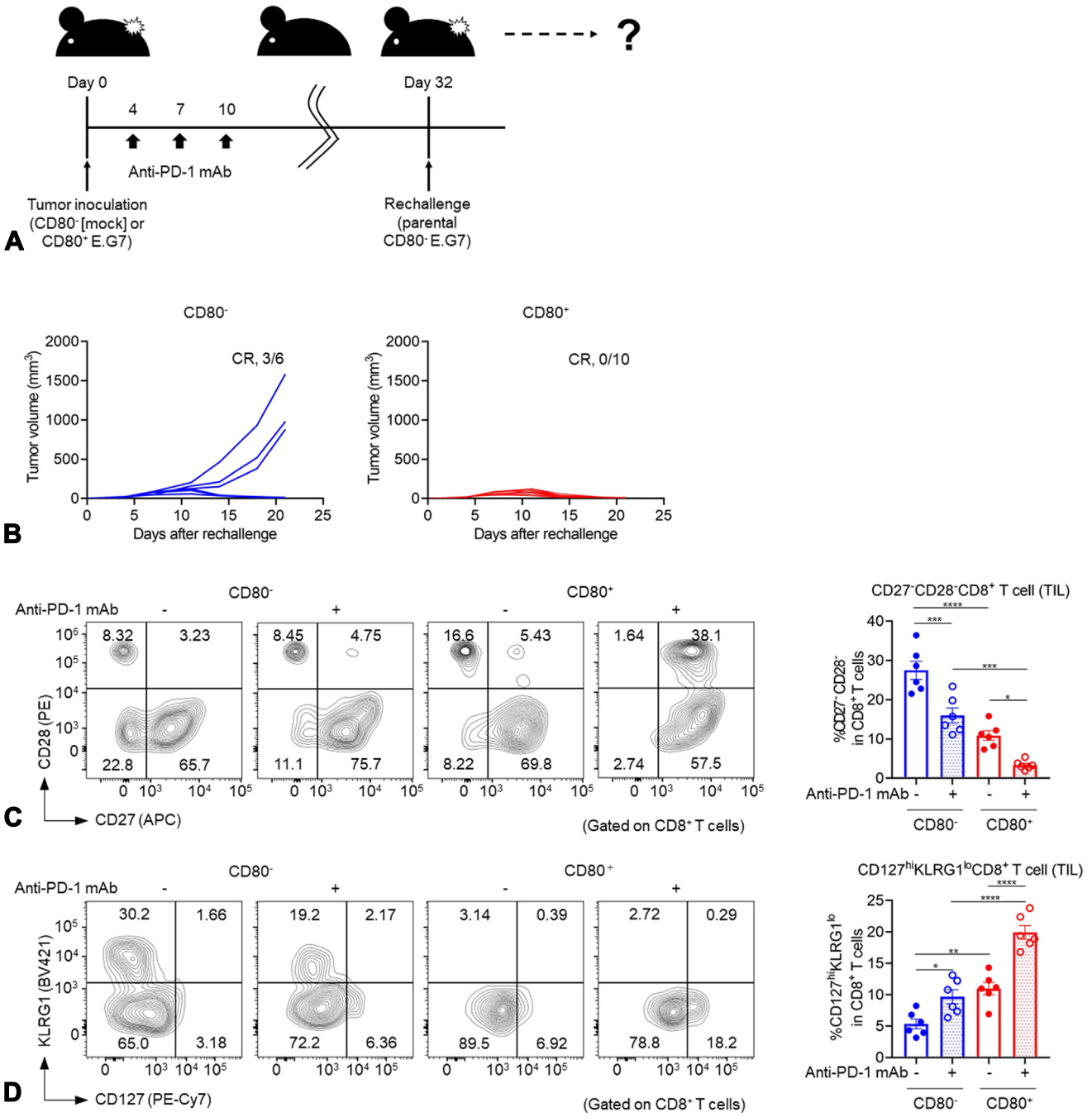

Figure 4. Parental tumor rechallenge in mice treated with PD-1 blockade. (*A*) Experimental schema. Mice that had completely eradicated the initial $CD80^{-}$ or $CD80^{+}$ tumors after anti-PD-1 monoclonal antibody were rechallenged with parental $CD80^{-}$ tumor cells on day 32. (*B*) Each rechallenged tumor volume. The means of the long and short diameters were used to generate tumor growth curves, and each tumor volume is found. The proportions of (*C*) $CD27^{-}CD28^{-}CD8^{+}$ T cells and (*D*) $CD127^{hi}KLRG\text{-}1^{lo}CD8^{+}$ T cells in TILs. Mouse experiments were performed as described in Figures 2 and 3 (n = 6 per group). Representative flow cytometry staining (left) and the summaries (right) are found. All in vivo experiments were performed in duplicate, with similar results. One-way ANOVA with Bonferroni corrections was used in (*C*) and (*D*) for the statistical analyses. The mean and SEMs are found. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. ANOVA, analysis of variance; CR, complete rejection; mAb, monoclonal antibody; PD-1, programmed cell death protein 1; TIL, tumor-infiltrating lymphocyte.

proportions of effector T cells ($CD44^{hi}CD62L^{lo}CD8^{+}$), cytokine-producing T cells (IFN-$\gamma^{+}CD8^{+}$), PD-$1^{+}CD8^{+}$ T cells, granzyme B $(GZM)^{+}CD8^{+}$ T cells, and perforin $(PRF)^{+}CD8^{+}$ T cells in TILs increased in CD80-expressing tumors (Fig. 3*B* and *C* and Supplementary Fig. 6*A–C*). In addition, CD28, which binds to CD80, expression in tumor-infiltrating $CD8^{+}$ T cells also significantly increased (Fig. 3*D*). Anti–PD-1 monoclonal

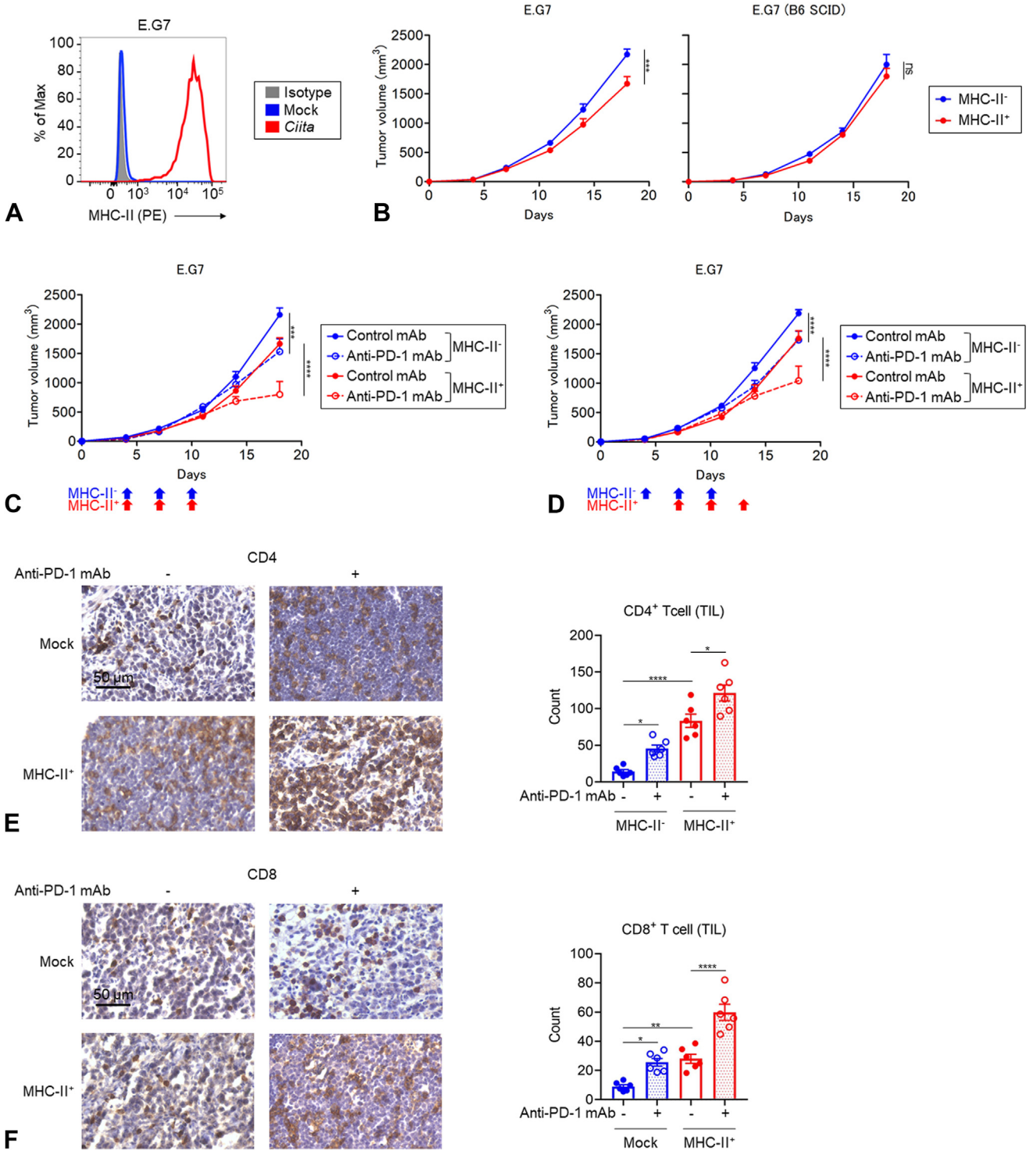

Figure 5. In vivo efficacy of PD-1 blockade against MHC-II^{-} or MHC-II^{+} tumors. (*A*) MHC-II expression in mouse cancer cell lines. Mouse *Ciita* was subcloned into pBABE-puro and transfected into the E.G7 cell line retrovirally. Representative flow cytometry staining is illustrated from triplicate experiments. (*B*) Tumor growth in immunocompetent wild-type or immunodeficient B6 SCID mice and (*C* and *D*) efficacy of PD-1 blockade in immunocompetent mic and T-cell infiltration (CD4, *E* and CD8, *F*). Mouse experiments were performed as described in Figures 2 and 3 (n = 8 per group). T-cell infiltration was assessed by IHC (n = 6 per group), and representative staining (left) and the summaries (right) are found in *E* and *F*. All in vivo experiments were performed in duplicate, with similar results. Two-way ANOVA with or without Bonferroni corrections was used in (*B*), (*C*), and (*D*), and one-way ANOVA with Bonferroni corrections was used in (*E*) for statistical analyses. The mean and SEMs are illustrated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ANOVA, analysis of variance; IHC, immunohistochemistry; mAb, monoclonal antibody; MHC, major histocompatibility complex; ns, not significant; PD-1, programmed cell death protein 1.

antibody further increased the infiltration and proportions in the TME (Fig. 3*A–D* and Supplementary Fig. 6*A–C*). PD-1 blockade-mediated efficacy was not observed in immunodeficient mice (Supplementary Fig. 5*B*). Because CTLA-4 suppresses T-cell function by strong binding to CD80/CD86,[31,32] we tested the efficacy of CTLA-4 blockade using the same mouse models, revealing similar tendencies to PD-1 blockade (Supplementary Fig. 7). Overall, CD80/CD86-high tumors responded significantly to ICIs.

CD80 Expressed in Tumor Cells Induce Durable Response to ICIs

$CD28^-$ T cells are reportedly senescent and have limited proliferative capacity, whereas $CD28^+$ T cells can be a precursor subset.[33] Given that $CD28^+CD8^+$ T cells were highly infiltrated into the TME of CD80-expressing tumors, a durable response to ICIs could be achieved in CD80-expressing tumors. To investigate this durable response, we performed rechallenge mouse experiments. Mice that had completely eradicated the initial tumors ($CD80^-$ [mock] or $CD80^+$ E.G7) after anti–PD-1 monoclonal antibody were rechallenged with parental $CD80^-$ EG.7 cells (Fig. 4*A*). After the complete eradication of $CD80^+$ E.G7 tumors treated with PD-1 blockade, all rechallenged parental $CD80^-$ E.G7 tumors were completely rejected (Fig. 4*B*). In contrast, after the complete eradication of $CD80^-$ E.G7 tumors, half of the rechallenged parental $CD80^-$ E.G7 tumors were not rejected (10 of 10 versus three of six, $p < 0.05$) (Fig. 4*B*). We analyzed CD27 and CD28 expression in TILs to evaluate senescent T cells. In addition, we analyzed the proportion of memory precursor effector cells (MPECs) in TILs with CD127 and KLRG1 because MPECs generate long-lived $CD8^+$ memory T cells.[34] As found in Figure 4*C* and *D*, the proportion of $CD27^-CD28^-CD8^+$ T cells decreased and that of $KLRG1^{lo}CD127^{hi}CD8^+$ T cells increased in the TILs of $CD80^+$ E.G7 tumors compared with the control. Anti–PD-1 monoclonal antibody further decreased the proportion of $CD27^-CD28^-CD8^+$ T cells and increased that of $KLRG1^{lo}CD127^{hi}CD8^+$ T cells, respectively (Fig. 4*C* and *D*). These findings suggest that CD80 expression in tumor cells decreases senescent T cells and increases MPECs, which may be related to a durable response to ICIs.

MHC-II-Expressing Tumors Are Effective to PD-1 Blockade

We elucidated the efficacy of PD-1 blockade in MHC-II–expressing tumors. We created an MHC-II–expressing mouse cancer cell line using *Ciita* gene (Fig. 5*A*). As previously reported,[30,35] MHC-II–expressing tumors grew slower than the controls in immunocompetent mice, whereas no considerable growth difference was noted in immunodeficient mice (Fig. 5*B*). When anti–PD-1 monoclonal antibody was administered at the same time on day 4, MHC-II–expressing tumors responded dramatically compared with controls (Fig. 5*C*). Because the average size was different between MHC-II–expressing tumors and the controls, as was observed in CD80-expressing tumors, we next administered anti–PD-1 monoclonal antibody when the tumor size reached the same size of approximately 100 mm^3 ($MHC\text{-}II^-$ [mock], day 4; $MHC\text{-}II^+$, day 7). Similarly, the anti–PD-1 monoclonal antibody also exhibited significant efficacy against MHC-II–expressing tumors compared with the controls (Fig. 5*D*). IHC revealed that both $CD4^+$ and $CD8^+$ T cells highly infiltrated the TME of MHC-II–expressing tumors and that PD-1 blockade further increased infiltration (Fig. 5*E* and *F*). These results suggest that MHC-II–expressing tumors are sensitive to ICIs.

Patients With CD80-High Thymic Carcinoma Have Longer PFS by Anti-PD-1 monoclonal antibody

Finally, we analyzed seven patients with thymic carcinoma who received nivolumab after platinum-based chemotherapy in the PRIMER study.[16] We analyzed FFPE samples from these patients at the time of diagnosis using IHC. Both CD80 and CD86 expressions were high in the tumor cells of four patients who achieved stable disease, whereas CD80 expression was low in the three patients who experienced progressive disease (PD) (CD80 high, four of four stable disease versus zero of three PD, $p = 0.029$) (Supplementary Table 3 and Fig. 1). In particular, a patient who achieved a long stable disease with PFS of 16.8 months had high CD80 expression (Fig. 1*D*). The PFS of patients with high CD80 expression was significantly longer than that of patients with low expression (Fig. 1*E*).

Discussion

Treatment of TETs may be complex when surgery alone is insufficient, and systemic treatment may be required in inoperable cases. Platinum-based chemotherapy is the standard first-line treatment for patients with TET who have unresectable disease or those who progress after surgery and radiotherapy.[1–6] Nevertheless, owing to the rarity of the disease, the available data are mainly derived from prospective phase 2 trials or retrospective analyses, and the prognosis remains poor, necessitating more effective therapies on the basis of their biology.[1–6] In small retrospective analyses using different positive cutoffs and antibody clones, PD-L1 expression in TETs seems to be high.[36–38] Thus, the efficacy of anti–PD-1 or PD-L1 monoclonal antibodies has

been evaluated in TETs, mainly thymic carcinoma.[14–17] Clinical responses have been observed in some studies, and patients with tumors with high PD-L1 levels may experience clinical benefits.[14,15,17] Nevertheless, the response rate of approximately 20% is insufficient, and all studies have revealed that a considerable number of patients, especially those with thymoma, develop severe irAEs.[14–17] Although the NCCN Guidelines recently added pembrolizumab to the treatment of refractory thymic carcinoma, predictive biomarkers are warranted to increase efficacy without severe irAEs. Here, we suggest that CD80/CD86– or MHC-II–high tumors can respond markedly to ICIs. In particular, CD80/CD86-CD28, a co-stimulatory molecule, associated with not only increased efficacy but also a durable response to ICIs. Thus, CD80/CD86 and MHC-II could be predictive biomarkers, promoting the development of ICIs against CD80/CD86– or MHC-II–high tumors.

CD80/CD86 are mainly expressed in antigen-presenting cells and bind to CD28, a co-stimulatory molecule, leading to T-cell activation.[20] Thus, hematological malignancies sometimes express these molecules,[39] but solid tumors rarely express these molecules, as observed in the CCLE data sets. In contrast, because of their origin, half of the TETs in our cohort highly expressed these molecules in tumor cells, and such TETs had high T-cell infiltration. Especially, patients with type B3 thymoma exhibited high CD80/CD86 expression, whereas patients with thymic carcinoma (type C) exhibited lower expression levels. CD80/CD86-expressing tumors can stimulate T cells through CD28, leading to tumor regression, as observed in our present and previous studies.[40,41] In addition, we found the significantly increased efficacy of ICIs in mouse models. Furthermore, senescent T cells with loss of CD27 and CD28 decreased in the TME of CD80-expressing tumors, along with increased infiltration of MPECs. Accordingly, the rechallenged tumors were completely rejected after the initial eradication of CD80-expressing tumors by PD-1 blockade, suggesting that patients with CD80/CD86-high tumors could respond and achieve a durable response to ICIs.

Several studies have reported MHC-II expression in several cancers.[42] Previous studies have revealed that MHC-II expression in tumor cells increases ICI-mediated efficacy.[30,36,43,44] In addition to $CD4^+$ T-cell-mediated help, we have previously reported the importance of cytotoxic $CD4^+$ T cells in antitumor immunity against MHC-II–expressing tumors.[30,35] In line with these previous studies, our present study revealed that MHC-II–expressing mouse tumors significantly responded to PD-1 blockade with increased T-cell infiltration, which is consistent with increased T-cell infiltration in MHC-II–expressing TETs in our cohort. These findings also suggest that patients with MHC-II–high tumors could potentially respond to ICI therapy.

The efficacy of lenvatinib in patients with advanced thymic carcinomas was recently revealed in the phase 2 REMORA trial.[7] Preclinical and clinical data suggest that the combination of ICIs with antiangiogenic agents exerts synergistic antitumor activity in several cancer types.[45,46] Indeed, a phase 2 trial of avelumab in combination with axitinib in TETs found promising antitumor effects.[47] Considering these previous studies and our findings, CD80/CD86 and MHC-II expression can be used to predict the efficacy of such combination treatments in the future.

This study has several limitations. Because it is difficult to obtain TET cell lines derived from mice, the cell lines used in our present study were E.G7, thymic T-cell lymphoma cells, and MC-38 cells, colorectal cancer cells. Consequently, the outcomes of our mouse models might not substantiate our hypothesis regarding ICI response in TET. From the PRIMER study,[16] however, patients with CD80-high thymic carcinoma actually had a significantly longer PFS with nivolumab, and a patient who achieved a durable SD had high CD80 expression in tumor cells, which is consistent with our findings from mouse models. We could only analyze less than 100 TETs because of the rarity of the disease, and all were obtained from patients with early TETs who underwent surgical resection. Because most patients did not experience any events, we could not evaluate the prognosis according to these molecules. In addition, ICIs have not been approved for TETs in Japan, and we could therefore only analyze these molecules in a few ICI-treated samples from the PRIMER study.[16] To obtain further insights into antitumor immunity against TETs, larger cohorts including ICI-treated patients should be analyzed.

In summary, approximately half of the TETs had high expression of CD80/CD86 and MHC-II in tumor cells, and this expression was related to T-cell infiltration. Our mouse experiments revealed that these molecules could increase the efficacy of ICIs. In particular, the CD80/CD86-CD28 co-stimulatory molecule axis not only increases efficacy but also induces a durable response to ICIs. Thus, CD80/CD86 and MHC-II could be predictive biomarkers, promoting the development of ICIs against CD80/CD86– or MHC-II–high TETs.

CRediT Authorship Contribution Statement

Hideki Ikeda: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Roles/Writing—original draft, Writing—review and editing.

Joji Nagasaki: Formal analysis, Investigation, Methodology, Writing—review and editing.

Daiki Shimizu: Collection of clinical samples and data, Writing—review and editing.

Yuki Katsuya: Collection of clinical samples and data, Writing—review and editing.

Hidehito Horinouchi: Collection of clinical samples and data, Writing—review and editing.

Yukio Hosomi: Collection of clinical samples and data, Writing—review and editing.

Etsuko Tanji: Formal analysis, Investigation, Methodology, Writing—review and editing.

Takekazu Iwata: Collection of clinical samples and data, Writing—review and editing.

Makiko Itami: Collection of clinical samples and data, Writing—review and editing.

Masahito Kawazu: Funding acquisition, Writing—review and editing, Supervision.

Yuichiro Ohe: Collection of clinical samples and data, Writing—review and editing, Supervision.

Takuji Suzuki: Writing—review and editing, Supervision.

Yosuke Togashi: Conceptualization and Project administration, Data curation, Funding acquisition, Methodology, Writing—Original draft, Writing—review and editing.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2023.100573.

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