

# PD-1 blockade restores impaired function of ex vivo expanded CD8<sup>+</sup> T cells and enhances apoptosis in mismatch repair deficient EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells

Rajeev Kumar<sup>1,2</sup>  
 Fang Yu<sup>1</sup>  
 Yuan-Huan Zhen<sup>3</sup>  
 Bo Li<sup>2</sup>  
 Jun Wang<sup>1</sup>  
 Yuan Yang<sup>1,2</sup>  
 Hui-Xin Ge<sup>4</sup>  
 Ping-Sheng Hu<sup>1,2</sup>  
 Jin Xiu<sup>1,2</sup>

<sup>1</sup>Clinical Research Centre, The Affiliated Hospital of Guizhou Medical University, Guiyang, People's Republic of China; <sup>2</sup>Cancer Immunology and Immunotherapy Centre, The Affiliated Hospital of Guizhou Medical University, Guiyang, People's Republic of China; <sup>3</sup>Department of Colorectal Surgery, The Affiliated Hospital of Guizhou Medical University, Guiyang, People's Republic of China; <sup>4</sup>Department of Surgery, The Affiliated Cancer Hospital of Guizhou Medical University, Guiyang, People's Republic of China

Correspondence: Rajeev Kumar  
 Clinical Research Center, The Affiliated Hospital of Guizhou Medical University, 28 Guiyi Street, Guiyang, Guizhou Province 550004, People's Republic of China  
 Tel/fax +86 851 674 8718  
 Email rajeev.bioraj@gmail.com

**Background:** Adoptive T cell therapy has been proven to be a promising modality for the treatment of cancer patients in recent years. However, the increased expression of inhibitory receptors could negatively regulate the function and persistence of transferred T cells which mediates T cell anergy, exhaustion, and tumor regression. In this study, we investigated increased cytotoxic activity after the blockade of PD-1 for effective immunotherapy.

**Methods:** The cytotoxic function of expanded CD8<sup>+</sup> CTLs and interactions with tumor cells investigated after blocking of PD-1. Ex vivo expanded CD8<sup>+</sup> CTLs were co-cultured with mismatch repair (MMR) stable or deficient (high microsatellite instability [MSI-H]) EpCAM<sup>+</sup> tumor cells. The levels of IFN- $\gamma$  and GrB were detected by enzyme-linked immunosorbent spot assay. Flow cytometry and confocal microscopy were used to assess CD107a mobilization, cytosolic uptake, and cell migration.

**Results:** A dramatic increase in PD-1 expression on the surface of CD8<sup>+</sup> CTLs during ex vivo expansion was observed. PD-1 level was downregulated by approximately 40% after incubation of the CD8<sup>+</sup> CTLs with monoclonal antibody which enhanced the secretion of IFN- $\gamma$ , GrB, and CD107a. Additionally, PD-1 blockade enhanced cell migration and cytosolic exchange between CD8<sup>+</sup> CTLs and MMR deficient (MSI-H) EpCAM<sup>+</sup>PD-L1<sup>+</sup> tumor cells.

**Conclusion:** The blockade of PD-1 enhanced the cytotoxic efficacy of CD8<sup>+</sup> CTLs toward MMR deficient tumor cells. In conclusion, we propose that blocking of PD-1 during the expansion of CD8<sup>+</sup> CTLs may improve the clinical efficacy of cell-based adoptive immunotherapy.

**Keywords:** PD-1, CTLs, MSI-H, EpCAM<sup>+</sup>PD-L1<sup>+</sup>, cancer immunotherapy

## Introduction

Following the success and approval of anti-CTLA-4 with ipilimumab and anti-PD-1 immunotherapy with nivolumab to treat both metastatic melanoma<sup>1,2</sup> and pre-treated metastatic squamous non-small-cell lung cancer (NSCLC),<sup>3,4</sup> there has been interest in shifting the focus to controlling immunosuppressive mechanisms.<sup>5-7</sup> One of the most important mechanisms underlying the significant clinical benefits of immune checkpoint blockade is associated with activating anti-tumor T cell immunity and attenuating T cell exhaustion.<sup>8,9</sup> The expression of inhibitory receptors (iRs) by CD8<sup>+</sup> T cells is generally considered a hallmark of impaired T cell function, especially during chronic viral infection and cancer.<sup>10,11</sup> Over the last decade, the family of iRs has been identified in T cell exhaustion, including PD-1, CTLA-4, mucin domain protein-3, LAG3, BTLA, and TIGIT.<sup>8,12</sup>

Although the mechanism of underlying T cell exhaustion is complex, an increasing amount of evidence indicates that blocking PD-1 is thus far the most effective means of restoring CD8<sup>+</sup> T cell function. Recently, the results of a study of 46 patients with advanced melanoma who received a single agent, pembrolizumab, a PD-1 monoclonal antibody (mAb), showed that the presence of CD8<sup>+</sup> T cells at the invasive tumor margin was associated with the expression of the PD-1/PD-L1 immune inhibitory axis, and these cells are predictive biomarkers for PD-1 mAb treatment.<sup>13</sup> Moreover, treatment with PD-1 mAb successfully attenuates T cell exhaustion, resulting in the proliferation of intra-tumoral CD8<sup>+</sup> T cells and restoring potent CTL-mediated responses in patients who experienced tumor regression. The presence and localization of CD8<sup>+</sup> T cells within the tumor microenvironment has been associated with prognostic significance in a number of solid tumors including melanoma, NSCLC,<sup>14</sup> colorectal cancer,<sup>15,16</sup> renal cell carcinoma, triple-negative breast cancer,<sup>17</sup> ovarian cancer,<sup>18</sup> glioma,<sup>19</sup> and urothelial carcinoma,<sup>20</sup> in addition, the successful treatment of anti-PD-1 induces robust clinical responses in these cancer types. Furthermore, a genetic biomarker for the effectiveness of anti-PD-1 treatment called mismatch repair (MMR)-deficiency was recently identified.<sup>21</sup> These findings provide additional evidence indicating that PD-1 blockade counteracts T cell exhaustion, resulting in endogenous anti-tumor immune responses in the form of CTL activity toward mutation-associated neoantigen recognition.

In contrast to using anti-PD-1 antibodies which target endogenous PD-1 on T cells within the human body, adoptive immunotherapy involves the infusion of autologous ex vivo activated and expanded T cells into patients with the aim of combating the tumor.<sup>22</sup> Although the T cells used in adoptive immunotherapy can be derived from different sources, such as tumor infiltrating lymphocytes (TILs), peripheral blood mononuclear cells (PBMCs), and tumor draining lymph nodes, the general procedure involves T cell isolation, ex vivo activation/genetic manipulation, expansion, and then transfusion.<sup>23</sup> Previous studies have shown that during the chronic ex vivo activation and expansion of tumor-specific T cells<sup>24,25</sup> or MHC-independent chimeric antigen receptor (CAR) T cells,<sup>25</sup> the expression of iRs is significantly upregulated on the expanded T cells, leading to exhaustion and poor in vivo persistence. The autologous transfusion of T cells with high levels of iRs into patients might explain the poor clinical efficacy of adoptive immunotherapy against solid tumors. In the present study, we aimed to determine the effect of PD-1 blockade on ex vivo expanded CD8<sup>+</sup> T cells

which were subjected to the standard activation and expansion procedure for adoptive immune cell therapy.

## Materials and methods

### Patient samples and cell lines

The ethics committee of The Affiliated Hospital of Guizhou Medical University, People's Republic of China approved this research. Human colon cancer cell lines were procured from American Type Cell Collection (ATCC, Manassas, VA, USA). Primary tumor samples—peripheral blood specimens, were obtained from the colorectal surgical department or from healthy volunteers after written informed consent which was obtained from the patients and donors. PBMCs were separated by density gradient centrifugation using Polymorphprep™ (AXIS-SHIELD PoC AS, Oslo, Norway) cell separation media. Microsatellite instability (MSI) was used to investigate the standard Bethesda microsatellite markers (Bat-25, Bat-26, D5S346, D17S250, and D2S123) by using fragment analysis of polymerase chain reaction products which were labeled with fluorescent dyes. Differences in the length of two or more markers were indicative of MSI status. Five high MSI (MSI-H) tumor specimens were selected for tumor epithelial cells' (TECs') isolation (Table 1).

### Primary TECs and cell lines

The following TECs and colon cancer cell lines were selected for noted expression of cytokeratin 20 (CK20): MMR deficient (MSI-H) TECs, DLD-1, and MMR proficient (MSS) SW480, SW620.<sup>26</sup> TECs were isolated from post-surgically excised colon cancer tissues as previously

**Table 1** Disease characteristics of five colorectal patients selected for TEC isolation

Characteristics	Total
Age	62
Sex	
Male	3
Female	2
AJCC stage	
II	3
III	2
Differentiation	
T3N0 M0	4
T3N1 M0	1
Chemotherapy	–
Radiotherapy	–
Biomarkers	
MMR (MSI-H)	5
Cytokeratin 20 (CK20)	5

**Abbreviations:** TECs, tumor epithelial cells; AJCC, American Joint Committee on Cancer; MMR, mismatch repair; MSI-H, high microsatellite instability.

described.<sup>27</sup> Briefly, tumor tissues were collected in RPMI 1640 medium supplemented with 100 U/mL penicillin/streptomycin, 10% heat-inactivated FBS, and 50 µg/mL gentamycin. The tumor tissues were further minced into small pieces in a dissociation solution containing collagenase, DNase, and trypsin (Thermo Fisher Scientific, Waltham, MA, USA) for 48 hrs at 37°C. The suspensions were washed using PBS and centrifuged at 1,200 rpm for 3 min. The cell pellets were resuspended in RPMI 1640 medium supplemented with 10% FBS, gentamycin, hydrocortisone, and insulin and then seeded into a T25 flask. EpCAM<sup>+</sup> TECs were purified using microbeads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) as described by the manufacturer, and the viability of purified TECs was determined by trypan exclusion assay. The isolated EpCAM<sup>+</sup> TECs and colon cancer cell lines were utilized for further experiments. For the DLD-1 cells, RPMI 1640 medium was used for culture and passage, while Leibovitz media (Thermo Fisher Scientific) was used for culture and passage of SW480 and SW620. Both media were supplemented with 10% FBS (Thermo Fisher Scientific), and the cells were maintained in a humidified, 5% CO<sub>2</sub> atmosphere at 37°C.

## Generation of CTLs

PBMCs (1.5×10<sup>6</sup>) were cultured overnight in T175 cell culture flasks in AIM V medium (Thermo Fisher Scientific) supplemented with 1% FBS and 500 U/mL IFN-γ. After 18 to 24 hrs, 50 ng/mL anti-CD3/28 antibody (OKT3) coated microbeads and 500 U/mL rIL-2 were added to the media. The cells were incubated at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere. Following the standard procedure of large-scale manufacturing, on day 7, the cell culture from each T175 flask was transferred to a disposable and gas-permeable cell expansion bag (CultiLife, Takara Bio, Otsu, Japan) in a final volume of 4–5 L. Flow cytometry was performed to evaluate the PD-1 levels on the T cells before and after 2 weeks of ex vivo expansion.

## Apoptosis assay

The CD8<sup>+</sup> T cells were harvested on day 14 and were sequentially co-cultured for another 48 hrs with cancer cells at an effector (CD8<sup>+</sup> T cells)-to-target (EpCAM<sup>+</sup> cancer cells) ratio of 25:1 in the presence of 1 µg/mL of anti-PD-1 purified antibody (eBioscience, San Diego, CA, USA) along with an isotype control.<sup>28</sup> The co-culture was incubated with neutralizing PD-1 (nPD-1 mAb; BPS Bioscience, San Diego, CA, USA) antibody for 20 minutes before adding blocking PD-1 for neutralization assay. Cells were harvested and washed

in 2× PBS and centrifuged at 1,500 rpm for 10 min. The cells were pre-stained with EpCAM PE (BD Biosciences, San Jose, CA, USA) for 30 min and 5 µL of Annexin V per 0.5×10<sup>6</sup> was used to reconstitute cells in ice-cold binding buffer for 15 min at 4°C. The cells were acquired using flow cytometry (FC500, Beckman Coulter, Brea, CA, USA) and analyzed by CXP software.

## Phenotyping and the CD107a-mobilization assay by flow cytometry

The antibodies used for CTL phenotyping (anti-CD3-ECD, CD4-PC5, CD8-PC7, PD-1-PE, CTLA-4PE, PD-L1 FITC; IgG1-FITC, IgG1-PE) were all obtained from BD Biosciences. To investigate competitive binding of staining antibody with blocking PD-1 mAb, flow cytometric analysis was performed using two different clones of staining antibody after 48 hrs: primary PD-1 (MIH4), secondary IgG1 FITC and PD-1 conjugated with PE.

Positive staining for PD-1 and PD-L1 was determined by comparing with the respective isotype control. The cell suspensions were incubated with mAb for 30 min at 4°C and then washed twice in PBS. PD-1 mAb-treated co-cultures were incubated with an anti-CD107a antibody and degranulation assay was performed.<sup>29</sup> The cells were acquired on an FC 500 (Beckman Coulter) and analyzed by the CXP analysis software.

## Enzyme-linked immunosorbent spot (ELISpot) assay

To measure IFN-γ and GrB secretion during the co-culturing of CD8<sup>+</sup> T and cancer cells, an ELISpot experiment was conducted using MultiScreen 96-well plates (Mabtech, Nacka Strand, Sweden) in triplicate. Wells that contained only cancer cells served as negative controls, and those that contained anti-CD28 (1:1,000) served as positive controls. The spots were visualized and counted on an Immunospot Imaging Analyzer (Cellular Technology Ltd, Shaker Heights, OH, USA).

## Chemotaxis assay

The migration of CD8<sup>+</sup> T cells was evaluated in vitro using a transwell insert (Corning Incorporated, Corning, NY, USA) with an 8.0 µm-pore size, as described previously.<sup>30</sup> Briefly, T cells were pre-treated with anti-PD-1 antibody for 48 hrs and then washed once with PBS. The cell count was then adjusted to 5×10<sup>5</sup> cells/mL using RPMI 1640 medium, and a 100 µL aliquot of the suspension was placed in the top chamber of the transwell insert. The cancer cells were

cultured in RPMI 1640 medium, and conditioned medium was added to the bottom chamber of the transwell plate prior to the addition of the CTLs to the top chamber. After the cells were incubated for 90 min at 37°C in a 5% CO<sub>2</sub> atmosphere, the top chamber was removed. The cells were stained with Giemsa, and the number of T cells which had migrated into the bottom chamber was counted using the trypan blue exclusion assay.

## Statistical analysis

All quantitative data are presented as the means with standard errors of at least three or more independent experiments. Student's *t*-tests were performed to analyze statistical significance. *P*-values <0.05 were considered significant.

## Results

### Extracellular level of EpCAM and PD-L1 in the colon cancer cell cultures

Primary tumors (5 µm paraffinized sections) were chromogenically immunostained for PD-L1, and found positive at the invasive front (Figure S1A). MHC-I/II expression levels were assessed by flow cytometry. We found a variable range of MHC-I (mean 94.6%, range 96.6%–90.1%) and MHC-II (mean 4%, range 2%–5%) expression level (Figure S1B). The purified TECs and colon cancer cell lines, including DLD-1, SW480, and SW620, were tested to determine cell viability, EpCAM, and intracellular CK20 levels. The viability of purified TECs was found to be 92.9% (range 89.62%–96.47%; *n*=5) before further experiments were pursued. The TECs and colon cancer cell lines were observed to be ~100% positive for EpCAM, and >90% for CK20 (Figure S2A and B). We further analyzed PD-L1 expression in these cells. We found a significant variation in PD-L1 expression levels on the TECs and colon cancer cell lines: TECs (range 92%–97%, mean =94.33%); DLD-1 (range 13.3%–21.0%, mean =17.23%); SW480 (<1%); SW620 (<1%); and K562 (<0.5%) (Figure S2C).

### PD-I levels on CD8<sup>+</sup> T cells before and after ex vivo expansion

The CD8<sup>+</sup> T cells were expanded using classic methods for 2 weeks using patients' PBMCs via the timed addition of IFN-γ, anti-CD3/CD28 coated microbeads, and rIL-2. The median expansion of the T cells was ~1,000-fold, and the majority of the cells were CD3<sup>+</sup>CD8<sup>+</sup> T cells (range 64%–87%, mean =79%) after expansion.<sup>31,32</sup> The presence of other cytotoxic cell types, including CD3<sup>+</sup>CD56<sup>+</sup> NK cells (range 0.1%–5.4%; mean =1.72%) and CD3<sup>+</sup>CD56<sup>+</sup> NKT cells (range 0.3%–15.1%; mean =6.34%) was also determined

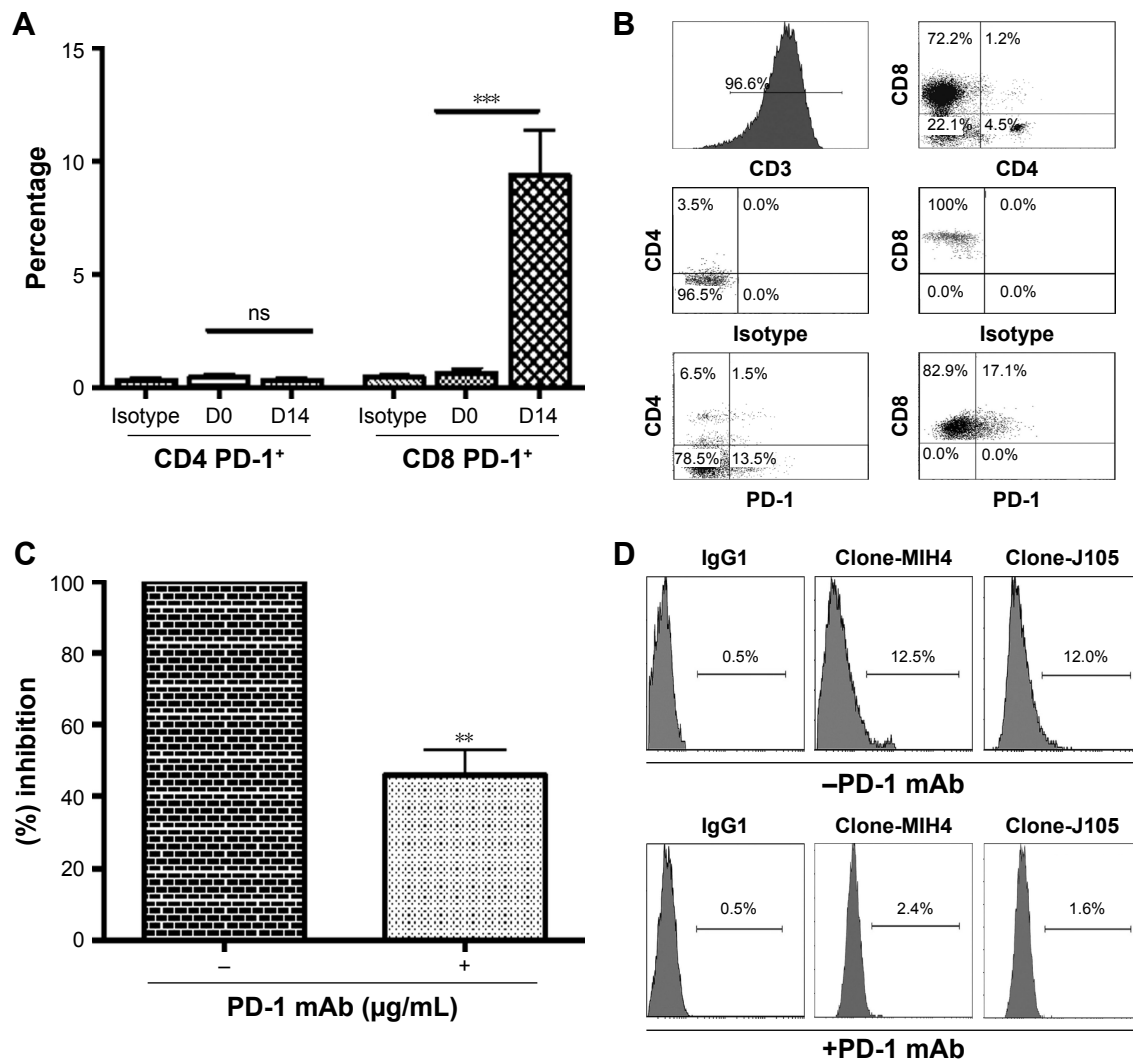
(Figure S3A) and found to be unchanged. CTLA-4 and PD-1 expression was determined and we observed a significant increase in the surface expression of PD-1 in most of the CD8<sup>+</sup> T cells (range 1.6%–31%, mean =9.4% *P*<0.0001) (Figure 1A and B), whereas the changes in CTLA-4 expression were unremarkable (Figure S3B). PD-1 expression was significantly lower (*P*=0.0037) (Figure 1C) after incubating with a PD-1 mAb for 48 hrs. Flow cytometric analysis revealed that anti-PD-1 (J105) binds a different epitope than the MIH-4 epitope of purified PD-1 mAb (Figure 1D).

### PD-I blockade enhances the cytotoxic effect of CD8<sup>+</sup> T cells against MMR deficient EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells

To test the effect of the PD-1 blockade on the anti-tumor activity of ex vivo expanded CD8<sup>+</sup> T cells, we evaluated cytotoxicity in colorectal cancer cells in the presence of a PD-1 mAb. We observed an insignificant apoptotic effect in MMR proficient TECs in the presence of PD-1 mAb after 48 hrs (Figure S1C). Therefore, MMR proficient TECs were excluded for further experiments. A dose dependent treatment was carried out at 0.5, 1.0, 2.5, and 5.0 µg/mL to select the optimal dose of PD-1 mAb for the subsequent experiments. The best killing activity of CD8<sup>+</sup> T cells was achieved at doses from 1.0–2.5 µg/mL (Figure 2A). Therefore, 1.0 µg/mL was chosen as the concentration for the following experiments. We further selected an effector-to-target (ET) ratio of 25:1 based on the co-incubation of TECs with CD8<sup>+</sup> T cells at different ET ratios (Figure 2B). Flow cytometric analysis detected a significant increase in apoptosis achieved with or without PD-1 mAb (Figure 2C) in EpCAM<sup>+</sup>PD-L1<sup>+</sup> cells: MMR deficient TECs (*P*=0.005) and DLD-1 cells (*P*=0.0025) (Figure 2D). Indeed, no noteworthy apoptotic effect in MMR proficient SW480 (*P*=0.350) and SW620 (*P*=0.050) were observed. We observed dose-dependent (5, 10, 15, and 30 nM) apoptosis to select suitable concentration of nPD-1 mAb, and found no significant effect of blocking PD-1 mAb in the presence of nPD-1 mAb (Figure 2E) in TECs.

### PD-I blockade increases cytotoxic cytokine secretion and degranulation of CD8<sup>+</sup> T cells against MMR deficient EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells

We performed quantitative ELISpot assays for both IFN-γ and GrB after cells were incubated with the PD-1 mAb at 1.0 µg/mL (Figure 3A and B). IFN-γ secretion was higher in CD8<sup>+</sup> T cells which were co-cultured with TECs (*P*=0.0256)



**Figure 1** The PD-1 expression on ex vivo expanded CD8<sup>+</sup> T cells obtained from patient's blood before surgery.

**Notes:** (A) Mean PD-1 level of CD4 and CD8 cells on day 0 and day 14 (n=11). (B) Representative dot plots of flow cytometry on day 14. (C) Percent normalized PD-1 expression after 48 hrs treatment with PD-1 mAb (n=5). (D) Different clones (MIH4 and J105) used for PD-1 staining on CD8<sup>+</sup> T cells after treatment by flow cytometry. \*\* $P < 0.005$ ; \*\*\* $P < 0.0005$ .

**Abbreviations:** mAb, monoclonal antibody; ns, not significant.

or DLD-1 cells ( $P=0.0361$ ) than in the control (Figure 3C). Similar results were also observed in GrB<sup>+</sup> CD8<sup>+</sup> T cells co-cultured with TECs ( $P=0.0072$ ) or DLD-1 cells ( $P=0.0461$ ) (Figure 3D). To determine the level of degranulation, CD107a mobilization was studied using flow cytometry (Figure 3E). PD-1 blockade enhanced the surface CD107a mobilization on CD8<sup>+</sup> T cells which were co-cultured with TECs ( $P=0.018$ ) or DLD-1 cells ( $P=0.0782$ ), as shown in Figure 3F.

### PD-1 blockade enhanced the interaction and cytosolic uptake between CD8<sup>+</sup> T cells and EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells

To analyze the chemotactic interactions between CD8<sup>+</sup> T cells and tumor cells after PD-1 blockade, tumor cells were stained with the non-invasive dye CMFDA (5 µM), for 20 min

at 37°C and then co-cultured with CD8<sup>+</sup> T cells for 48 hrs.<sup>33</sup> After culture, the cells were analyzed by flow cytometry. PD-1 blockade significantly increased the proportion of CD8<sup>+</sup>CMFDA<sup>+</sup> cells among T cells co-cultured with TECs ( $P=0.0002$ ) or DLD-1 cells ( $P=0.05$ ) (Figure 4A). We evaluated chemotactic attraction using a transwell migration assay. Pre-treatment with PD-1 mAb at 1.0 µg/mL significantly enhanced the number of CD8<sup>+</sup> T cells which migrated to the lower chamber of the transwell insert in the presence of TECs ( $P < 0.0001$ ) or DLD-1 cells ( $P=0.0015$ ), as shown in Figure 4B.

### Discussion

In recent years, cancer immunotherapy has emerged as an efficacious treatment modality which produces clinically valid outcomes in certain types of cancer.<sup>34,35</sup> The most

promising strategies for cancer immunotherapy include a variety of approaches, such as the use of therapeutic antibodies which target immune checkpoint molecules, dendritic cell-based vaccines, adoptive transfer of ex vivo activated T cells, and the combinations of these strategies with or without chemo/radiotherapy.<sup>36-38</sup>

Although the impressive treatment outcomes have been obtained using TILs and CD19 targeted CAR T cells, demonstrating the promise of T cell-based adoptive immunotherapy, these successes have not been translated beyond early stage clinical studies.<sup>35</sup> It has become apparent that tumors develop multiple inhibitory networks to effectively evade immune surveillance.<sup>39</sup> The immunosuppressive microenvironment has been recognized as one of the hallmarks of tumors.<sup>40</sup> Strategies aimed at attenuating the endogenous immunosuppressive environment using non-myeloablative chemotherapy, irradiation or therapeutic mAbs against immune checkpoint inhibitors have been shown to improve the clinical efficacy of adoptive T cell immunotherapy for solid tumors.<sup>41</sup> However, lower effectiveness of ex vivo expanded T cells which express high levels of iRs is often ignored.

Adoptive T cell therapy generally requires the ex vivo expansion of a large number of T cells to allow an infusion to achieve therapeutic benefits. The CD3/TCR complex

stimulation of tumor reactive T cells mediated by co-stimulatory signals to retain proliferation and functional properties of antigen specific T cells.<sup>32</sup> This procedure is similar to that used to expand tumor-specific T cells, non-specific cytotoxic cells (eg, NK, NKT, CD8<sup>+</sup> T), and MHC-independent CAR T cells. Chronic exposure to cytokines or tumor antigens leads to the expansion of T cells and causes them to develop artificial exhaustion prior to transfusion.<sup>42,43</sup> In our study, we demonstrate that surface expression of PD-1 on the expanded CD8<sup>+</sup> T cells was dramatically increased (by 10-fold) during the classic ex vivo expansion procedure with OKT3 and IL-2. This finding supports a previous study which showed that transfused TILs produced a high PD-1 level in a melanoma animal model.<sup>44</sup> We showed that blocking PD-1 interaction after ex vivo expansion significantly enhanced the cytotoxicity of CD8<sup>+</sup> T cells toward EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells, and this increased the release of the cytotoxic IFN- $\gamma$  and GrB, as well as CD8<sup>+</sup> T cell degranulation. Moreover, PD-1 blockade improved the interaction and cytosolic exchange between CD8<sup>+</sup> T cells and EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells.

The expression of PD-L1 on cancer cells has been associated with poor prognosis in cancer, demonstrating that PD-1/PD-L1 pathway-mediated immune tolerance has clinical

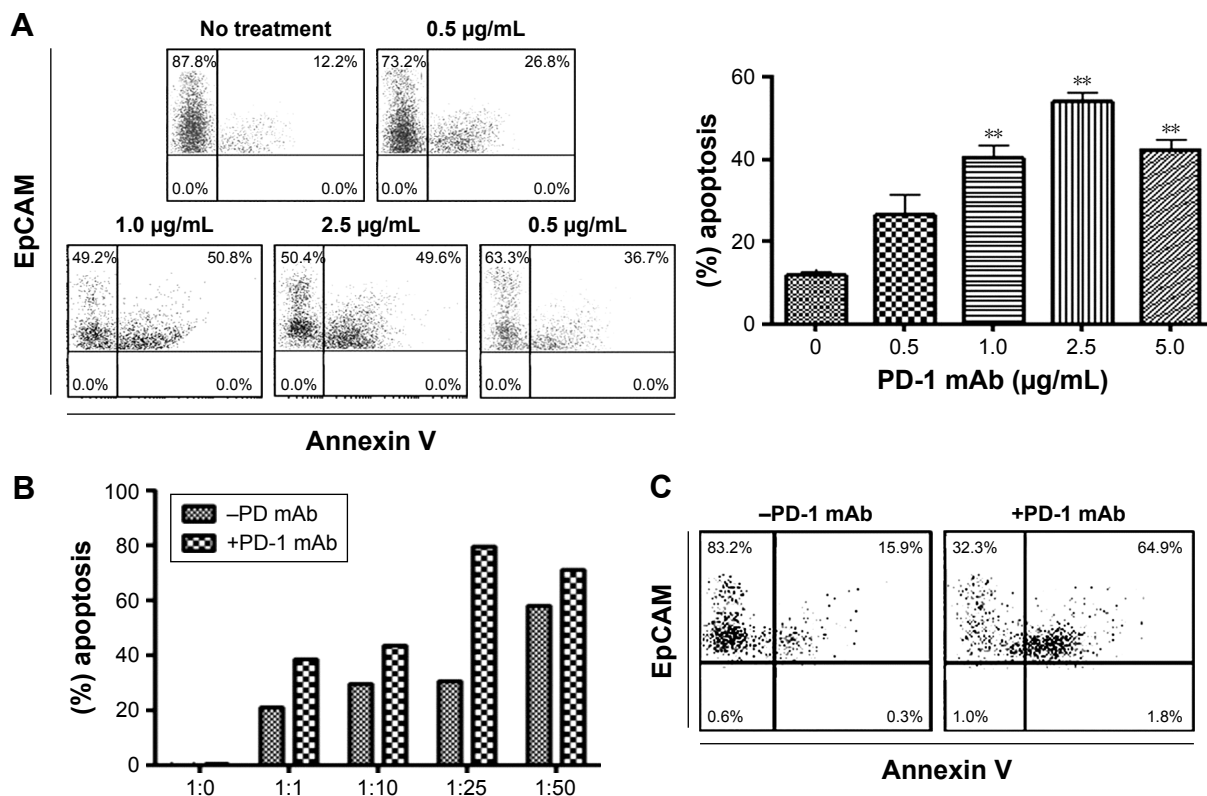
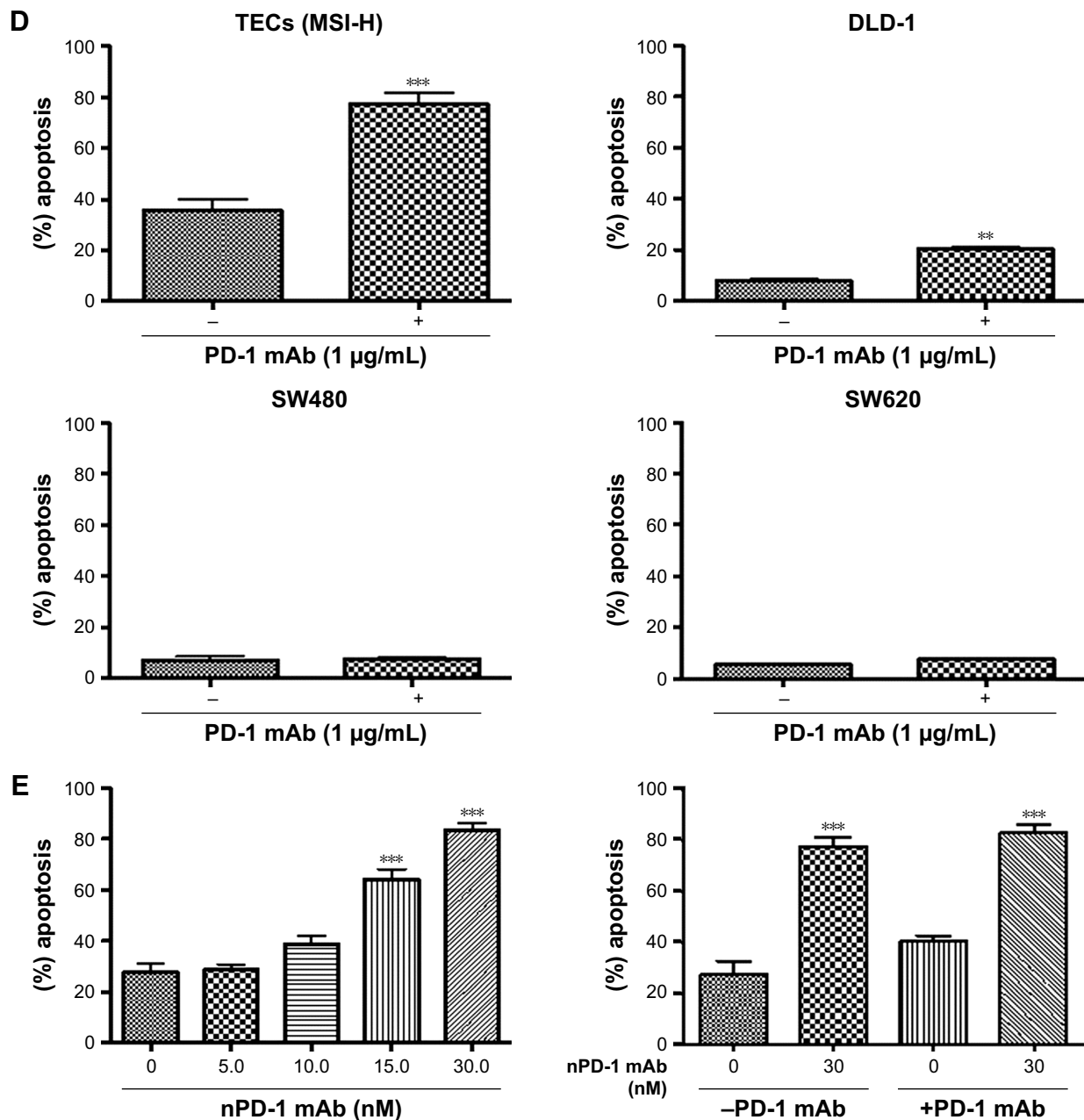


Figure 2 (Continued)



**Figure 2** Apoptotic effect of CD8<sup>+</sup> T cells on EpCAM<sup>+</sup> tumor cells after PD-1 blockade for 48 hrs.

**Notes:** (A) Different concentrations of PD-1 mAb used to optimize the dose (n=3). (B) Apoptosis measured at different effector-to-target ratios, ranging from 1:1 to 50:1. (C) Representative dot plot of apoptosis in EpCAM<sup>+</sup> TECs (D) Differential change of apoptosis in tumor cells; TECs (MSI-H), DLD-1, SW480, and SW620 after PD-1 mAb treatment (n=5). (E) Cell death induced in dose-dependent manner after PD-1 epitope blockade of PD-1 protein (left) at 1.0 µg/mL, and PD-1 mAb neutralized (right) in presence of nPD-1 antibody (n=3). \*\**p*<0.005; \*\*\**p*<0.0005.

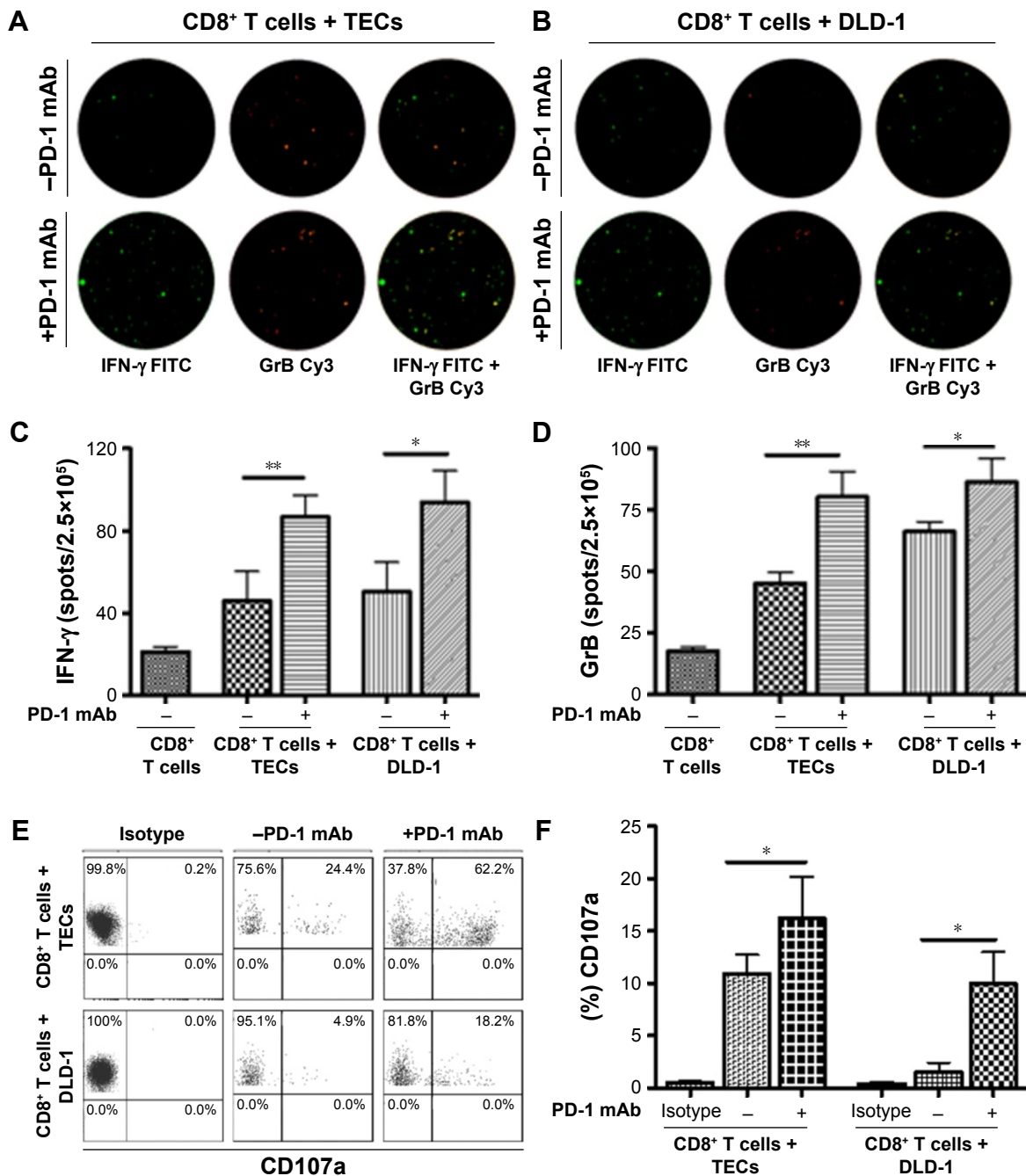
**Abbreviations:** mAb, monoclonal antibody; TECs, tumor epithelial cells; MSI-H, high microsatellite instability.

significance.<sup>39,40</sup> The results of a previous study demonstrated that PD-1 blockade partially enhanced the cytotoxicity of PD-1<sup>+</sup> CD8<sup>+</sup> T cells against lymphoma.<sup>41</sup>

The CK20 identified as a diagnostic and prognostic biomarker that classified in colorectal cancer by MMR status, and the inverse correlations have observed in the higher tumor grade. However, the association between MMR status and tumor grades has not been specifically investigated.<sup>45</sup> In our

study we found typical expression of CK20 in MMR deficient TECs, which were isolated from the primary tumor site.

The clinical trial of PD-1 mAb reached the primary endpoint, showing striking immune related response and significant disease control rate. Perhaps the increased mutation-associated tumor antigens need to be further demonstrated in MMR deficient colon cancer.<sup>21,46</sup> In the current study, we have shown that PD-1 blockade induced a high



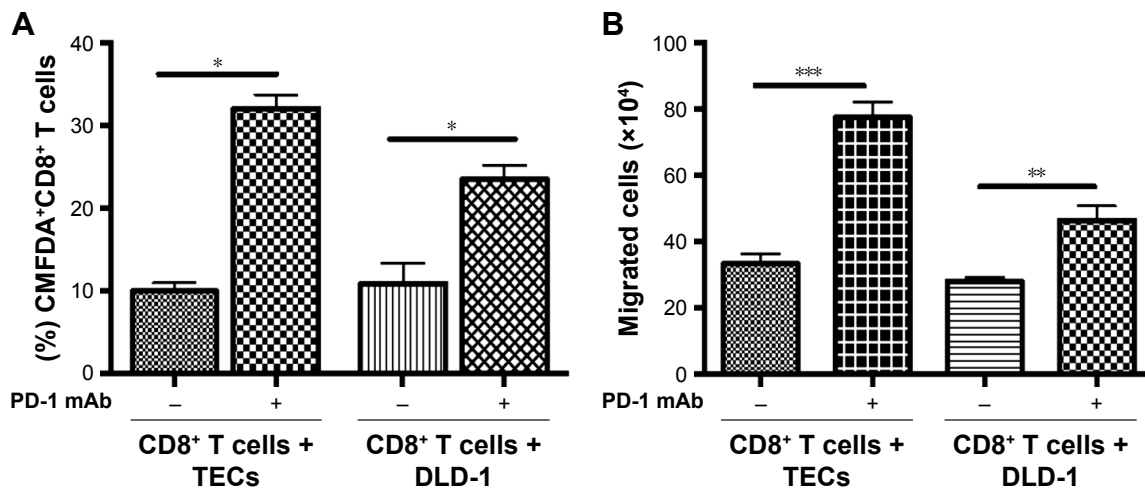
**Figure 3** Effect of PD-1 mAb on cytolytic IFN- $\gamma$ , GrB, and CD107a. **Notes:** Mobilization after 48 hrs. Representative spots of (A) IFN- $\gamma$  and (B) GrB from CD8<sup>+</sup> T cells co-cultured with TECs and DLD-1 respectively. Histogram of (C) IFN- $\gamma$  in CD8<sup>+</sup> T cells + TECs and CD8<sup>+</sup> T cells + DLD-1 and (D) GrB in CD8<sup>+</sup> T cells + TECs and CD8<sup>+</sup> T cells + DLD-1 (n=5). (E) Dot plot of CD107a mobilization and (F) histogram of CD8<sup>+</sup> T cells + TECs and CD8<sup>+</sup> T cells + DLD-1 (n=4). \*P<0.05; \*\*P<0.005. **Abbreviations:** mAb, monoclonal antibody; TECs, tumor epithelial cells.

killing effect in MMR deficient (MSI-H) EpCAM<sup>+</sup>PD-L1<sup>+</sup> cancer cells. Mechanistically, we have shown that EpCAM<sup>+</sup>PD-L1<sup>+</sup> TECs and DLD-1 cells engage PD-1<sup>+</sup> T cells via the PD-1 ligand. Our results demonstrate that PD-1 blockade enhanced cancer cell apoptosis, the production of cytolytic molecules, and CD8<sup>+</sup> T cell degranulation. Moreover, it was previously shown that blocking PD-1 promoted

the migration of transfused T cells to tumor sites.<sup>38</sup> Our data also indicate that PD-1 blockade promotes the migration and cytosolic exchange between CD8<sup>+</sup> T cells with EpCAM<sup>+</sup>PD-L1<sup>+</sup> TECs and DLD-1 cancer cells.

Our results offer a viable means of restoring T cell exhaustion during the traditional expansion procedure and obtaining highly functional CD8<sup>+</sup> T cells which can be used





**Figure 4** Cellular interactions, cytosolic uptake, and CD8<sup>+</sup> T cells' migration after 48 hrs.

**Notes:** (A) Cytosolic uptake by CD8<sup>+</sup> T cells from TECs and DLD-1 cells validated by flow cytometry (n=3). (B) Chemotactic attraction increased after pre-treatment with PD-1 mAb; conditioned medium from TECs and DLD-1 cells was used in the lower chamber of the transwell plate (n=3). \*P<0.05; \*\*P<0.005; \*\*\*P<0.0005.

**Abbreviations:** mAb, monoclonal antibody; TECs, tumor epithelial cells.

for adoptive immunotherapy. Furthermore, this function can be restored after blocking PD-1 using mAb, peptides, small molecule inhibitors and advance knock-out technologies during ex vivo expansion prior to T cell transfusion.<sup>47–49</sup> The transfusion of effective CD8<sup>+</sup> CTLs with lower iRs might be associated with better clinical efficacy, but verification of this hypothesis awaits further human studies.

## Conclusion

One of the challenges of adoptive T cell transfusion is the lack of efficient tumor-killing T cells, which results in poor clinical responses. Here, we investigated whether blocking of PD-1 on CD8<sup>+</sup> CTLs during ex vivo expansion could potentiate the tumor-killing activity and therefore, achieve effective adoptive immunotherapy. We demonstrated that after ex vivo expansion, the level of PD-1 on CD8<sup>+</sup> CTLs increased dramatically. PD-1 blockade could significantly potentiate the cytotoxic activity of CD8<sup>+</sup> CTLs against MMR deficient EpCAM<sup>+</sup>PD-L1<sup>+</sup> tumor cells in addition to increasing the secretion of IFN- $\gamma$ , GrB, and CD107a. Moreover, PD-1 blockade enhanced cell migration and cytosolic exchange between CD8<sup>+</sup> CTLs and EpCAM<sup>+</sup>PD-L1<sup>+</sup> tumor cells. Our findings might provide a new insight that may improve the clinical efficacy of cell-based adoptive immunotherapy.

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## Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

## Disclosure

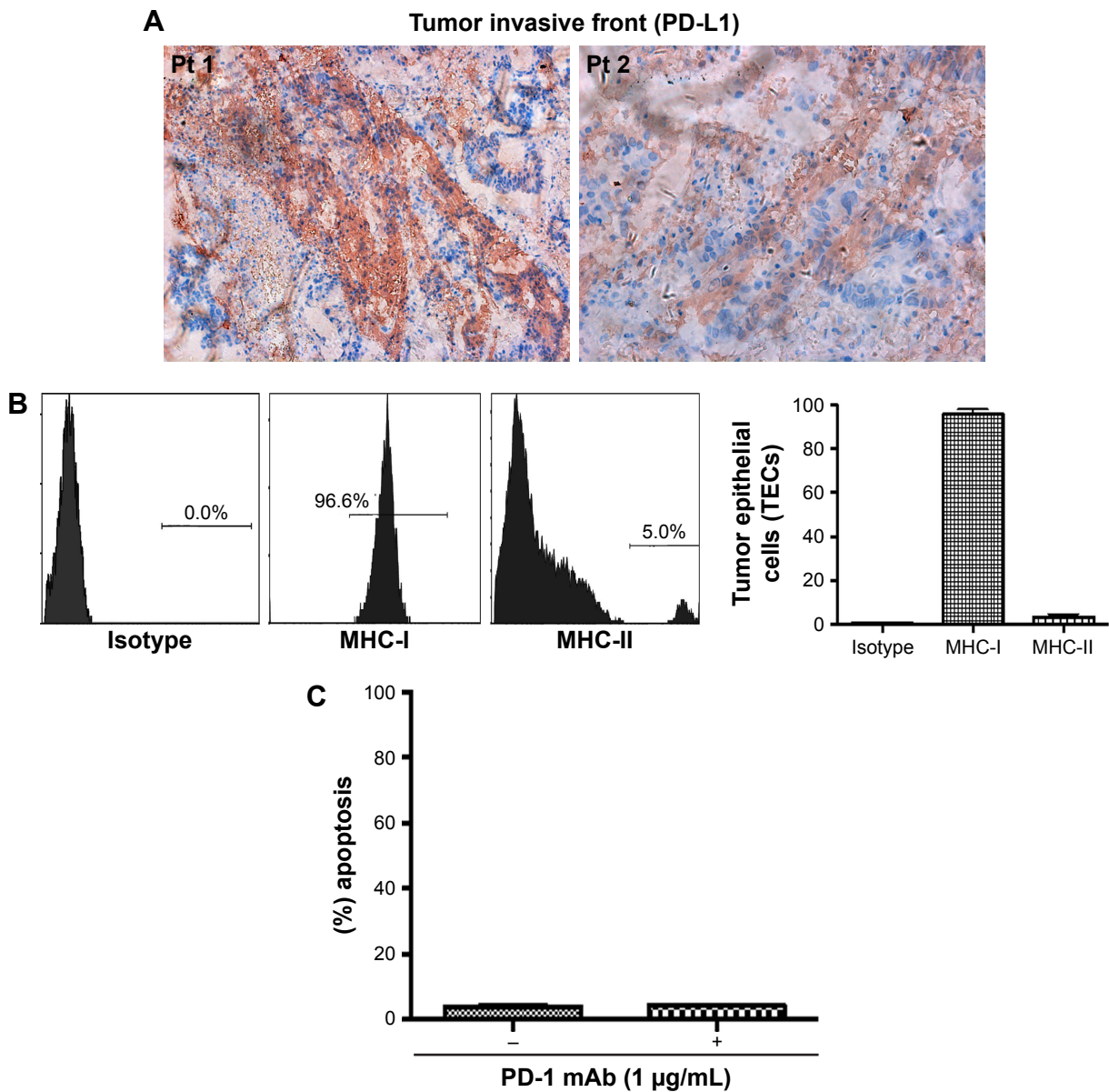
The authors report no conflicts of interest in this work.

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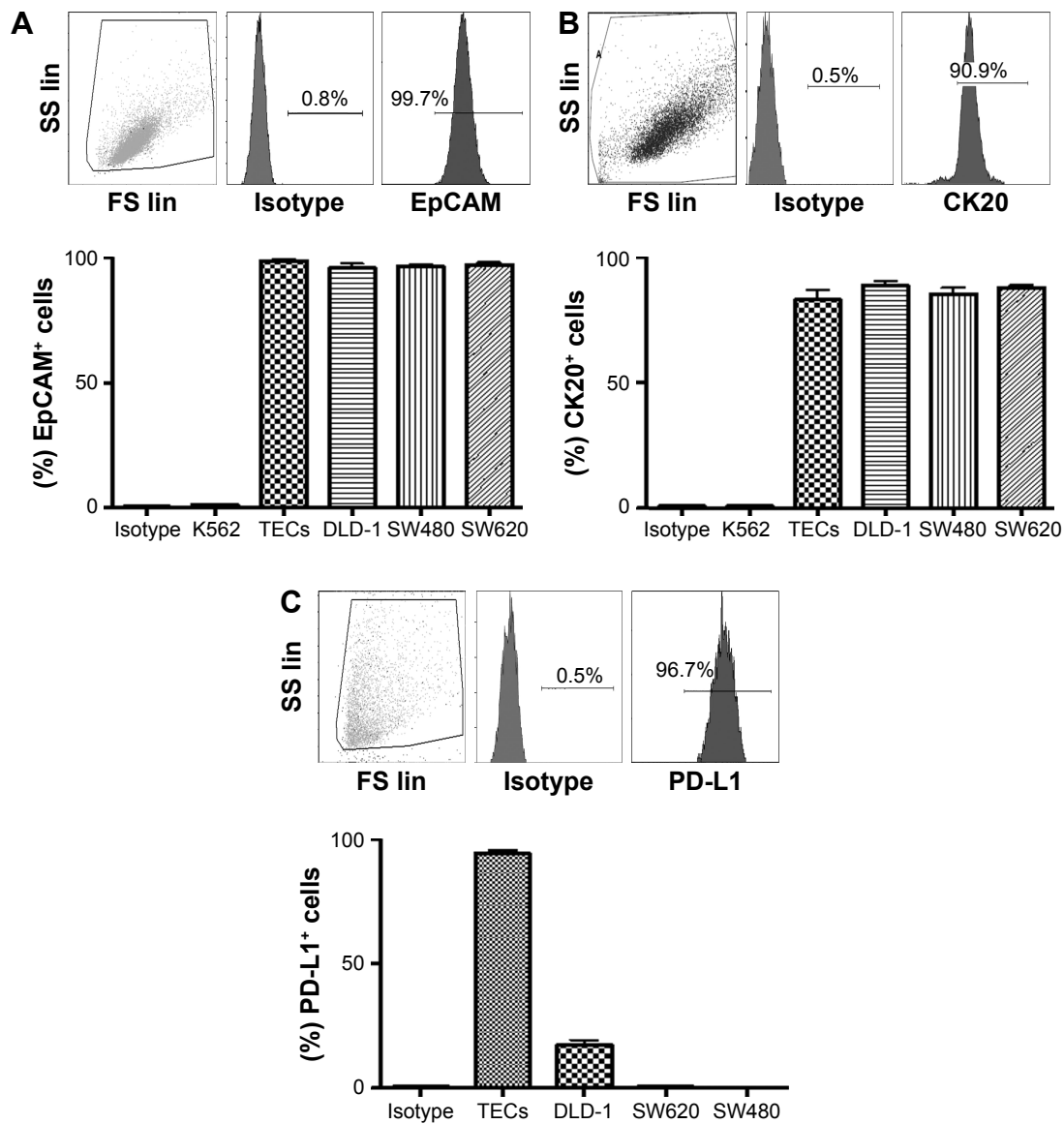
## Supplementary materials



**Figure S1** Information on primary tumor.

**Notes:** (A) PD-L1 staining on 5 µM tumor sections utilized for TECs' isolation. (B) MHC-I/II staining of isolated TECs shown by flow cytometry, and (C) apoptotic effect of CD8<sup>+</sup> T cells on TECs (MSS).

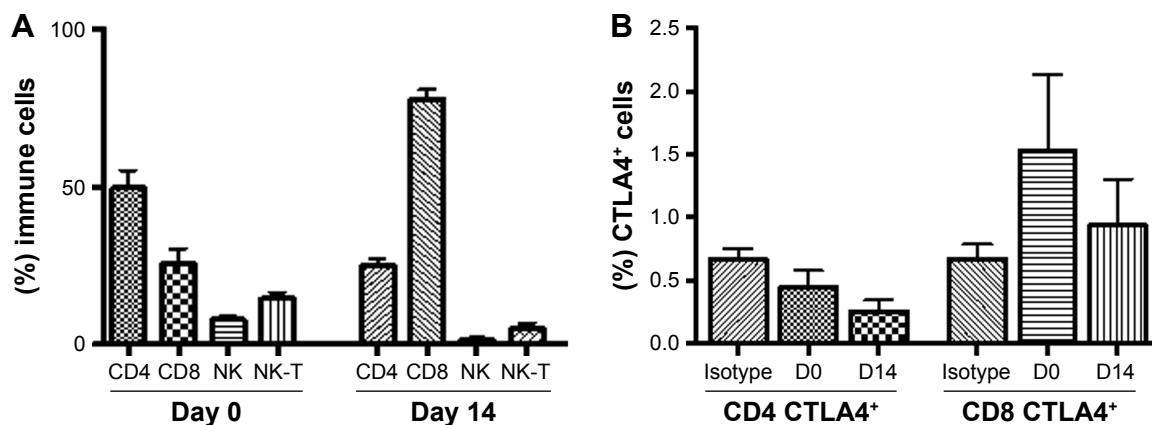
**Abbreviations:** TECs, tumor epithelial cells; Pt, patient; MSS, MMR proficient; MMR, mismatch repair.



**Figure S2** The representative dot plot of flow cytometry staining of tumor antigens, and average level on TECs, DLD-1, SW480, and SW620 along with K562 as a negative control (n=3).

**Note:** (A) Extracellular EpCAM, (B) intracellular CK20, and (C) intracellular PD-L1.

**Abbreviations:** TECs, tumor epithelial cells; CK20, cytokeratin 20; SS lin, side scatter linear; FS lin, forward scatter linear.



**Figure S3** Percentages of (A) killer immune cells, and (B) intracellular CTLA4 before and after ex vivo T cell expansion by flow cytometry (n=11).

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