



Disruption of CTLA-4 expression on peripheral blood CD8 + T cell enhances anti-tumor efficacy in bladder cancer

Wei Zhang¹ · Long Shi³ · Zhilong Zhao⁴ · Pingping Du⁵ · Xueshuai Ye⁵ · Dongbin Li⁶ · Zhenhua Cai⁷ · Jinsheng Han⁸ · Jianhui Cai^{1,2}

Received: 1 October 2018 / Accepted: 22 February 2019 / Published online: 8 March 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Activation of programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T cells leads to T cell exhaustion and ultimately facilitates tumor progression. Recent success of using immune cell checkpoint inhibitors offers a great promise to treat various cancers, including bladder cancer. However, the expression pattern and therapeutic value of PD-1 and CTLA-4 in peripheral blood T cells remain largely unexplored. In this study, we presume that disruption of the potential dysregulated checkpoint molecules in peripheral blood T cells may improve the anti-tumor efficacy of cytotoxic T cells in bladder cancer. We showed that both PD-1 and CTLA-4 expression were specifically elevated on CD8 + T cells but not CD4 + T cells in peripheral blood of patients with bladder cancer compared with that in healthy donors. Notably, CTLA-4 expression was significantly higher in muscle-invasive bladder cancer (MIBC) and correlated with tumor size. By blocking CTLA-4 with anti-CTLA-4 antibody and CRISPR-Cas9-mediated CTLA-4 disruption, we revealed that CTLA-4-disrupted CTLs had enhanced cellular immune response and superior cytotoxicity to the CD80/CD86-positive bladder cancer cells in vitro. Moreover, the CTLA-4-disrupted CTLs exhibited a pronounced anti-tumor effect in vivo as demonstrated by prophylactic assay and therapeutic assay in the subcutaneous xenograft model. Collectively, our findings confirm improved therapeutic efficacy of CTLA-4-disrupted CTLs and provides the potential strategy for targeting immune checkpoints to enhance the promising immunotherapy.

Keywords Immune checkpoint blockade · PD-1 · PD-L1 · CTLA-4 · Bladder cancer

✉ Jianhui Cai
jianhuicai2001@163.com

¹ Graduate school of Hebei Medical University, Shijiazhuang 050017, Hebei Province, People's Republic of China

² Department of Surgery, Department of Oncology and Immunotherapy, Hebei General Hospital, 348 Heping West Road, Shijiazhuang 050000, Hebei Province, People's Republic of China

³ Department of Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei Province, People's Republic of China

⁴ Department of Surgery, The Third Affiliated Hospital of Jinzhou Medical University, Jinzhou 121000, Liaoning Province, People's Republic of China

⁵ Center of Cell Therapy Engineering Technology, Hebei NOFOY Bio-Tech Co. Ltd., 238 Changjiang Avenue, High-tech Zone, Shijiazhuang 050035, Hebei Province, People's Republic of China

⁶ Department of Gastrointestinal Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang 050011, Hebei Province, People's Republic of China

⁷ Handan Central Hospital, Handan 056001, Hebei Province, People's Republic of China

⁸ Cangzhou Sino-Western Integrated Hospital, Cangzhou 061000, Hebei Province, People's Republic of China

Introduction

Bladder cancer is a prevalent urogenital malignant tumor in the US and is the most frequently diagnosed urological cancer in China, accounting for approximately 3% of total cancer-related death [1]. Clinically, bladder cancer can be divided into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Although most patients present with NMIBC (Ta–T1), approximately 30–40% of patients have muscle-invasive disease (T2–T4a). Muscle invasion is closely correlated with the clinical outcome of bladder cancer patients, presumably due to the presence of metastasis at the time of diagnosis. Therefore, it is indispensable for us to develop new therapies for bladder cancer [2].

The recent success with using inhibitors targeting immune checkpoint molecules PD-1, PD-L1, and CTLA-4 offers great promise for various cancer therapies [3–5]. In bladder cancer, atezolizumab and nivolumab, two immune checkpoint inhibitors, have been approved for treatment in the second-line setting [4, 6]. Programmed cell death1 (PD-1) is a major immune checkpoint molecules expressed on both activated and exhausted T cells, and mediates inhibition of cytokine secretion and lytic activity [7, 8]. The interactions between PD-1 on T cells and PD ligand-1 (PD-L1) on tumor cells or antigen presenting cells (APCs) can suppress T cell activation and T-cell-mediated tumor cell killing [9, 10]. Numerous reports have examined PD-1 and CTLA-4 expression on tumor-infiltrating T-cells, and their correlation with patients' prognosis has been discussed [11–14]. In bladder cancer, several immune checkpoint inhibitors that target PD-1, its ligand PD-L1, and CTLA4 have already been approved for use, representing the most important change to the urological oncologist's tool-kit in the past decade [15]. However, PD-1 and CTLA-4 expression on the peripheral blood T cells of cancer patients, particularly in those with bladder cancer, remain largely unknown.

In this paper, we analyzed PD-1 and CTLA-4 expression on peripheral blood T-cells of bladder cancer patients and found that both PD-1 and CTLA-4 expression were specifically elevated on CD8+ T cells. Specifically, CTLA-4 but not PD-1 expression was highly expressed in MIBC and closely associated with the tumor size. Subsequently, we blocked CTLA-4 on CTLs and tested whether CTLA-4 disruption on CTLs can enhance cytotoxic activity in vitro and anti-tumor efficiency in vivo.

Materials and methods

Cell culture and reagents

The human bladder cancer cell lines (UM-UC-3, TCCSUP, J82, T24 and 5637) were all purchased from Cell Resource

Center of the Chinese Academy of Medical Sciences (Shanghai, China). All cells were all cultured in Dulbecco modified Eagle medium (DMEM, Invitrogen, USA) containing 10% fetal bovine serum (FBS), and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma) at 37 °C in humidified air with 5% CO₂. All cell lines were tested for free of mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza). Neutralizing anti-CTLA-4 antibody was purchased from LifeSpan BioSciences, Inc.

Clinical specimens

A total of 62 patients met the histological criteria for bladder cancer was enrolled in this study (Table 1). Tumor size was determined by histopathologic method. Peripheral blood specimens from these patients were obtained, and informed consents were signed for sample collection according to a protocol approved by the Ethics Committee of HeBei General Hospital. Additionally, 32 normal donors were also obtained from the HeBei General Hospital. All the healthy human donors were provided with written informed consent before enrollment.

Flow cytometric analysis

Isolated PBMCs from patients or healthy donors were suspended in PBS. Briefly, 4 ml of human venous blood sample in heparinised vials (BD biosciences, USA) was gently mixed by inverting the tube several times. Then, 4 ml of Ficoll Histopaque (Sigma-Aldrich, #10771) was prepared in a 15 ml centrifuge tube, followed by layering the blood on the top of Ficoll Histopaque. Centrifuged the tubes for 30 min at 100 g in 4 °C and aspirated the whitish buffy coat (PBMCs) formed in the interphase between histopaque and medium. Finally, washing twice with 10 ml of sterile Dulbecco's modified eagle medium (centrifuge in 100×g for 10 min). PBMCs were immediately stained or frozen for posterior analysis. Samples were incubated for 30 min on ice with appropriate dilution of antibodies. The antibodies used in this study were shown as follows: anti-CD4-FITC (clone RPA-T4), anti-CD8-PerCP-Cy5.5 (clone RPA-T8), anti-PD-1-APC (clone MIH4), anti-CTLA-4-APC (Franklin Lakes, NJ, USA). Meanwhile, the APC mouse IgG1 (BD Biosciences) and PE mouse IgG2b (BioLegend) were used as negative controls. All the stained cells were analyzed using FACS Ariar (BD Bioscience). The detailed methods were available elsewhere as previously reported [16, 17].

CRISPR-Cas9 mediated CTLA-4 disruption

Cas9 RNP-mediated CTLA-4 disruption on CTLs was carried out similar to the previous article reported by Schumann et al. with modifications [18]. Three single guided

Table 1 Correlation of PD-1 and CTLA-4 expression on CD8+CTLs with clinical parameters of 62 bladder cancer patients

Clinical parameter	N	PD-1 expression		p value	CTLA-4 expression		p value
		Low	High		Low	High	
Age (years)				0.798			0.124
< 60	27	13	14		10	17	
≥ 60	35	18	17		21	14	
Gender				0.799			0.309
Male	33	16	17		19	14	
Female	29	15	14		12	17	
Histological grade				0.793			0.600
High	39	20	19		18	21	
Low	23	11	12		13	10	
Tumor size				0.445			0.002
< 3 cm	33	15	18		23	10	
≥ 3 cm	29	16	13		8	21	
Tumor category				0.309			0.002
NMIBC	32	14	18		22	10	
MIBC	30	17	13		9	21	

NMIBC non-muscle-invasive bladder cancer, MIBC muscle-invasive bladder cancer

RNAs and a negative control were designed. The sequence was shown as follows, sg1: CTCAGCTGAACCTGGCTACC; sg2: CCTTGGATTTCAGCGGCACA; sg3: GCACAA GGCTCAGCTGAACC, and control: ACGGAGGCTAAG CGTCGCAA. Briefly, PBMCs isolated from bladder cancer patients or healthy donors were transfected with the intended plasmids by Nucleo-factor II (Lonza). After washing, cells were resuspended in a 100 ml transfection buffer and then electroporation was performed.

Enzyme-linked immunosorbent assay (ELISA)

The production of IFN- γ and TNF- α was detected with a cytokine ELISA using a commercial ELISA kit (ExCell Bio, Shanghai, China). Briefly, 96-well plates coated with cytokine capture antibodies were firstly blocked with blocking buffer for 1 h at room temperature. After washing three times with the washing buffer, 100 μ l cell culture supernatant was added and incubated at room temperature for 2 h, followed by incubation with detection of antibody at room temperature for 1 h. Finally, 100 μ l diluted streptavidin peroxidase was added, followed by TMB substrate (Sigma, USA). After the reaction was stopped, enzyme activity was measured at 450 nm using a multi-functional microplate reader.

In vitro culture and induction of dendritic cells (DCs)

Freshly collected 50 ml of anticoagulated peripheral blood from healthy volunteers was diluted 1 time with 37 °C 1 \times PBS, and slowly poured into the centrifuge tube containing lymphocyte separation solution (Ficoll-Hypaque,

density 1.077 g/l) along the tube wall (volume and separation volume ratio is 1:1). Gradient centrifugation was performed at room temperature. The interface cells were taken, placed in a 50 ml centrifuge tube, and the cells were suspended with calcium-free magnesium PBS. Cells were then centrifuged (800 \times g, 10 min), washed twice, and centrifuged (250 \times g, 10 min) again. PBMCs were obtained after the platelets were washed out. The mononuclear cells were evenly dispensed into a culture flask, the suspension cells and culture solution were removed and the adherent cells were DC cells. Resuspend the cells in RPMI 1640 complete medium containing 10% fetal bovine serum, adjust the cell density to 1 \times 10⁶ cells/ml, and incubate in an incubator at 37 °C with a volume fraction of 5% CO₂. After incubation for 2 h, the supernatant was aspirated, and the non-adherent cells were washed away with a pre-warmed culture medium containing 1000 U/ml rhGM-CSF and 500 U/ml to obtain adherent cells. The growth status, morphology and quantity changes were observed daily under an inverted microscope and photographed. After 24 h of culture, 20 ng/ml TNF- α was added to each group to promote DCs maturation, and DCs were harvested on the 7th day. At this time, the cells were mature DCs, and the DCs phenotype was detected by flow cytometry. The supernatant was stored in a refrigerator at -80 °C and used for ELISA to detect IL-12 levels.

CD3 antibody-stimulated T cell activation

T lymphocytes express CD3 specifically, and are further divided into CD4⁺, CD8⁺ T cell subsets; therefore, the changes of surface molecules of T cells at different time points are detected. In brief, 1 \times 10⁶ T cells were collected

and washed twice with PBS containing 0.5% sodium azide and 0.1% BSA, centrifuged at 400 g/min for 6 min, then the supernatant was discarded, 10 μ l FITC-anti-CD3, FITC-anti-CD4, FITC-anti-CD8 antibody were added and incubated for 10 min in the dark at 4 °C in the refrigerator. The cells were washed twice with 2 ml of cold PBS buffer containing 0.5% sodium azide, 0.1% BSA, resuspended in cold 1% paraformaldehyde, and tested by an upflow tester, analyzed by CellQuest software.

Quantitative real-time PCR

RNA was isolated from bladder cancer cell lines using the RNeasy mini kit (Qiagen) and cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen, Shanghai, China). cDNA was amplified using SYBR Premix Ex Taq™ (Takara, Tokyo, Japan) in a 7500 RT-PCR System (Applied Biosystems, USA). The following primers were used: CD80 forward, 5'-GGCCCGAGTACAAGAACC G-3'; CD80 reverse, 5'-TCGTATGTGCCCTCGTCAGAT-3'; CD86 forward, 5'-GGACAAGCAGTGACCATCAAG-3'; CD86 reverse, 5'-CTGCTCATCTATACACGGTTACC-3'; β -actin forward, 5'-CATGTACGTTGCTATCCAGGC-3'; β -actin reverse, 5'-CTCCTTAATGTCACGCACGAT-3'; quantitative differences between cDNA samples were normalized by including β -actin in all experiments.

Cytotoxicity assay

The cytotoxic activity of CTLs was determined by carboxy-fluorescein succinimidyl ester (CFSE)/propidium iodide (PI) staining assay and analyzed by flow cytometry. Briefly, T24 cells were labeled with 4 mM CFSE (Invitrogen, USA) for 15 min at room temperature in PBS. After washing with PBS for two times, CFSE-labeled T24 cells were then incubated with CTLs with indicated treatment by different effector-to-target ratio (5:1 or 20:1) for 6 h. PI solution (Sigma, Shanghai, China) was added to determine the apoptotic cells.

Animal studies

For generation of immune reconstruction mice, 5-week-old male SCID mice (Chinese Academy of Sciences at Shanghai, China) were intraperitoneally injected with 4×10^7 periphery blood lymphocyte (PBL). For prophylactic assay, 1×10^7 CTL-treated CTLA-4 blocking antibody or control IgG were first transferred by tail vein in 200 μ l saline, followed by subcutaneous transplantation of T24 cells at a dose of 1×10^6 cells suspended in 100 μ l PBS ($n = 5$ per group). For therapeutic assay, T24 cells were first inoculated in SCID mice. When tumors grew to near 100 mm³, mice were randomly divided into two groups and transferred with 1×10^7 CTLs treated CTLA-4 blocking antibody or control

IgG via tail vein ($n = 5$ per group). The housing conditions followed the guidelines of the animal center: temperature, 20–22 °C; humidity, 50–70%; normal diet, and specific pathogen-free. Primary tumor volume was monitored with a ruler every 3 days. After 27 days, the mice were sacrificed using 30 psi CO₂ for <20 s until cardiac arrest. Tumor burden was monitored by calculation of tumor volume. Tumor volume was calculated as follows: volume = (Length) \times (Width)²/2. All animal procedures were approved and monitored by The Ethics Committee of HeBei General Hospital (License no. 201811). All manipulations were performed according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Statistical analysis

Data are reported as the mean \pm SD from at least three separate experiments. Statistical analysis was performed using SPSS (v13.0, Chicago, IL, USA) or GraphPad Prism (version 6.0; GraphPad Software). Correlation between PD-1 expression and clinical parameters was compared through χ^2 test. Intergroup differences in in vitro and in vivo assays were assessed by Student's *t* test. One-way ANOVA was used to make comparisons among multiple groups. *p* value less than 0.05 (two-sided) was considered statistically significant.

Results

Expression pattern of PD-1 and CTLA-4 in peripheral blood of patients with bladder cancer

Sixty-two cases of newly diagnosed bladder cancer patients were included in this study. Table 1 shows their characteristics. Expression of two major co-inhibitory molecules, PD-1 and CTLA-4, on lymphocytes of the isolated PBMCs from 62 bladder cancer patients and 32 healthy donors was examined. The frequency of PD-1 + cells among CD4 + T cells from bladder cancer patients was comparable to that from healthy donors (mean frequency 14.7% \pm 3.5% vs. 13.6% \pm 4.6%, Fig. 1a). In contrast, the expression of PD-1 on CD8 + T cells was significantly higher in bladder cancer patients than that in healthy donors (mean frequency 12.8% \pm 4.5% vs. 8.1% \pm 2.9%, Fig. 1b). The frequency of CTLA-4 + cells among CD4 + T was comparable between bladder cancer patients and healthy donors (Fig. 1c). Meanwhile, the expression of CTLA-4 on CD8 + T cells was also higher in bladder cancer patients than that in healthy donors (mean frequency 10.2% \pm 3.2% vs. 7.5% \pm 2.8%, Fig. 1d). Collectively, these data above indicate that the checkpoint

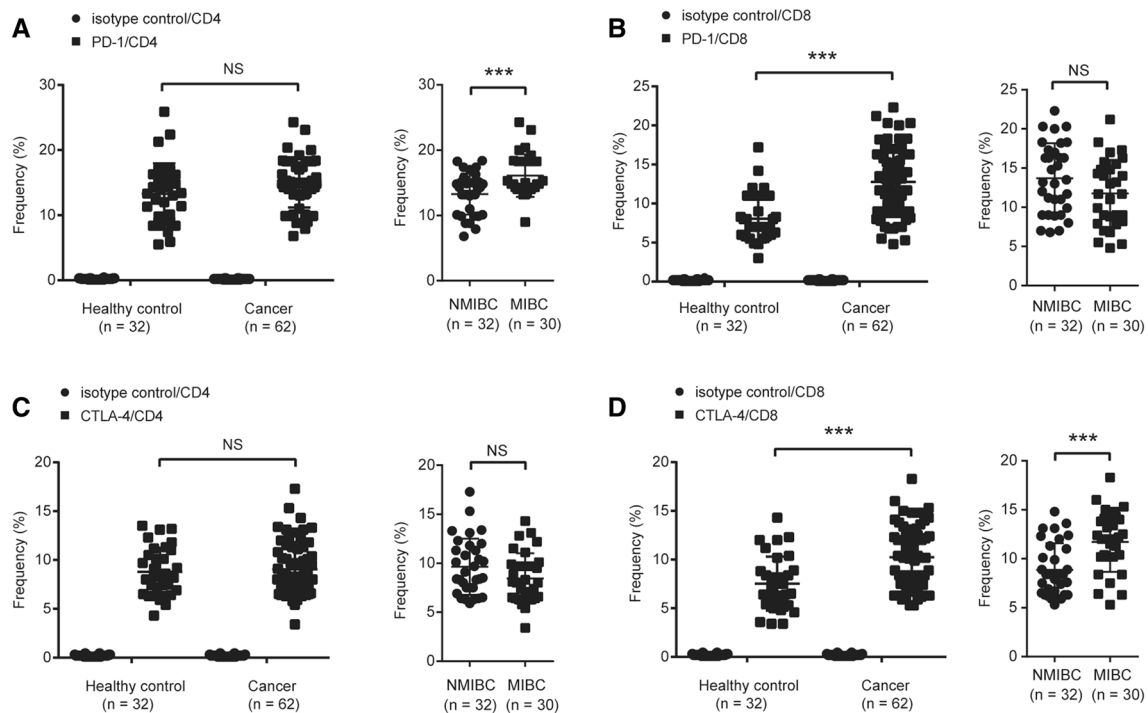


Fig. 1 Expression pattern of PD-1 and CTLA-4 in peripheral blood of patients with bladder cancer. PBMC isolated from patients with bladder cancer ($n=62$) or normal donors (healthy control, $n=32$) was used for quantification of PD-1 and CTLA-4 expression on CD4+ and CD8+ T cells. **a** PD-1 expression in CD4+ T

cells. **b** PD-1 expression in CD8+ T cells. **c** CTLA-4 expression in CD4+ T cells. **d** CTLA-4 expression in CD8+ T cells. For determining the PD-1 and CTLA-4 staining, an isotype-matched control was used. The comparison of each molecule in CD4+ and CD8+ T cells between NMIBC and MIBC is shown in the right panel. *** $p < 0.001$

molecules PD-1 and CTLA-4 in blood CD8+ T cells are often dysregulated in bladder cancer patients. Notably, PD-1/CD4+ and CTLA-4/CD8+ expression in MIBC were significantly higher than that in NMIBC (Fig. 1).

Furthermore, we determined the correlation of PD-1 and CTLA-4 expression on CD8+ T cells with clinical parameters of bladder cancer patients, including age, gender, histological grade, tumor size, and tumor category. Using the mean expression frequency as a cutoff, we divided the samples into low-expression and high-expression group. As a result, no significant correlation between PD-1 expression on CD8+ T cells and any clinical parameters was found (Table 1). However, a close correlation was found between CTLA-4 expression and tumor size; in addition, CTLA-4 expression was preferentially found in the MIBC tumors (Table 1).

CTLA-4 disruption on CTLs enhances cellular immune response and cytotoxicity toward bladder cancer cells in vitro

T-cell inactivation induced by PD-1 and CTLA-4 is thought to be a mechanism underlying immunosuppression. Previously, several reports have demonstrated that CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor effects [19,

20]. In this study, we focused on testing whether disruption of CTLA-4 in CD8+ T cells improves the anti-tumor activity in bladder cancer cells. To test this hypothesis, we first disrupt CTLA-4 expression on CD8+ T cells by CTLA-4 blocking antibodies. We first measured the CTLA-4 expression in control CTLs and anti-CTLA-4-treated CTLs upon stimulation with autologous DCs loaded with epitopes from T24 cells at three time points for a total period of 21 d. As shown in Fig. 2a, the expression of CTLA-4 on CTLs increased from 9.1 to 17.6% in the control group; in the CTLA-4-blocked group, CTLA-4 expression increased from 4.1 to 9.2%. Notably, during the culture period, we found that the proliferation of CTLs was not influenced by CTLA-4 blockage (Fig. 2b). Meanwhile, we noticed higher level of IFN- γ and TNF- α produced by the CTLA-4-blocked CTLs as compared with control CTLs by ELISA (Fig. 2c). Moreover, we sought to determine whether the cytotoxic activity of the modified CTLs is enhanced or not. By quantitative real-time PCR, we found that the ligands for CTLA-4, CD80/CD86, were highly expressed in the T24 bladder cancer cells (Fig. 2d). Then, we co-cultured the CTLA-4-blocked CTLs or control CTLs from five donors with T24 cells. As a result, the cytotoxicity of the CTLA-4-blocked CTLs was significantly improved in a dose-dependent manner toward T24 cells (Fig. 2e).

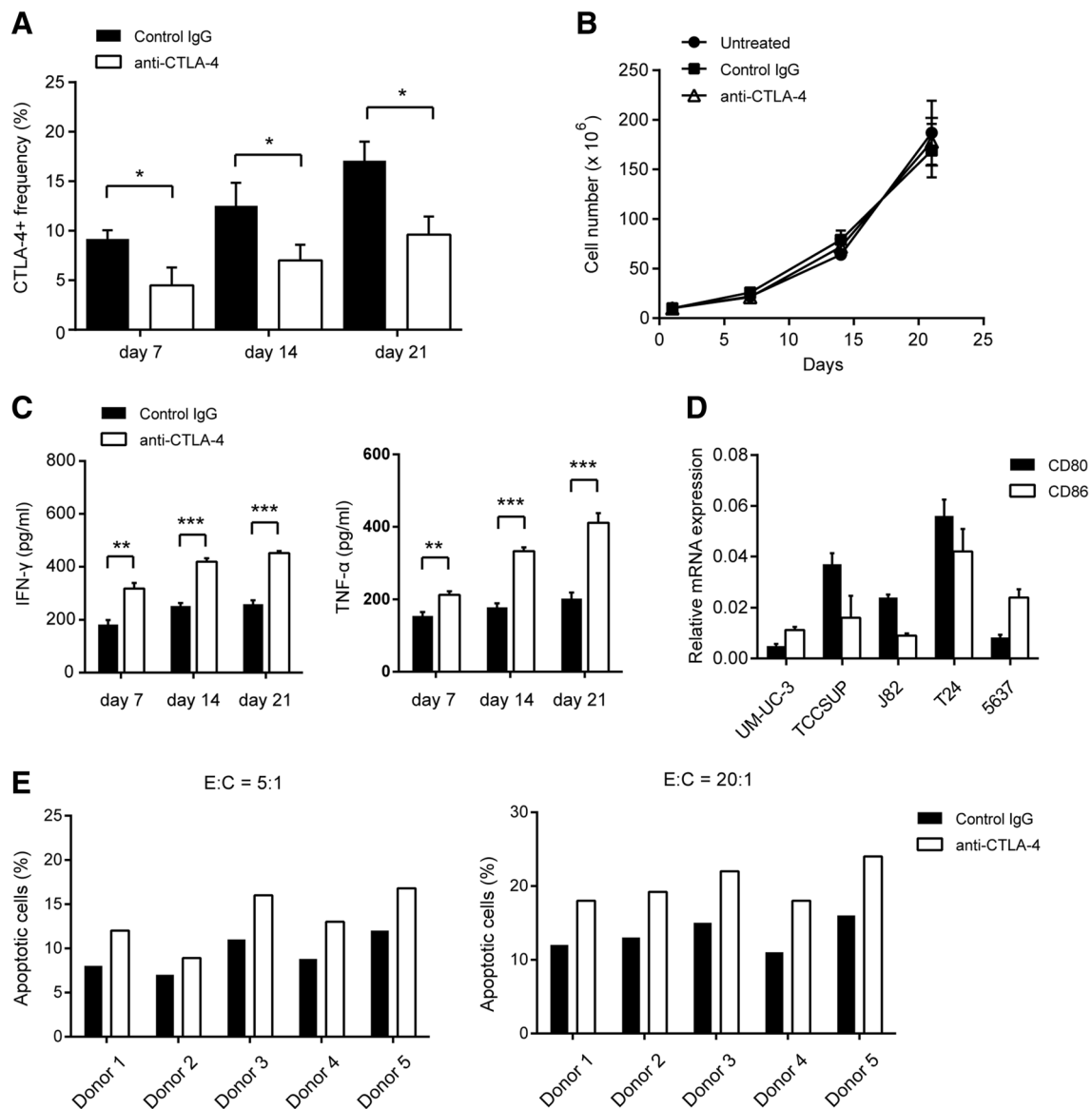


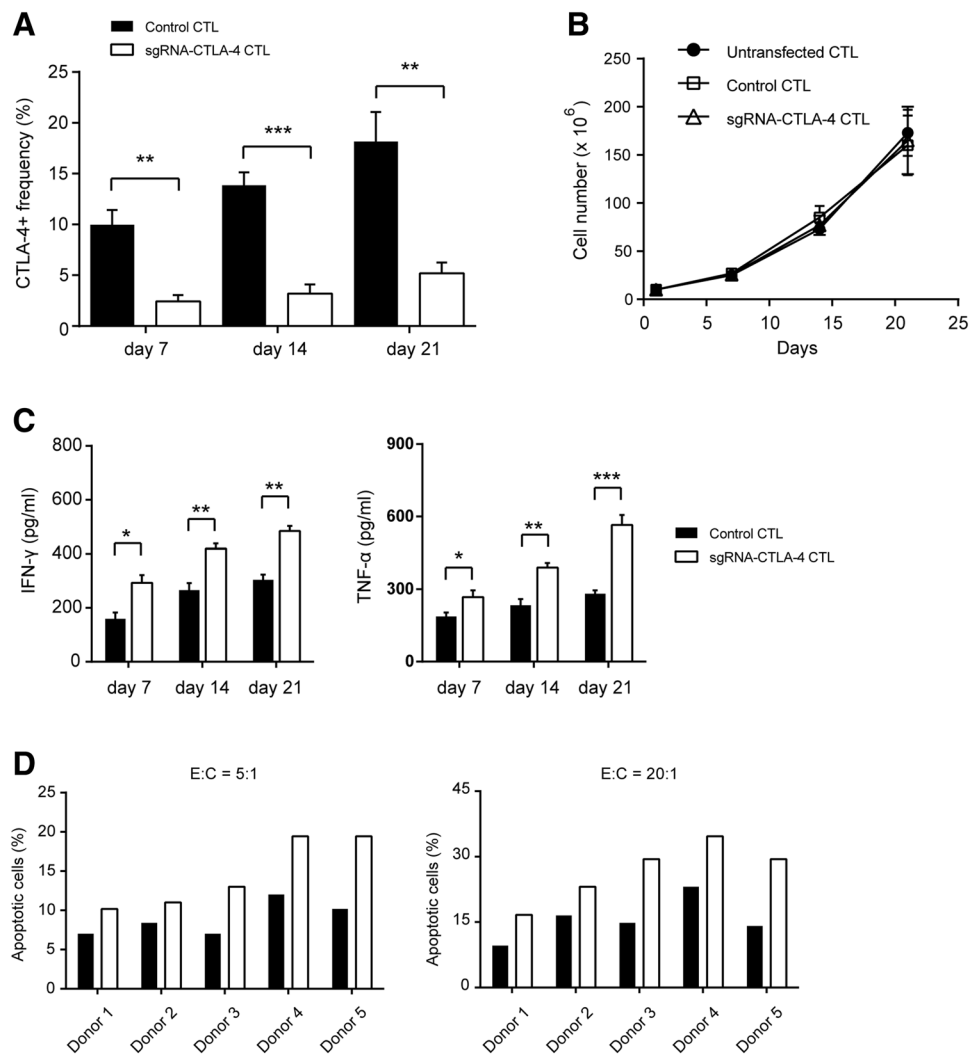
Fig. 2 Blocking antibody-mediated CTLA-4 disruption on CTLs enhances cellular immune response and cytotoxicity toward bladder cancer cells in vitro. **a** The CTLA-4 expression on CTLs was determined by flow cytometry 7, 14, and 21 days after anti-CTLA-4 treatment. **b** The cell proliferation of untreated CTLs, control CTLs, and anti-CTLA-4 treated CTLs was measured every 7 days for a total period of 21 days. **c** Culture medium from anti-CTLA-4-treated

CTLs and control CTLs were assayed for IFN- γ and TNF- α using a cytokine ELISA assay. **d** The mRNA expression of CD80/86 in five bladder cancer cells. **e** Anti-CTLA-4 treated CTLs or control CTLs were co-cultured with CFSE-labeled T24 cells at ratio (E:T) of 5:1 and 20:1, respectively. After 6 h, propidium iodide (PI) was added and the apoptotic cells were analyzed by flow cytometry. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Furthermore, we performed CRISPR-Cas9-mediated gene editing to disrupt CTLA-4 expression on CD8+ T cells. As a result, the expression of CTLA-4 on CTLs drastically reduced and this inhibitory effect was maintained until day 21 (Fig. 3a). Consistently, the proliferation of CTLs was neither influenced by CRISPR-Cas9-mediated CTLA-4 knockout, nor affected by the electroporation as

shown by the un-transfected control CTLs (Fig. 3b). And expectedly, CRISPR-Cas9-mediated CTLA-4 knockout was accompanied with higher level of IFN- γ and TNF- α production (Fig. 3c) and increased cytotoxicity toward T24 cells (Fig. 3d). Taken together, these findings suggest that CTLA-4 knockout on CTLs is sufficient to enhance anti-tumor activity in bladder cancer cells.

Fig. 3 SgRNA:Cas9-mediated CTLA-4 disruption on CTLs enhances cellular immune response and cytotoxicity toward bladder cancer cells *in vitro*. **a** The CTLA-4 expression on CTLs was determined by flow cytometry 7, 14, and 21 days post-transfection. **b** The cell proliferation of sgRNA:Cas9-treated CTLs, control CTLs, and un-transfected CTLs was measured every 7 days post-transfection for a total period of 21 days. **c** Culture medium from sgRNA:Cas9-treated CTLs and control CTLs were assayed for IFN- γ and TNF- α using a cytokine ELISA assay. **d** CTLA-4 knockout CTLs or control CTLs were co-cultured with CFSE-labeled T24 cells at ratio (E:T) of 5:1 and 20:1, respectively. After 6 h, propidium iodide (PI) was added and the apoptotic cells were analyzed by flow cytometry. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



CTLA-4 antibody blockage on CTLs shows superior anti-tumor activity in SCID mouse model of subcutaneous xenograft

Given that CTLA-4-disrupted CTLs exhibited enhanced cytotoxicity *in vitro*, we assumed whether the anti-CTLA-4 CTLs also display heightened cytotoxicity *in vivo*. To demonstrate this hypothesis, we generated SCID mice with immune reconstruction and established human xenograft models of T24 cells by subcutaneous injection. In this study, both prophylactic assay and therapeutic assay were performed. For the prophylactic assay, 1×10^7 control CTLs or anti-CTLA-4 CTLs were first transferred, followed by subcutaneous transplantation of T24 cells at a dose of 1×10^6 cells (Fig. 4a, $n = 5$ per group). As shown in Fig. 4b, the tumor growth was markedly slowed in anti-CTLA-4 CTLs group compared to the control CTLs group, suggesting that the latent period of grafted T24 was postponed by anti-CTLA-4 CTLs. Meanwhile, the PCNA-positive cells were also significantly reduced in the anti-CTLA-4 CTLs group

(Fig. 4c). For the therapeutic assay, T24 cells were first inoculated in SCID mice. When tumors grew to near 100 mm^3 , mice were randomly divided into two groups and transferred with 1×10^7 control CTLs or anti-CTLA-4 CTLs (Fig. 4d, $n = 5$ per group). As shown in Fig. 4e, the tumor volume was markedly reduced in anti-CTLA-4 CTLs group compared to the control CTLs group, suggesting the enhanced anti-tumor activity of anti-CTLA-4 CTLs *in vivo* ($n = 5$ per group). Similarly, the immunoreactivity of the proliferation index PCNA was also decreased in the anti-CTLA-4 CTLs group (Fig. 4f).

Discussion

It is well known that the function of tumor-specific CTLs may be influenced by the acquired immune resistance induced by the elevated PD-1/CTLA-4 signaling and blocking the PD-1 and CTLA-4 pathway could enhance immune response in cancer patients [21]. In this study, we detected

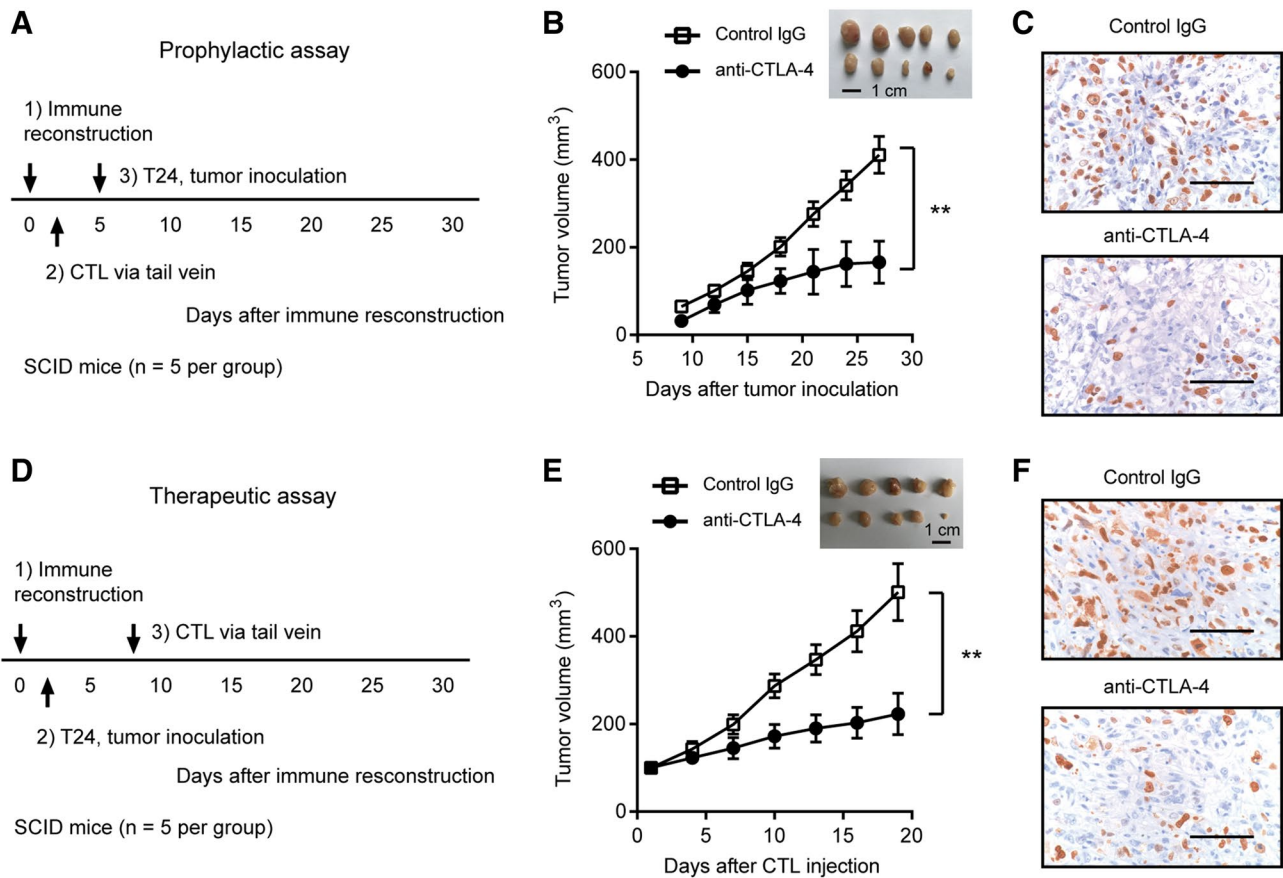


Fig. 4 CTLA-4 disruption on CTLs shows superior anti-tumor activity in SCID mouse model of subcutaneous xenograft. **a** The schema for prophylactic assay. **b** The tumor volume of the mice treated with CTLA-4-disrupted CTL or control CTL since the start of tumor inoculation ($n=5$). **c** In the prophylactic assay, the representative PCNA staining in the tumor tissues from treated with CTLA-4-disrupted CTL or control CTL. **d** The schema for therapeutic assay. **e** The tumor volume of the mice treated with CTLA-4-inhibition CTLs or control CTLs since the start of CTLs injection ($n=5$). **f** In the therapeutic assay, the representative PCNA staining in the tumor tissues from treated with CTLA-4-disrupted CTL or control CTL. $**p < 0.01$

the expression of immune checkpoint molecules PD-1 and CTLA-4 on the CD4 + T and CD8 + T cells in the PBMCs from bladder cancer patients and healthy donors and found that PD-1 and CTLA-4 expression was significantly elevated on the CD8 + T cells. We subsequently performed CRISPR-Cas9-mediated gene editing and blocking antibody-mediated CTLA-4-disrupted CTLs. CTLA-4-disrupted CTLs had increased cytokine secretion and lytic activity and ultimately contributed to enhanced anti-tumor activity to bladder tumors *in vivo*. These data reveal the inhibitory role of the CTLA-4 on the anti-tumor function of CTLs, and demonstrate CTLA-4 disruption is sufficient to rescue CTLs efficacy.

Recently, emerging studies have confirmed the expression profile of PD-1 and CTLA-4 expression in peripheral circulating blood lymphocytes, tumor-infiltrating lymphocytes and the tumor tissues [16, 19, 22–24]. The immune checkpoint inhibitors have been tested in clinical trials for various malignancies including metastatic urothelial carcinoma, with

major partial or complete responses and limited side effects [25, 26]. Actually, the expression status of checkpoint molecules is indeed important for immune checkpoint therapy but limited biomarker for the response to PD-1/CTLA-4 blockade, thus suggesting a role for tumor-infiltrating immune cells and other factors, which may better predict therapy response [27, 28]. In this study, we revealed the differentially expressed CTLA-4 on CD8 + T cells. Interestingly, a close correlation was found between the CTLA-4 expression on CD8 + T cells and the tumor size and MIBC category in bladder cancer patients. Indeed, high histological grades are likely accompanied with advanced TNM stage and larger tumor size. However, no significant correlation between histological grade and CTLA-4 expression was found. This may be due to the small number of samples used in this study. Due to the cohort we studied without immune checkpoint therapy and clinical follow-ups, we failed to evaluate the predictive role of CTLA-4 expression on CD8 + T cells in response to CTLA-4 blockade. Similarly, whether CTLA-4

expression on CD8 + T lymphocytes from peripheral blood correlate with CTLA-4 expression on tumor tissues of bladder cancer warrants further investigation.

Previously, Rupp et al. have demonstrated that CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells (anti-CD194-1BB ζ) and results in impaired tumor clearance in a subcutaneous xenograft model [19]. Similarly, Su et al. have revealed that CRISPR-Cas9-mediated disruption of PD-1 on human T cells for adoptive cellular therapies of Epstein–Barr virus-positive gastric cancer [20]. And CRISPR-Cas9-mediated efficient PD-1 disruption can enhance cellular immune response of human primary T cells from cancer patients [29]. In melanoma, a bolus of enhanced IL-21-primed polyclonal antigen-specific CTL combined with CTLA4 blockade boost anti-tumor efficacy [30]. In this study, by CRISPR-Cas9 system and anti-CTLA-4 blocking antibody, we showed that CTLA-4 disruption on CTLs significantly improved the cellular immune response of the CTLs as characterized by increased IFN- γ production and enhanced cytotoxicity towards bladder cancer cells. By two subcutaneous xenograft models, we confirmed the profound therapeutic efficacy of CTLA-4-disrupted T cells in vivo. Therefore, our data suggest that high expression of CTLA-4 can serve as a signature of exhausted CTLs, and CTLA-4 blockade can rescue the exhausted phenotypes of CTLs and enhance their anti-tumor activities.

In conclusion, our data, as proof of principle, suggest that CTLA-4 disruption on CTLs can act as the brake of immune tolerance in terms of its cellular immune responses and in vivo anti-tumor activity in bladder cancer. In addition, our findings lead us to recommend combining first-line therapy with immune checkpoint-modified CTLs for clinical bladder cancer treatment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- Siegel RL, Miller KD, Jemal A (2016) Cancer statistics, 2016. *CA Cancer J Clin* 66:7–30
- Miyamoto DT, Mouw KW, Feng FY, Shipley WU, Efsthathiou JA (2018) Molecular biomarkers in bladder preservation therapy for muscle-invasive bladder cancer. *Lancet Oncol* 19:e683–e695
- Zhang J, Bu X, Wang H, Zhu Y, Geng Y, Nihira NT, Tan Y, Ci Y, Wu F, Dai X, Guo J, Huang YH, Fan C, Ren S, Sun Y, Freeman GJ, Sicinski P, Wei W (2018) Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance. *Nature* 553:91–95
- Kim HS, Seo HK (2018) Immune checkpoint inhibitors for urothelial carcinoma. *Investig Clin Urol* 59:285–296
- Powles T, Morrison L (2018) Biomarker challenges for immune checkpoint inhibitors in urothelial carcinoma. *Nat Rev Urol* 15:585–587
- El Rassy E, Assi T, Bakouny Z, Pavlidis N, Kattan J. Beyond first-line systemic treatment for metastatic urothelial carcinoma of the bladder. *Clin Transl Oncol* 2018 21:280–288
- Zhao R, Song Y, Wang Y, Huang Y, Li Z, Cui Y, Yi M, Xia L, Zhuang W, Wu X (2019) Zhou Y PD-1/PD-L1 blockade rescue exhausted CD8 + T cells in gastrointestinal stromal tumours via the PI3K/Akt/mTOR signalling pathway. *Cell Prolif*. <https://doi.org/10.1111/cpr.12571>
- Tan J, Chen S, Lu Y, Yao D, Xu L, Zhang Y, Yang L, Chen J, Lai J, Yu Z, Zhu K, Li Y (2017) Higher PD-1 expression concurrent with exhausted CD8 + T cells in patients with de novo acute myeloid leukemia. *Chin J Cancer Res* 29:463–470
- Jiang X, Wang J, Deng X, Xiong F, Ge J, Xiang B, Wu X, Ma J, Zhou M, Li X, Li Y, Li G, Xiong W, Guo C, Zeng Z (2019) Role of the tumor microenvironment in PD-L1/PD-1-mediated tumor immune escape. *Mol Cancer* 18:10
- Fu C, Jiang A (2018) Dendritic cells and CD8 T cell immunity in tumor microenvironment. *Front Immunol* 9:3059
- Li J, Shayan G, Avery L, Jie HB, Gildener-Leapman N, Schmitt N, Lu BF, Kane LP, Ferris RL (2016) Tumor-infiltrating Tim-3(+) T cells proliferate avidly except when PD-1 is co-expressed: evidence for intracellular cross talk. *Oncoimmunology* 5:e1200778
- Dejaegher J, Verschuere T, Vercalsteren E, Boon L, Cremer J, Sciot R, Van Gool SW, De Vleeschouwer S (2017) Characterization of PD-1 upregulation on tumor-infiltrating lymphocytes in human and murine gliomas and preclinical therapeutic blockade. *Int J Cancer* 141:1891–1900
- Siddiqui I, Schaeuble K, Chennupati V, Fuertes Marraco SA, Calderon-Copete S, Pais Ferreira D, Carmona SJ, Scarpellino L, Gfeller D, Pradervand S, Luther SA, Speiser DE, Held W (2019) Intratumoral Tcf1(+)/PD-1(+)/CD8(+) T cells with stem-like properties promote tumor control in response to vaccination and checkpoint blockade immunotherapy. *Immunity* 50:195–211 e110
- Kurtulus S, Madi A, Escobar G, Klapholz M, Nyman J, Christian E, Pawlak M, Dionne D, Xia J, Rozenblatt-Rosen O, Kuchroo VK, Regev A, Anderson AC (2019) Checkpoint blockade immunotherapy induces dynamic changes in PD-1(-)/CD8(+) tumor-infiltrating T cells. *Immunity* 50:181–194 e186
- Felsenstein KM, Theodorescu D. Precision medicine for urothelial bladder cancer: update on tumour genomics and immunotherapy. *Nat Rev Urol* 2017 15:92–111
- Kamphorst AO, Pillai RN, Yang S, Nasti TH, Akondy RS, Wieland A, Sica GL, Yu K, Koenig L, Patel NT, Behera M, Wu H, McCausland M, Chen Z, Zhang C, Khuri FR, Owonikoko TK, Ahmed R, Ramalingam SS (2017) Proliferation of PD-1 + CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. *Proc Natl Acad Sci USA* 114:4993–4998
- Zhang W, Bai JF, Zuo MX, Cao XX, Chen M, Zhang Y, Han X, Zhong DR, Zhou DB (2016) PD-1 expression on the surface of peripheral blood CD4(+) T cell and its association with the prognosis of patients with diffuse large B-cell lymphoma. *Cancer Med* 5:3077–3084
- Hultquist JF, Hiatt J, Schumann K, McGregor MJ, Roth TL, Haas P, Doudna JA, Marson A, Krogan NJ (2019) CRISPR-Cas9 genome engineering of primary CD4(+) T cells for the interrogation of HIV-host factor interactions. *Nat Protoc* 14:1–27
- Rupp LJ, Schumann K, Roybal KT, Gate RE, Ye CJ, Lim WA, Marson A (2017) CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. *Sci Rep* 7:737

20. Su S, Zou Z, Chen F, Ding N, Du J, Shao J, Li L, Fu Y, Hu B, Yang Y, Sha H, Meng F, Wei J, Huang X, Liu B (2017) CRISPR-Cas9-mediated disruption of PD-1 on human T cells for adoptive cellular therapies of EBV positive gastric cancer. *Oncoimmunology* 6:e1249558
21. Davarpanah NN, Yuno A, Trepel JB, Apolo AB. Immunotherapy: a new treatment paradigm in bladder cancer. *Curr Opin Oncol* 2017 29:184–195
22. Zhou G, Sprengers D, Boor PPC, Doukas M, Schutz H, Mancham S, Pedroza-Gonzalez A, Polak WG, de Jonge J, Gaspersz M, Dong H, Thielemans K, Pan Q, JNM IJ Bruno MJ Kwekkeboom J (2017) Antibodies against immune checkpoint molecules restore functions of tumor-infiltrating T cells in hepatocellular carcinomas. *Gastroenterology* 153:1107–1119
23. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G (2013) Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. *Cancer Res* 73:3591–3603
24. Lussier DM, Johnson JL, Hingorani P, Blattman JN (2015) Combination immunotherapy with alpha-CTLA-4 and alpha-PD-L1 antibody blockade prevents immune escape and leads to complete control of metastatic osteosarcoma. *J Immunother Cancer* 3:21
25. Di Nunno V, De Luca E, Buttigliero C, Tucci M, Vignani F, Gatto L, Zichi C, Ardizzoni A, Di Maio M, Massari F (2018) Immune-checkpoint inhibitors in previously treated patients with advanced or metastatic urothelial carcinoma: a systematic review and meta-analysis. *Crit Rev Oncol Hematol* 129:124–132
26. Tripathi A, Plimack ER (2018) Immunotherapy for urothelial carcinoma: current evidence and future directions. *Curr Urol Rep* 19:109
27. Buchbinder E, Hodi FS (2015) Cytotoxic T lymphocyte antigen-4 and immune checkpoint blockade. *J Clin Investig* 125:3377–3383
28. Sheik Ali S, Goddard AL, Luke JJ, Donahue H, Todd DJ, Werchaniak A, Vleugels RA (2015) Drug-associated dermatomyositis following ipilimumab therapy: a novel immune-mediated adverse event associated with cytotoxic T-lymphocyte antigen 4 blockade. *JAMA Dermatol* 151:195–199
29. Su S, Hu B, Shao J, Shen B, Du J, Du Y, Zhou J, Yu L, Zhang L, Chen F, Sha H, Cheng L, Meng F, Zou Z, Huang X, Liu B (2016) CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients. *Sci Rep* 6:20070
30. Chapuis AG, Lee SM, Thompson JA, Roberts IM, Margolin KA, Bhatia S, Sloan HL, Lai I, Wagener F, Shibuya K, Cao J, Wolchok JD, Greenberg PD, Yee C (2016) Combined IL-21-primed polyclonal CTL plus CTLA4 blockade controls refractory metastatic melanoma in a patient. *J Exp Med* 213:1133–1139

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.