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ORIGINAL ARTICLE

Efficacy of an ALDH peptide-based dendritic cell vaccine targeting cancer stem cells

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Abstract

Cancer immunotherapies may be limited by their failure to target cancer stem cells (CSCs). We previously described an approach to target these cells using a dendritic cell (DC) vaccine primed with lysates of CSCs identified by aldehyde dehydrogenase (ALDH). However, its clinical application is limited by the difficulty of obtaining adequate amounts of tumor from patient to make CSC lysate for vaccine preparation. To address this issue, we evaluated targeting ALDH^{high} CSCs using two antigenic peptides derived from ALDH in D5 melanoma model in both protection and therapeutic settings. ALDH 1A1 or 1A3 peptide-DC vaccines primed cytotoxic T lymphocytes (CTLs) that specifically killed ALDH^{high} D5 CSCs, with ALDH 1A1 + 1A3 dual peptides-DC vaccine mediating an additive CTL effect compared to single peptide-DC vaccines. In a tumor challenge model, ALDH peptide-DC vaccines induced significant protective immunity suppressing D5 tumor growth with the dual peptides-DC vaccine being superior to each peptide individually. In a therapeutic model, dual peptide-DC vaccine resulted in significant tumor growth suppression with anti-PD-L1 administration significantly augmenting this effect. Immune monitoring studies revealed that ALDH dual peptides-DC vaccination elicited strong T cell (CTL & IFNγ Elispot) and antibody immunity targeting ALDH^{high} CSCs, resulting in significant reduction of ALDH^{high} D5 CSCs. ALDH dual peptides-DC vaccination plus anti-PD-L1 administration resulted in increased recruitment of CD3^{+} TILs in the residual tumors and further reduction of ALDH^{high} D5 CSCs. ALDH peptide(s)-based vaccine may allow for clinical translation via immunological targeting of ALDH^{high} CSCs. Furthermore, this vaccine augments the efficacy of immune checkpoint blockade.

Keywords Aldehyde dehydrogenase · Peptide · Dendritic cells · Vaccine · Cancer stem cells · Immunotherapy

Introduction

Cancer stem cells (CSCs) are intimately involved in tumor initiation and metastasis as well as in mediating resistance to chemotherapy/radiotherapy and evasion of immune surveillance [1]. Eliminating CSCs represents a significant

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challenge in cancer therapeutics. In previous studies, we reported that CSC lysate-pulsed dendritic cells (DC) vaccines were effective at preventing lung metastasis of murine melanoma D5 and subcutaneous tumor growth of murine head and neck squamous cell cancer (HNSCC) SCC7 by eliciting strong and specific anti-CSC immunity [2]. We demonstrated that this approach was particularly advantageous when deployed in the adjuvant setting [3, 4]. However, our previously reported methodology for preparing CSC vaccines involved isolating CSCs from bulk tumor and generating lysates utilized for priming DCs. This limits the clinical applicability of this approach due to the variable availability of tumor tissues obtainable from each patient. Targeting of shared CSC antigens instead of CSC cell lysates provides an approach amenable to development of an "off the shelf" therapeutic vaccine.

Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes [5]. High levels of ALDEFLUOR/ALDH activity have been successfully used as a marker to isolate CSC-enriched populations in a variety of solid tumors [2, 6, 7], including breast cancer, lung cancer, colon cancer, sarcoma and HNSCC. Although there are 19 members in the human ALDH family, the isoforms expressed in CSCs are primarily ALDH1A1 and ALDH1A3 [8]. In addition to serving as CSC markers, ALDH1A1 and ALDH1A3 also play important functional roles in these cells and thus have served as potential therapeutic targets [8, 9]. Disulfiram, an irreversible pan-ALDH inhibitor, blocked in vitro and in vivo irradiation-induced conversion of non-stem breast cancer cells into breast CSCs [10]. Moreover, $ALDH^{high}$ CSCs were recognized and eliminated in vitro by HLA-A2-restricted, $ALDH1A1_{88–96}$ peptide-specific $CD8^+$ T cells; and adoptive transfer of these ALDH1A1 peptide-specific $CD8^+$ T cells inhibited tumor growth and reduced lung metastases in vivo [11]. This demonstrated the feasibility of targeting CSC peptide via passive (T cell) immunotherapy *vs.* active (vaccine-based) immunotherapy as we report in this study. ALDH1A1 and ALDH1A3 are the predominant ALDH isozymes expressed in $ALDH^{high}$ CSCs of breast cancer, lung cancer, melanoma, and head and neck cancer [12]. Microarray analysis of $ALDH^{high}$ and $ALDH^{low}$ cells from melanoma tumor cells revealed that seventeen ALDH genes were up-regulated and 2 genes were down-regulated in $ALDH^{high}$ cells [12]. Specifically, $ALDH^{high}$ cells expressed over 20-fold more ALDH1A1 and ALDH1A3 than $ALDH^{low}$ cells. This study suggested that ALDH 1A1 and ALDH1A3 represent two ideal targets for immunological targeting of $ALDH^{high}$ CSCs. However, up to date, ALDH1A1 and ALDH1A3 inhibitors to target these two isoforms have shown significant limitations for clinical application. For example, CM037, a benzothienopyrimidine-based competitive ALDH1A1 inhibitor representing a potential therapeutic drug to target ALDH1A1 in CSCs in ovarian cancer and endometrial cancer, is ineffective in vivo likely due to its low solubility in aqueous solutions [13]. ALDH1A3 inhibitor, such as free citral, has also been found ineffective in vivo [14], while citral encapsulated by nanoparticle reduced the tumor growth [14]. In addition, while pan-ALDH inhibitor disulfiram was toxic to CSCs in a copper-dependent manner, disulfiram alone did not show inhibitory effect on the ALDH activity [15, 16]. We hypothesize that ALDH peptides can serve as CSC associated antigen(s) to prime dendritic cells to induce specific immunity against $ALDH^{high}$ CSCs. In the present study, we developed ALDH1A1 and 1A3 peptide(s)-DC vaccines and examined their efficacy and mechanism of action utilizing an immunocompetent murine model.

Cancer immunotherapy utilizing immune checkpoint blockade, including PD-1/PD-L1 inhibitors, represents a major advance in cancer therapeutics [17, 18]. However, the overall response rate for monoclonal antibodies targeting PD-1/PD-L1 is generally less than 30% in many solid malignancies, suggesting that many patients show primary resistance to checkpoint inhibitors. In addition, many of these responses are transitory. In that regard, our previous studies demonstrated that the efficacy of CSC lysate-DC vaccine was augmented significantly by the simultaneous administration of anti-PD-L1 monoclonal antibody [4]. We therefore examined the efficacy of adding PD-1/PD-L1 blockade to ALDH peptide DC vaccines.

Methods

Mice

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free environment and used at age 7–8 weeks or older. Principles of laboratory animal care (*National Institutes of Health*) were followed, and the University of Michigan Laboratory of Animal Medicine approved all animal protocols.

Murine cancer cells

The D5 cell line is a sub-clone of murine melanoma cell line B16 that is syngeneic to C57BL/6 mice. Subcutaneous inoculation of D5 cells into the flank of C57BL/6 mice was performed to establish both protection and established tumor therapy models. D5 cell lines are cultured in complete medium (CM) consisting of RPMI1640 (GIBCO, Gaithersburg, MD) and supplements in 5% CO_2 atmosphere.

ALDEFLUOR assay

The ALDEFLUOR™ Kit (StemCell Technologies, British Columbia, Canada) was used to isolate ALDEFLUOR$^+$/ALDH^{high} CSCs from D5 cells according to the manufacturer's instruction.

Preparation of ALDH^{high} CSC-targeted DC vaccines

DCs were prepared as we described previously [4]. Briefly, murine bone marrow-derived cells were cultured in 10 ml complete medium supplemented with 20 ng/ml GM-CSF (GenScript, NJ) at a concentration of $(2–4)\times10^5$ cells/ml in non-tissue culture petri dishes (Corning Incorporated, Corning, NY). Half of the CM with GM-CSF was refreshed on day 3, 6 and 8. On day 10, DCs were harvested and loaded with ALDH^{high} CSC lysate (control) or ALDH peptide(s). ALDH^{high} D5 CSCs were frozen and thawed 3 times to make CSC lysate. ALDH^{high} D5 CSC lysate was added to DCs at a 1:3 cell equivalent ratio. The DCs were then incubated at 37 °C for 24 h with 5% CO_2. After incubation, ALDH^{high} D5 lysate-pulsed DCs were used as vaccine (control) in the subsequent experiments.

The sequence of ALDH 1A1 peptide (*LLYKLADLI*) and 1A3 peptide (*LLHQLADLV*) was based on the study of Visus C et al. [8] as well as the information from GenBank accession no. NM_000689 and NM_053080. The two peptides were synthesized by Genscript (Piscataway, NJ). The ALDH 1A1 peptide and 1A3 peptide that exceeded 90% purity were analyzed by high-performance liquid chromatography and validated for identity by mass spectrometry. Lyophilized peptides were dissolved in ultrapure water at a concentration of 10 mg/ml and stored at -80 °C. ALDH 1A1 or/and 1A3 peptide(s) were added to DCs at a ratio of 0.8 mg single peptide or 1.6 mg dual peptides (0.8 mg each peptide)/2 million DCs, which were then incubated at 37 °C for 24 h with 5% CO_2 and used as ALDH peptide(s)-DC vaccines in the following experiments.

Tumor models

To test the protective effect of ALDH peptide(s)-DC vaccine, 2×10^6 DCs as vaccine per C57BL/6 mouse were administered (s.c.) once a week for two weeks, starting 14 days before D5 tumor cell challenge (5×10^5 D5 cells/mouse). For the D5 tumor treatment model, 5×10^4 D5 cells were injected (*s.c.*) into the flank of each C57BL/6 mouse. One day after tumor inoculation, 2×10^6 DCs were administered (*s.c.*) once a week for two weeks, and anti-PD-L1 monoclonal antibody (Clone number 80, provided by Dr. Elaine Hurt, MedImmune, Gaithersburg, MD) was administered (*i.p.*) at 0.05 mg/mouse, 3 times/week every other day starting from the next day of each vaccination for two weeks. The long and short diameters of tumor were measured twice a week. Tumor volumes were calculated as $(\text{width}^2\times\text{length})/2$. At the endpoint (when tumor was > 20 mm in diameter), mice were sacrificed, and various samples such as spleen, peripheral blood, and residual tumor were harvested for immune function assays.

Cytotoxic T lymphocytes (CTLs) cytotoxicity

Spleens were harvested from normal healthy or tumor-bearing treated B6 mice and made into splenocyte cell suspensions. Splenic T cells were isolated by MACS separator kits (MiltenyiBiotec. Inc. Auburn, CA) with anti-CD3-coupled microbeads, followed by anti-CD3/anti-CD28 (BD Pharmingen, San Diego, CA) activation and IL-2 (Prometheus Laboratories, San Diego, CA) expansion, which consistently resulted in > 90% of CD3$^+$ T cells[2]. We then co-cultured the ALDH^{high} CSCs and ALDH^{low} non-CSCs as target cells with the CTLs as generated above for 6 h. After that, we detected the cytotoxicity of CTLs by a lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI) according to the manufacturer's protocol.

T cell proliferation

T cell proliferation was determined using a carboxyfluorescein succinimidyl ester (CFSE)-based assay as previously described [19]. In brief, the isolated splenic CD3$^+$ T cells from C57BL/6 mice were labeled with 5 μM CFSE (eBioscience, San Diego, CA) and stimulated by ALDH 1A1, 1A3 single or dual peptide(s)-DC vaccine or ALDH^{high} CSC lysate-DC vaccine (as positive control) at a 1:1 cell ratio of DCs to T cells. Cell proliferation was detected by monitoring the changes in fluorescence intensity of the labeled cells after 7 days of co-culture.

Detection of CD3$^+$ tumor infiltrating lymphocytes (TILs)

At the end of treatment, residual tumors were resected and fixed in formalin and embedded in paraffin. Sections of 4 μm thickness were deparaffinized in xylene and rehydrated through graded alcohols. These deparaffinized slides were then processed in microwave heating in 10 mmol/L citrate buffer (pH 6.0) for 15 min for antigen retrieval, in the presence of 3% H_2O_2 for endogenous peroxidase inactivation, followed by incubation with anti-CD3 primary antibody (Abcam, Cambridge, MA) at 1:100 dilution, at 4 °C overnight. After washing three times in PBS, the slides were incubated for 30 min in biotinylated secondary goat anti-rabbit IgG antibody (1:2000) (Sigma, Indianapolis, IN). Immunostaining was performed using a DAB kit (3, 3′

diaminobenzidine, DAKO) and manufacturer's instructions were followed (Abcam, Cambridge, MA).

Intracellular IFN-γ staining and enzyme-linked immunospot (Elispot) assay

To determine IFN-γ intracellular secretion, the stimulated spleen T cells with ALDH peptide(s)-DCs as described above were permeabilized with pre-chilled perm buffer III (BD Bioscience, San Jose, CA) at 4 °C for 30 min. After washing once with PBS, the cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse IFN-γ at 4 °C for 30 min, followed by flow cytometry.

Extracellular IFN-γ secretion of splenic T cells primed with ALDH peptide(s)-DC vaccine in response to D5 $ALDH^{high}$ CSCs versus $ALDH^{low}$ non-CSCs was determined using a Mouse IFN-γ Elispot set (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. In brief, we collected splenic cells from ALDH peptide(s)-DC vaccinated mice and used MACS separator kits (MiltenyiBiotec. Inc. Auburn, CA) with anti-CD3-coupled microbeads to isolate $CD3^+$ T cells from the splenic cells. Then, 0.2×10^6 isolated $CD3^+$ T cells were added to the anti-IFN-γ-coated 96-well polyvinylidene fluoride (PVDF) ELISPOT plate. The $CD3^+$ T cells were re-stimulated with PBS (negative control), $ALDH^{high}$ CSCs, $ALDH^{low}$ non-CSCs, or phytohemagglutinin (PHA) (positive control) at 37 °C for 18 h. After cell removal, plates were developed at room temperature for 2 h in the presence of 0.4 U/ml IFN-γ-specific alkaline phosphatase-coupled mAb. Spot detection was performed following incubation for 6 min in the dark with a 1-step nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate (Thermo Fisher Scientific, Waltham, USA). IFN-γ-specific spot-forming cells (SFC) were counted using a Bioreader® 5000 Eα (BIO-SYS GmbH, Karbon, Germany).

IgG binding assay and antibody/complement–dependent cytotoxicity (CDC)

Plasma IgG was detected by mouse IgG ELISA quantitation set (Bethyl Laboratories, Montgomery, TX). $ALDH^{high}$ or $ALDH^{low}$ D5 cells were incubated with the equal quantity of IgG, followed by incubation with FITC-conjugated anti-mouse IgG antibody (BD Bioscience, San Jose, CA). Binding of plasma IgG to $ALDH^{high}$ D5 cells was assessed by flow cytometry. CDC against $ALDH^{high}$ CSCs versus $ALDH^{low}$ non-CSCs was measured as described previously [2]. Briefly, 10^5 viable $ALDH^{high}$ D5 CSCs were incubated with plasma harvested from animals subjected to various treatments for 1 h followed by cell culture in the presence of rabbit complement for another hour. Viable cells were then counted under a microscope after trypan blue staining to calculate cell lysis. % of viable cells = viable $ALDH^{high}$ CSCs after plasma and complement incubation/10^5. Lower % of.

viable $ALDH^{high}$ CSCs at the end of incubation indicates more cell lysis.

Detection of $ALDH^{high}$ CSCs and $CD3^+$ T cells in residual tumor

Residual tumors were harvested from all mice at the end of experiments and cut into small piece (1–8 mm^3) for digestion by 1 × collagenase/hyaluronidase (Stem Cell Technologies, Vancouver, BC, Canada) for 30–40 min to make single cell suspensions. These single cell suspensions were then used to detect the percentage of $ALDH^{high}$ D5 CSCs or $CD3^+$ TILs utilizing the ALDEFLUOR™ Kit and APC-conjunct anti-CD3 (BD Biosciences, San Jose, CA), respectively, followed by flow cytometry.

Flow cytometry analysis

Cell surface expression (CD3, PD-1, PD-L1) and intracellular expression (IFN-γ, ALDH) were analyzed by flow cytometry. All FITC- or phycoerythrin- or peridinin chlorophyll protein-conjugated antibodies and matched isotype controls were purchased from BD Biosciences. Flow cytometry was performed on a LSRII flow cytometer (BD Biosciences) and analyzed by FlowJo™ version 10 (Tree Star, Inc., Ashland, OR).

Statistical analysis

Statistical analyses of CFSE, FACS, Elispot, ELISA, immunohistochemistry and CTL cytotoxicity were performed using Student's t-test when comparing only 2 groups and using one-way ANOVA with Tukey's multiple comparisons when comparing > 2 groups. The growth curves were expressed as mean ± SE and compared by two-way ANOVA. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with GraphPad Prism 9.0.

Results

ALDH 1A1 and/or 1A3 peptide(s)-DC vaccines induced T cell proliferation and anti-$ALDH^{high}$ CSC activity in vitro

As described above, high levels of ALDEFLUOR/ALDH activity have been successfully used as a single marker to isolate cancer stem cell-enriched populations in a variety of tumors. Functional activity in CSCs is primarily mediated by two isoforms: ALDH 1A1 and 1A3. We examined the effect

of two peptides derived from ALDH 1A1 and 1A3, respectively, as described in the Materials and Methods (ALDH 1A1 and 1A3 peptides)-DC vaccine on T cell proliferation and function in vitro. Splenic $CD3^+$ T cells from the normal B6 mice were labeled with CFSE followed by stimulation with unloaded-DC (as negative control), ALDH 1A1 peptide-DC, ALDH1A3 peptide-DC, ALDH 1A1 + 1A3 peptides-DC. D5 $ALDH^{high}$ CSC lysate-DC was used as a positive control. As shown in Fig. 1a, the T cell proliferation rate was 20.2%, 26.1% and 31.5% after ALDH 1A1, 1A3 and 1A1 plus 1A3 peptides-DC in vitro stimulation, respectively, demonstrating significant increases compared with the negative control (9.51% after unloaded-DC stimulation). The T cell proliferation rate after stimulation with ALDH 1A1 + 1A3 peptides-DC increased to 31.5%, which was comparable to 35.5% generated by the positive control ($ALDH^{high}$ D5 CSC lysate-DC). Figure 1b indicates that significantly stronger $CD3^+$ T cell responses to the ALDH 1A1 and/or 1A3 peptide antigens could be elicited by the DCs presenting these peptide antigens than unloaded DCs.

We used these ALDH 1A1 and/or 1A3 peptides DC-stimulated T cells as CTLs and assessed their cytotoxicity to $ALDH^{high}$ CSCs *vs.* $ALDH^{low}$ non-CSCs as measured by an LDH release assay. As shown in Fig. 1c, CTLs primed with ALDH 1A1 and/or 1A3 peptide(s)-DCs exhibited significantly higher killing of $ALDH^{high}$ D5 cells compared to the negative control (unloaded-DC primed T cells).

at a 10:1 effector (E): target cell (T) ratio (all p values < 0.05). Importantly, the dual (ALDH 1A1 + 1A3) peptides-DC-activated T cells killed a higher proportion of $ALDH^{high}$ CSCs than did single peptide-DC activated T cells ($p = 0.0681$ and $p = 0.0206$, respectively). However, the cytotoxicity elicited by ALDH peptide(s)-DC primed T cells was not observed when $ALDH^{low}$ non-CSCs were used as a negative target control. These experiments demonstrated that ALDH 1A1 and/or 1A3 peptide(s)-DC vaccines could induce T cell anti-$ALDH^{high}$ CSC activity as well as T cell proliferation in vitro.

ALDH 1A1 plus 1A3 peptides-DC vaccines demonstrated additive effect against D5 tumor challenge in vivo

Based on the in vitro data as described above, we proceeded to test the effect of combined dual ALDH peptides-DC vaccine in a protective D5 tumor model. Two vaccinations were administered 14 and 7 days before tumor cell injection as shown in Fig. 2a. Treatment consisted of PBS control, single

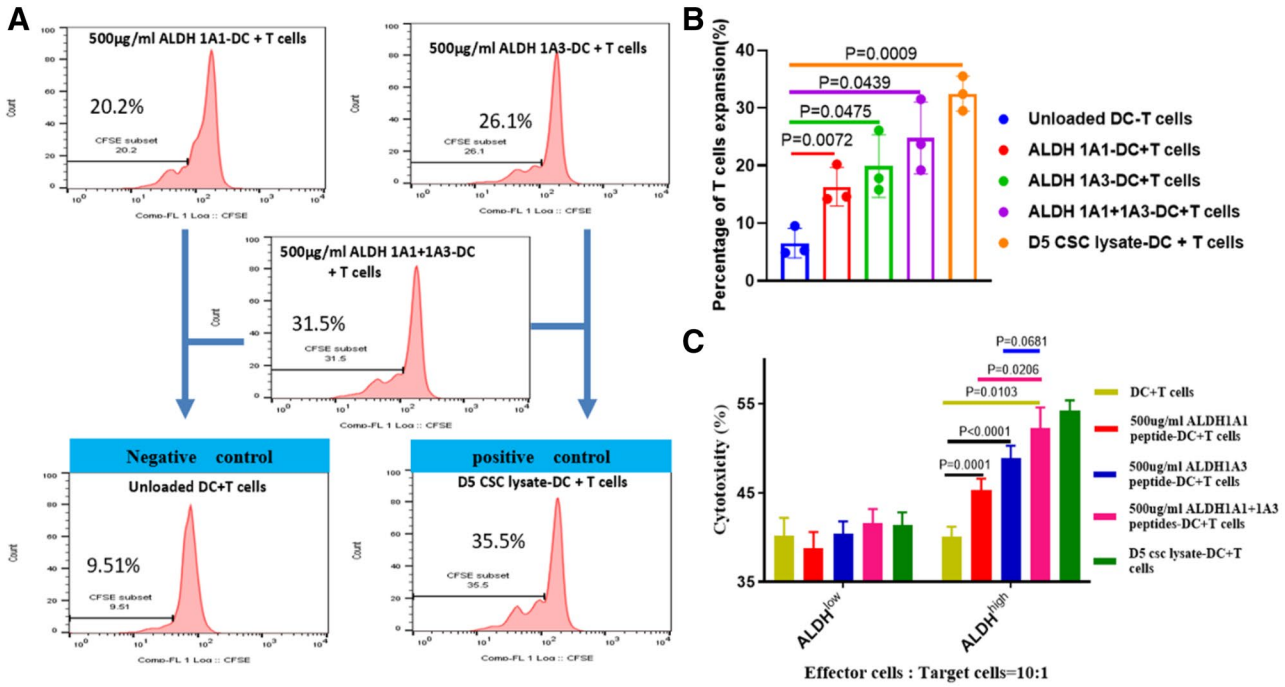

Fig. 1 ALDH peptide(s)-DC vaccine promoted the in vitro proliferation and anti-$ALDH^{high}$ CSC function of T cells. **a**: Flow cytometry indicated proliferation of CFSE labeled $CD3^+$ T cells from the spleen of normal C57BL/6 mice stimulated by DCs loaded with ALDH 1A1 and/or 1A3 peptide(s). Representative results of the CFSE assay after 7-day co-culture of peptide(s)-DC: T cells at a 1:1 ratio are shown. **b**: The CFSE assays were performed three times. Statistical analyses are shown for T cell expansion in response to different ALDH peptide(s)-DC vaccines as indicated. **c**: The $CD3^+$ T cells isolated from the spleen of normal B6 mice and stimulated by DCs loaded with ALDH 1A1 and/or 1A3 peptide(s) were co-incubated with D5 $ALDH^{high}$ versus $ALDH^{low}$ target cells at a 10:1 E/T ratio. Data were representative of three experiments performed

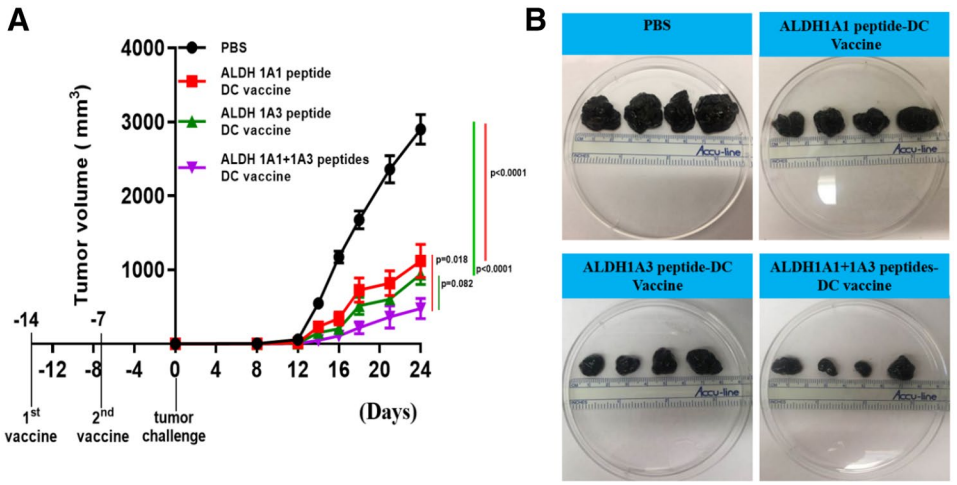

Fig. 2 ALDH dual peptides-DC vaccine significantly inhibited D5 tumor growth in the protection model. **a**: D5 growth curves in mice treated with PBS, single ALDH peptide-DC vaccines and the dual peptides-DC vaccine. This experiment was repeated twice. The growth curves were expressed as mean ± SE and compared by two-way ANOVA. **b**: The resected residual tumors from hosts treated with the ALDH peptide(s)-DC vaccines

ALDH peptide-DC vaccine or dual peptides-DC vaccine. The ALDH 1A1 or 1A3 peptide-DC vaccine significantly inhibited subcutaneous tumor growth ($p < 0.0001$) compared to PBS control. Importantly, the ALDH 1A1 plus 1A3 dual peptides-DC vaccine inhibited tumor growth more than vaccination with single peptide vaccines ($p = 0.018$ and $p = 0.082$, respectively).

The representative pictures of residual subcutaneous tumors at the end of the experiment after resection are displayed (Fig. 2b), indicating that the dual ALDH peptides-DC vaccine could induce more potent suppression of tumor growth than single ALDH peptide-DC vaccines.

ALDH peptide(s)-DC vaccine elicited T cell anti-CSC immune responses

We collected the spleens, peripheral blood and residual tumors at the end of the experiments in Fig. 2 and used them to analyze the anti-CSC immune function in vitro. The primed splenic T cells isolated from ALDH 1A1, 1A3 or dual peptide-DC vaccinated mice exhibited increased cytotoxicity of $ALDH^{high}$ D5 CSCs than splenic T cells from PBS treated mice at a E/T = 10:1 ratio ($p = 0.0449$, $p = 0.0063$ and $p = 0.0147$, respectively, Fig. 3a). Of note, dual ALDH peptides-DC vaccine induced higher ($p = 0.0111$) cytotoxicity to $ALDH^{high}$ CSCs than ALDH1A1 peptide-DC vaccine. These elevated cytotoxicities were not observed against $ALDH^{low}$ D5 non-CSC target cells. Importantly, dual ALDH peptides-DC vaccine induced significantly ($p = 0.0302$) higher cytotoxicity to $ALDH^{high}$ CSCs than to $ALDH^{low}$ non-CSCs (Fig. 3a). These data indicate that ALDH peptide(s)-DC vaccine elicits specific anti-$ALDH^{high}$ CSC CTL activity. Using the same CTLs, we performed intracellular staining of IFN-γ to evaluate the cytokine response against $ALDH^{high}$ CSCs versus $ALDH^{low}$ non-CSCs by flow cytometry. Compared with the 1.79% intracellular IFN-γ producing splenic T cells from PBS treated mice, increased intracellular IFN-γ secreting splenic T cells were conferred by ALDH peptide 1A1 (2.76%), 1A3 (3.83%) and dual 1A1 + 1A3 (7.18%)-DC vaccines in response to $ALDH^{high}$ CSCs (Fig. 3b). In contrast, augmented T cell responses were not elicited to $ALDH^{low}$ non-CSCs (Fig. 3c), confirming ALDH peptide (s)-DC vaccines induced specific T cell immune responses to $ALDH^{high}$ CSCs. As shown in Fig. 3d, compared with PBS treated CTLs, CTLs primed by ALDH 1A1, 1A3 or dual peptide-DC vaccine secreted significantly more intracellular IFN-γ ($p = 0.0255$, $p = 0.0330$, and $p = 0.0359$, respectively) in response to $ALDH^{high}$ CSCs, but not $ALDH^{low}$ non-CSCs. Of note, CTLs activated by dual ALDH peptides-DC vaccine secreted more intracellular IFN-γ ($p = 0.0334$) in response to $ALDH^{high}$ CSCs than to $ALDH^{low}$ non-CSCs.

Furthermore, Elispot assays demonstrated that the single peptide-DC vaccine and dual peptides-DC vaccine increased extracellular IFN-γ-producing splenic T cells in response to $ALDH^{high}$ D5 CSCs *vs.* $ALDH^{low}$ D5 non-CSCs (Fig. 4a). As shown in Fig. 4b, the number of IFNγ-producing T cells from ALDH 1A3 peptide-DC and dual peptides-DC treated mice was significantly greater in response to D5 $ALDH^{high}$ CSCs than that in response to $ALDH^{low}$ D5 non-CSCs ($p = 0.0374$ and $p = 0.0116$, respectively). In addition, ALDH 1A1, 1A3 and dual peptide(s)-DC elicited significantly more IFN-γ-producing T cells in response to $ALDH^{high}$ CSCs ($p = 0.0250$, $p = 0.0241$ and $p = 0.0212$, respectively) compared to PBS. These results verified that ALDH peptide(s)-DC vaccine stimulates specific T cell immune responses against $ALDH^{high}$ CSCs.

ALDH peptide(s)-DC vaccine elicited humoral anti-CSC immune responses

To explore whether ALDH peptide(s)-DC vaccination generates B cell immune responses against $ALDH^{high}$ CSCs, we evaluated the binding to $ALDH^{high}$ CSCs by immune sera collected from the animals subjected to ALDH

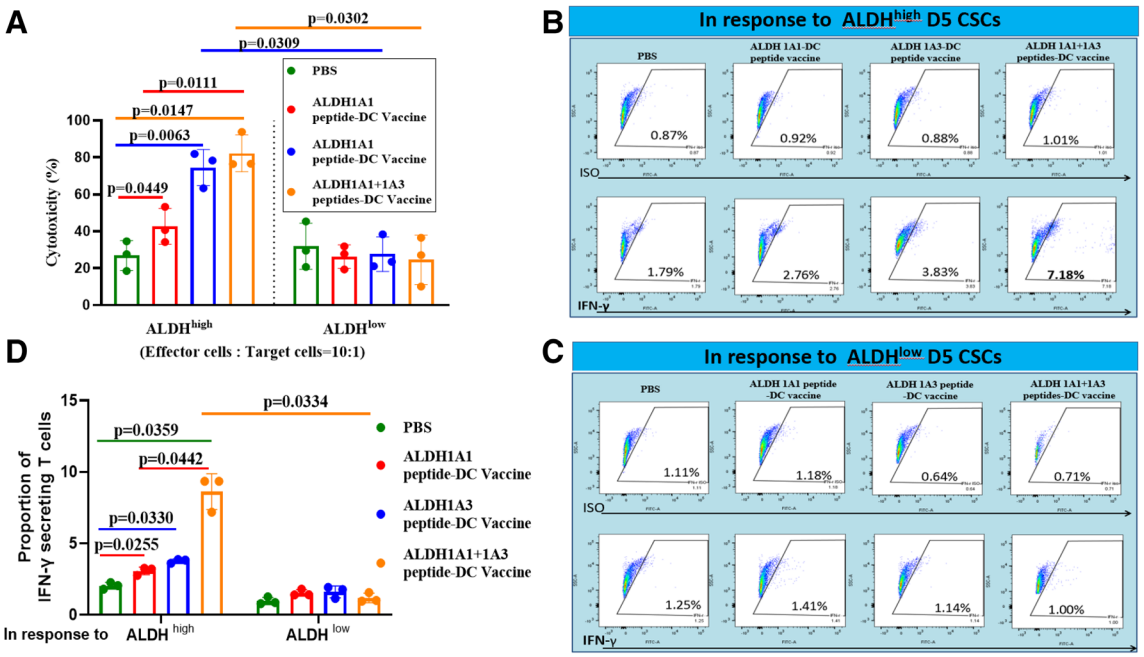

Fig. 3 ALDH peptide(s)-DC vaccine elicited T cell immune responses against ALDH^{high} CSCs. **a**: Cytotoxicity of spleen T cells isolated from D5-bearing mice treated with ALDH 1A1 and/or 1A3 peptides-DC vaccines. This experiment was repeated twice. **b**: Flow cytometry showed intracellular staining of IFN-γ secreting T cells induced by ALDH 1A1 and/or 1A3 peptide(s)-DC vaccine in response to ALDH^{high} D5 CSCs. The top row shows flow cytometry scatter plots using the isotype control for anti-IFN-γ mAb. **c**: Flow cytometry showing the intracellular staining of IFN-γ secreting T cells primed by ALDH 1A1 and/or 1A3 peptide(s)-DC vaccine in response to ALDH^{low} D5 non-CSCs. **d**: The flow cytometry experiments for the detection of intracellular IFN-γ secreting T cells were repeated twice. Bar graph showing the statistical difference of IFN-γ secretion in response to ALDH^{high} cells versus ALDH^{low} cells by T cells collected from animals subjected to various ALDH peptide(s)-DC vaccine treatment as indicated

peptide(s)-DC treatment. As shown in Fig. 5a**,** plasma IgG isolated from ALDH 1A1, 1A3 or dual peptide-DC vaccine-treated mice bound to ALDH^{high} D5 CSCs significantly higher than plasma IgG collected from PBS-treated mice ($p = 0.0028$, $p < 0.0001$, $p < 0.0001$, respectively). The representative binding rates as revealed by flow cytometry were 5.3% for PBS control, and 12.2%, 15.9%, 16.2% for ALDH 1A1, 1A3 and dual peptide-DC vaccine treatment (Fig. 5a). To assess the potential immunological consequence of such binding, we conducted complement-dependent cytotoxicity(CDC)assays. The CDC assay showed that ALDH 1A1 or 1A3 peptide-DC vaccine-primed immune plasma killed ALDH^{high} D5 CSCs significantly more than the control ($p = 0.0007$ and $p < 0.0001$, respectively). Importantly, dual ALDH peptide-DC vaccine-primed immune plasma demonstrated a significantly increased ALDH^{high} CSCs lysis compared with plasma primed by each single peptide-DC vaccine ($p < 0.0001$, $p = 0.0086$, respectively) (Fig. 5b). In contrast, such lysis was not observed when ALDH^{low} non-CSCs were used as control targets. These data indicate that ALDH peptide(s)-DC vaccine can confer host humoral anti-ALDH^{high} CSC specific immunity.

To confirm the in vivo targeting of ALDH^{high} CSCs by the ALDH peptide(s)-DC vaccine, we harvested residual tumors from the treated animals. The tumors were made into single cell suspensions and were stained by the ALDEFLUOR assay to quantify residual ALDH^{high} CSCs. The proportion of ALDH^{high} D5 cells in residual tumors was decreased by ALDH 1A1 (4.18%), 1A3 (3.42%) and dual peptide-DC 1A1/1A3-DC (1.96%) vaccines, compared with the PBS control (14.1%) (Fig. 5c), indicating that dual peptide DC vaccination results in effective reduction of the ALDH^{high} CSCs from nearly 15% to less than 2%.

In addition, immunohistochemistry assays on residual tumors showed that both ALDH 1A1, ALDH 1A3 and ALDH 1A1 + 1A3 peptide(s)-DC vaccines induced more CD3$^+$ tumor-infiltrating lymphocytes (TILs) than PBS (Fig. 5d**).** Moreover, dual 1A1 + 1A3 peptide-DC vaccines recruited more CD3$^+$ T cells to infiltrate D5 tumor than each single peptide-DC vaccine ($p = 0.0122$, $p = 0.0071$, respectively) (Fig. 5e).

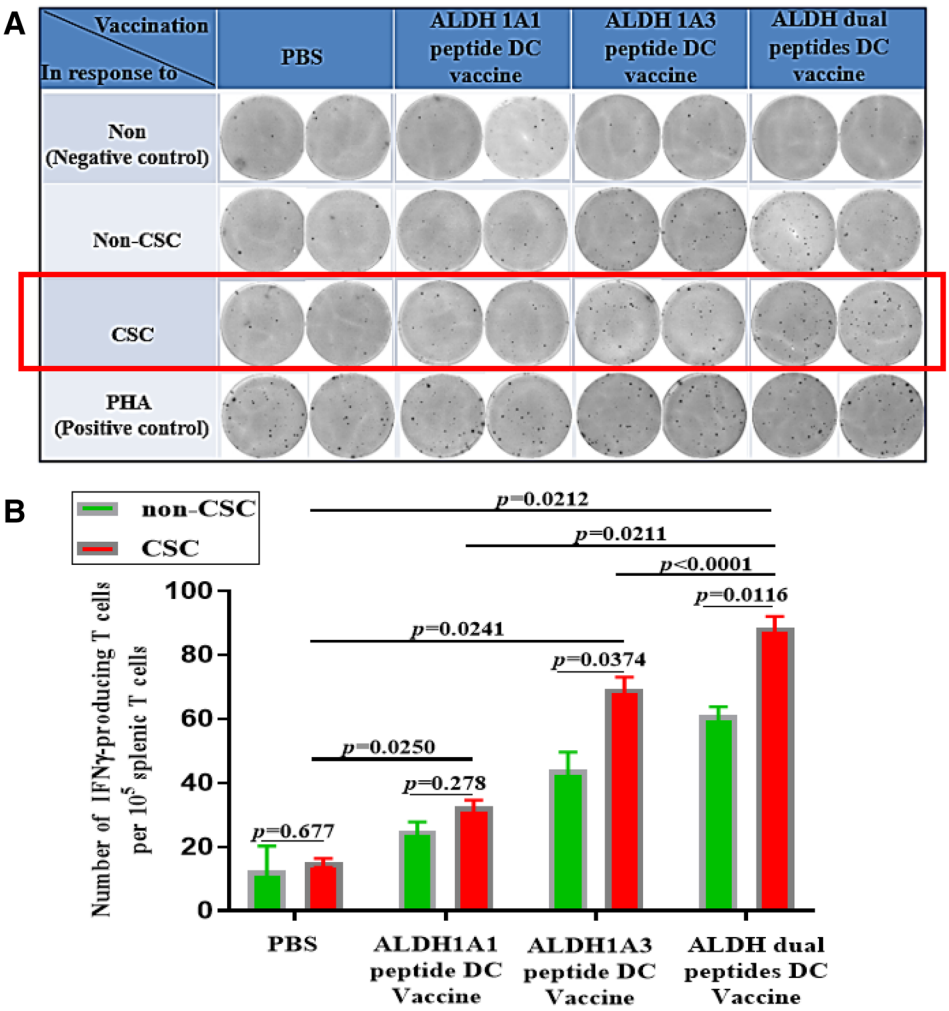

Fig. 4 ALDH peptide(s)-DC vaccine increased extracellular IFN-γ secretion by T cells against ALDH^{high} CSCs. **a**: Representative Elispot results showing the by splenic T cells primed with ALDH peptide(s)-DC vaccination in response to D5 ALDH^{high} CSCs versus ALDH^{low} non-CSCs. **b**: The histogram graph showing the statistics of the Elispot data in **a**. The experiment was repeated twice

Anti-PD-L1 treatment significantly enhanced the anti-tumor effect of ALDH peptides-DC vaccine

We detected the expression of PD-L1 on D5 ALDH^{high} CSCs, ALDH^{low} non-CSCs and unsorted D5 cells by flow cytometry. ALDH^{high} D5 CSCs showed higher PD-L1 expression than ALDH^{low} D5 non- CSCs or unsorted D5 cells ($P < 0.0001$, $P = 0.0032$, respectively) (Fig. 6a and b). We therefore hypothesized that blockade of PD-1/PD-L1 pathway may enhance the efficacy of ALDH peptides-DC vaccines. To test this hypothesis, we examined the potential synergism of anti-PD-L1 treatment and dual ALDH peptides-DC vaccine in the D5 therapeutic model. In this D5 therapy model, therapy was not begun until after tumor implantation. Dual peptide-DC vaccine was administered (s.c.) once a week for two weeks starting 24 h after tumor inoculation with PBS as a control ($n = 5$), and anti-PD-L1 mAb was administered (i.p.) 3 times following each vaccination for a total of 6 times in 2 weeks with an anti-PD-L1 isotype (iso) control. As shown in Fig. 6c, dual peptide-DC vaccine significantly inhibited the growth of D5 tumor compared with PBS control ($p < 0.0001$) as we observed in our previous experiments. The addition of anti-PD-L1 resulted in a significantly greater inhibition of D5 tumor growth compared with dual peptide-DC vaccine alone ($p = 0.0235$) or anti-PD-L1 alone ($p < 0.0001$).

We tested the potential immune mechanism which may be involved in this effect. CTLs from the mice vaccinated with dual peptides-DC with or without anti-PD-L1 exerted greater cytotoxicity of ALDH^{high} CSCs than CTLs from the mice treated with PBS ($p = 0.0402$ and $p = 0.0454$, respectively) (Fig. 6d). Interestingly, although anti-PD-L1 alone did not increase the cytotoxicity of CTLs against ALDH^{high} CSCs compared with the CTLs from the PBS group, dual peptides-DC vaccine plus anti-PD-L1 significantly enhanced the cytotoxic effect of CTLs to ALDH^{high} CSCs compared with the dual peptide-DC vaccine alone ($p = 0.0362$) or anti-PD-L1 administration alone ($p = 0.0091$). However, these effects were not observed when ALDH^{low} non-CSCs were used as target controls in the same CTL assays (Fig. 6d), confirming the specificity

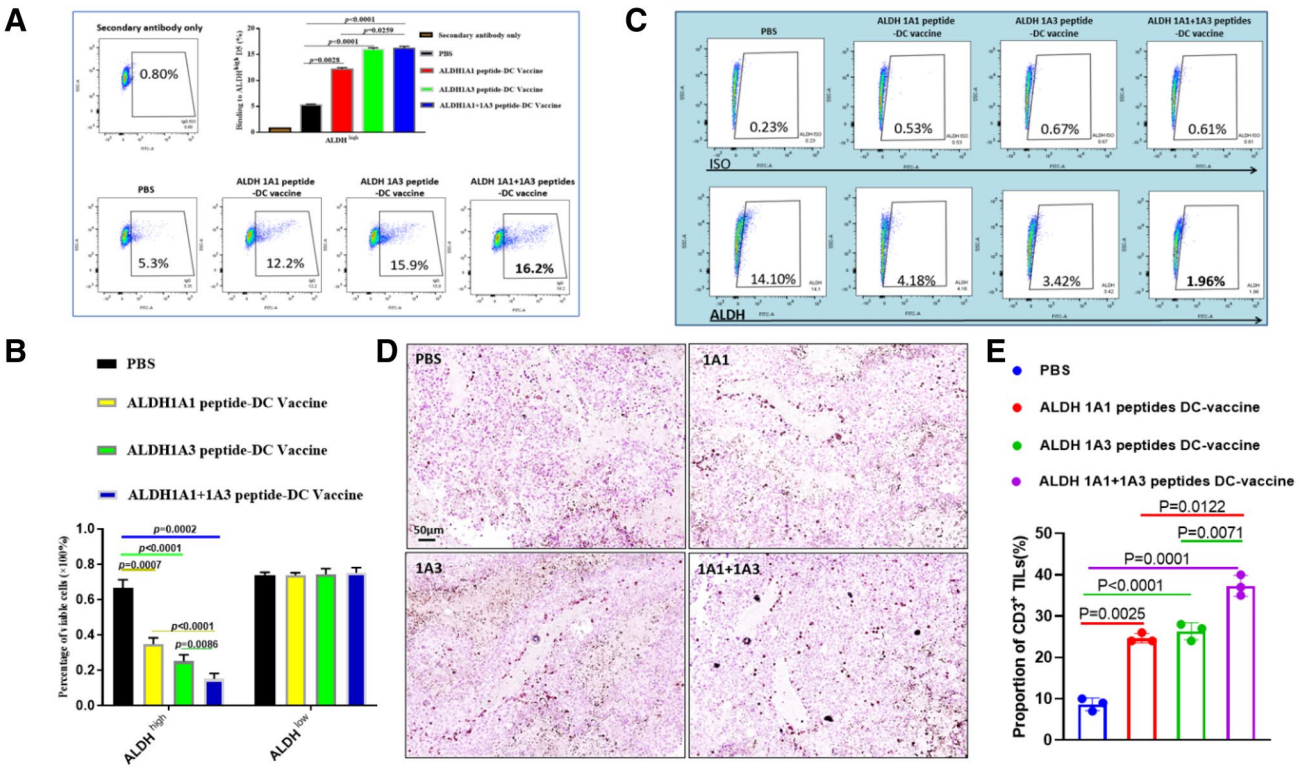

Fig. 5 ALDH peptide(s)-DC vaccine induced humoral immune responses and $CD3^+$ TILs **a**: ALDH peptide(s)-DC vaccine primed mice to produce plasma antibody which bound to $ALDH^{high}$ D5 CSCs. Flow cytometry scatter plots demonstrated the representative binding rate of plasma IgG to $ALDH^{high}$ D5 CSCs, and the histogram graph showed the mean ± standard error of the plasma binding rate to $ALDH^{high}$ D5 CSCs. This experiment was repeated three times. **b**: ALDH peptide(s)-DC vaccine-primed plasma antibody lysed $ALDH^{high}$ D5 CSCs via CDC. $ALDH^{low}$ D5 non-CSCs were used as target control. This experiment was repeated twice. **c**: Flow cytometry showing the percentage of $ALDH^{high}$ CSCs in residual D5 tumors post-treatment as indicated. **d**: ALDH peptide-DC vaccination treatments induced $CD3^+$ TILs. Representative IHC results showing $CD3^+$ TILs in residual tumors harvested from the treated hosts. Tumor cells and background were stained with red and amaranth, respectively. The experiment was repeated twice. **e**: Bar graph comparing the $CD3^+$ TILs induced by different treatments as indicated

of ALDH dual peptides-DC vaccine induced immunity against $ALDH^{high}$ CSCs..

In addition, we measured IFN-γ secretion via Elispot (Fig. 6e). We found that the number of IFN-γ-producing T cells per 10^5 splenic T cells was significantly increased in mice subjected to dual peptides-DC vaccine in response to $ALDH^{high}$ CSCs ($p = 0.0491$) compared with PBS controls. Moreover, T cells from mice treated with dual peptide-DC vaccine plus anti-PD-L1 induced significantly stronger IFN-γ secretion in response to $ALDH^{high}$ CSCs than T cells from mice treated with dual peptide-DC vaccine ($p = 0.0470$) or anti-PD-L1 alone ($p = 0.0211$). Of note, we detected significantly fewer numbers of the IFN-γ-producing T cells in response to $ALDH^{low}$ non-CSCs than to $ALDH^{high}$ CSCs by T cells obtained from mice treated with dual peptide-DC vaccine ($p = 0.0369$) or dual peptide-DC plus anti-PD-L1 ($p = 0.0382$), demonstrating ALDH peptides-DC vaccine elicited anti-$ALDH^{high}$ CSC specificity. Additionally, flow cytometry showed that the proportion of intracellular IFN-γ-producing T cells from the spleen of mice treated with PBS, dual peptide-DC and dual peptide-DC plus anti-PD-L1 was 0.9%, 4.3% and 9.9%, respectively (data not shown). Collectively, this group of data suggest that co-administration of anti-PD-L1 mAb significantly augmented the immune effect of ALDH peptides-DC vaccination by eliciting T cell immune responses against $ALDH^{high}$ CSCs.

Accumulating evidence suggests that TILs may serve as a biomarker to assess the efficacy of cancer immunotherapy [20, 21]. We found that ALDH 1A1 and/or 1A3 peptide(s)-DC vaccination increased $CD3^+$ TILs in residual tumors harvested from the vaccine-treated hosts (Fig. 5d and e). To evaluate the immune response in tumors after the combined use of anti-PD-L1 with the vaccine, we examined $CD3^+$ TILs by flow cytometry and histology from mice in the D5 therapy experiments in Fig. 6c. Representative immunohistochemistry (IHC) of the $CD3^+$ TILs is shown in Fig. 7a. Both dual peptides-DC vaccine and anti-PD-L1 alone induced $CD3^+$ TILs in residual tumors. Importantly, the combination of these two treatments recruited more $CD3^+$

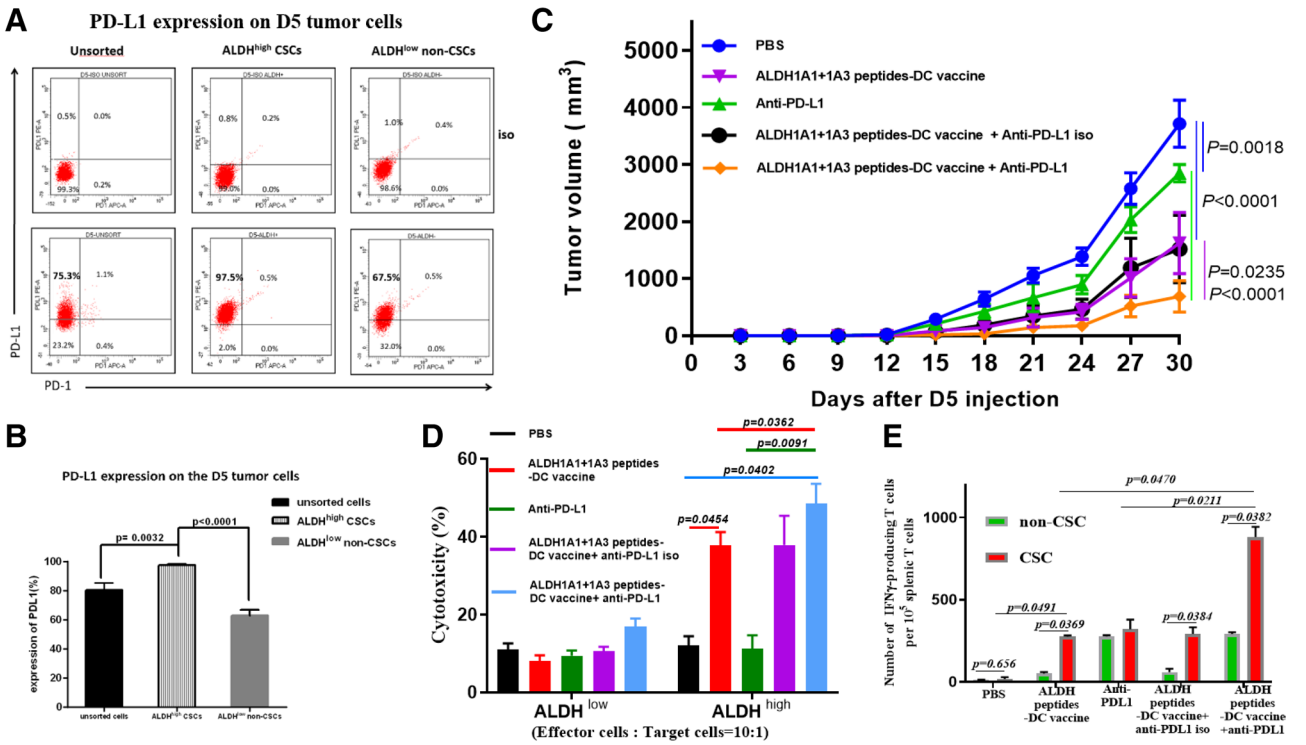

Fig. 6 Anti-PD-L1 significantly enhanced the anti-tumor effect of ALDH dual peptide-DC vaccine in the D5 therapy model. **a**: PD-L1 expression of unsorted D5, ALDH^{high} D5 CSCs and ALDH^{low} D5 non-CSCs as determined by flow cytometry. **b**: The expression of PD-L1 on unsorted D5 cells, ALDH^{high} CSCs and ALDH^{low} non-CSCs. Data graph was generated using the flow plot data in three independent experiments. **c**: Tumor growth in the day 1 D5 therapy model in mice (n = 5 for each group) treated with PBS, dual peptide-DC with or without anti-PD-1 or anti-PD-L1 iso. Data are representative of three experiments. **d**: Cytotoxicity of splenic T cells isolated from D5-bearing mice treated in **C** (E/T = 10:1 ratio) against D5 ALDH^{high} CSCs *vs.* ALDH^{low} non-CSCs. This experiment was repeated twice. **e**: IFNγ-producing splenic T cells from animals in **C** were assayed by Elispot in responses to D5 ALDH^{high} CSCs versus ALDH^{low} non-CSCs. Data are presented as a histogram graph. This experiment was repeated twice

T cells to infiltrate the D5 tumor than each single treatment ($p < 0.0001$, $p = 0.0041$) (Fig. 7b). These data suggest that blockade of the PD-1/PD-L1 pathway enhanced the efficacy of ALDH dual peptides-DC vaccine (Fig. 6c) by increasing local CD3$^+$ TILs in addition to enhancing systemic T cell and antibody immune responses to ALDH^{high} CSCs as demonstrated above.

By flow cytometry, CD3$^+$ TILs from mice after treatment were: PBS (4.58%), ALDH dual peptide-DC (7.02%) and dual peptide-DC plus anti-PD-L1 (10.5%) (Fig. 8a). Statistical analysis of three flow cytometry experiments showed that dual peptide-DC and anti-PD-L1 combined treatment group induced more TILs than monotherapy group ($p = 0.0005$ and $p = 0.0178$) (Fig. 8b). In addition, we examined the amount of residual CSCs at the end of the therapy experiments from Fig. 6c. By flow cytometry, both ALDH dual peptide-DC vaccine and anti-PD-L1 administration reduced the proportion of the ALDH^{high} D5 cells in residual tumors (0.38% and 0.43%, respectively) compared with the PBS control (2.03%) (Fig. 8c). Of note, combined therapy of the two approaches reduced CSCs (0.18%) with the highest efficacy, indicating their interactive roles as evident by the reduction of ALDH^{high} D5 cells by more than 90% compared to the PBS control.

Discussion

Cancer stem cells promote the growth, metastasis and contribute to relapse of tumors. Furthermore, CSCs are relatively resistant to chemotherapy and radiotherapy [6]. Although immunotherapy represents an important breakthrough in cancer therapy, the limitation of these therapies may relate to their inability to effectively target the CSC population. The development of CSC targeting cancer vaccines has the potential to overcome this limitation. Over the past 50 years, many attempts have been made to generate cancer vaccines with limited success. However, it is important to note that the vast majority of cancer vaccines have focused on targeting differentiation associated antigens. These antigens are not expressed on CSCs, potentially limiting their clinical efficacy. Ex vivo-generated DCs loaded

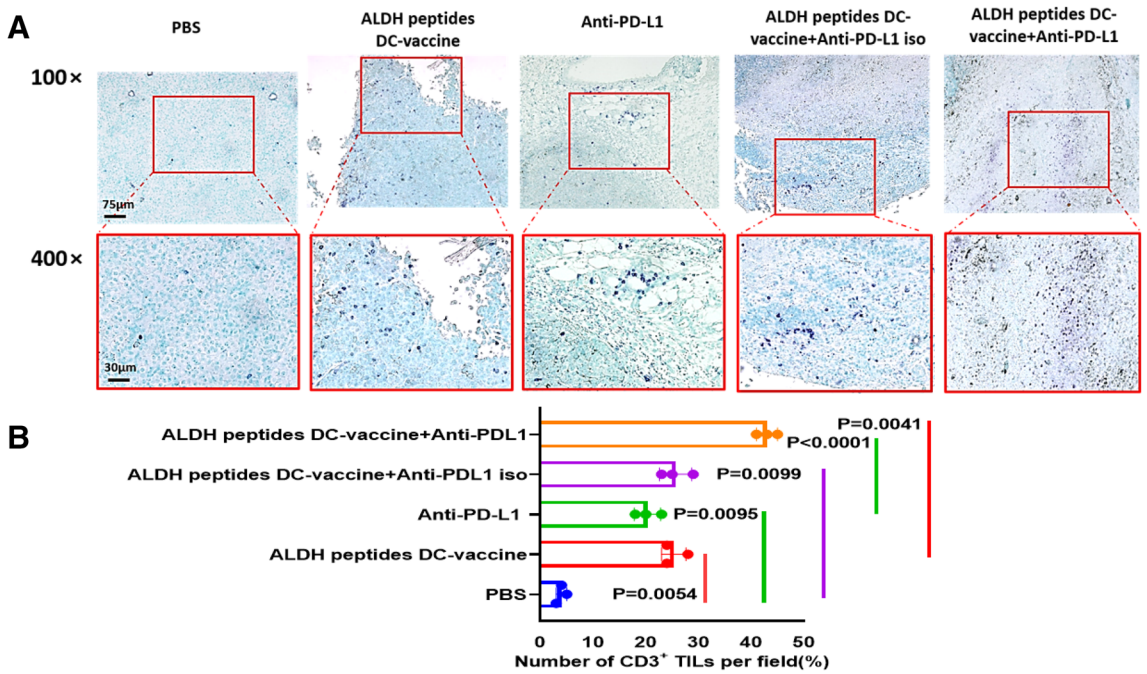

Fig. 7 **a**: The representative IHC results confirming increased $CD3^+$ TILs in residual tumors. Tumor cells and background were stained with green color, and the purple-stained cells indicate the $CD3^+$ TILs. **b**: Bar graph showing the statistical difference of $CD3^+$ TILs post-treatments as indicated. The experiment was repeated twice

with tumor cell lysate or molecular antigens have been tested in the clinic and proven to be effective [22]. However, the clinical responses have been confined to a limited number of patients. In a phase I trial, Rudnick et al. evaluated the safety and potential synergy of surgical resection with Gliadel Wafer implantation, followed by autologous tumor lysate-pulsed DC vaccine in patients with malignant glioma. While the adjuvant autologous DC vaccine was safe, elicited modest immunogenicity, comparisons between vaccine responders and non-vaccine responders were not statistically significant [23]. Based on 173 published trials, Neller et al. described that 138 of 1711 patients treated with tumor cell lysate-based DC vaccines exhibited an objective response rate of 8.1%, and 63 of 1733 patients who were treated with molecularly defined antigen-based DC vaccines showed a response rate of 3.6% [24]. One of the major reasons responsible for these low response rates was the inability of these vaccines to induce anti-CSC immunity.

CSC-targeted immunotherapies represent promising advances in cancer treatment. We previously developed a vaccination strategy utilizing cell lysates of $ALDH^{high}$ CSCs to pulse DCs and found that vaccination of animals with these $ALDH^{high}$ CSC-targeted DCs resulted in significant anti-tumor immunity in prevention models [2]. Furthermore, we demonstrated in the adjuvant setting that $ALDH^{high}$ CSC lysate-DC vaccine significantly inhibited local recurrence after surgical resection of established tumor, compared with either $ALDH^{low}$ or unsorted tumor cells lysate-DC vaccine [4]. In addition, using a radiation therapy model for established tumors, we reported that $ALDH^{high}$ CSC lysate-DC vaccination significantly inhibited tumor growth, reduced development of metastases and prolonged mice survival [3]. These results were consistent with two other studies in which pancreas and breast CSC lysate-pulsed DC vaccines, respectively, exhibited cytotoxic effect to CSCs [25, 26].

Although we have shown significant efficacy of vaccination with DCs pulsed with cell lysate of $ALDH^{high}$ CSCs (CSC-DC), obtaining adequate amounts of tumor from patients to isolate and make CSC lysate for vaccine production is labor intensive and often not feasible, thus representing a significant limitation for the clinical translation of this approach. Shared CSC antigens could serve as specific targets for cancer immunotherapy. For example, cytokine-induced killer cells armed with anti-CD3/anti-CD133 bispecific antibody significantly killed pancreatic and hepatic $CD133^{high}$ CSCs [27]. In addition, a specific chimeric antigen receptor (CAR) was designed to target CSC marker EpCAM, and the CAR therapy exerted significant anti-tumor activity against prostate cancer [28]. Studies have found that several isoforms of ALDH such as 1A1 and 1A3 not only regulate CSC function but also serve as markers for CSCs [29]. Visus et al. identified ALDH 1A1 as a novel $CD8^+$ T cell-defined tumor antigen in head and neck squamous cell carcinoma [8], and HLA-A2-restricted,

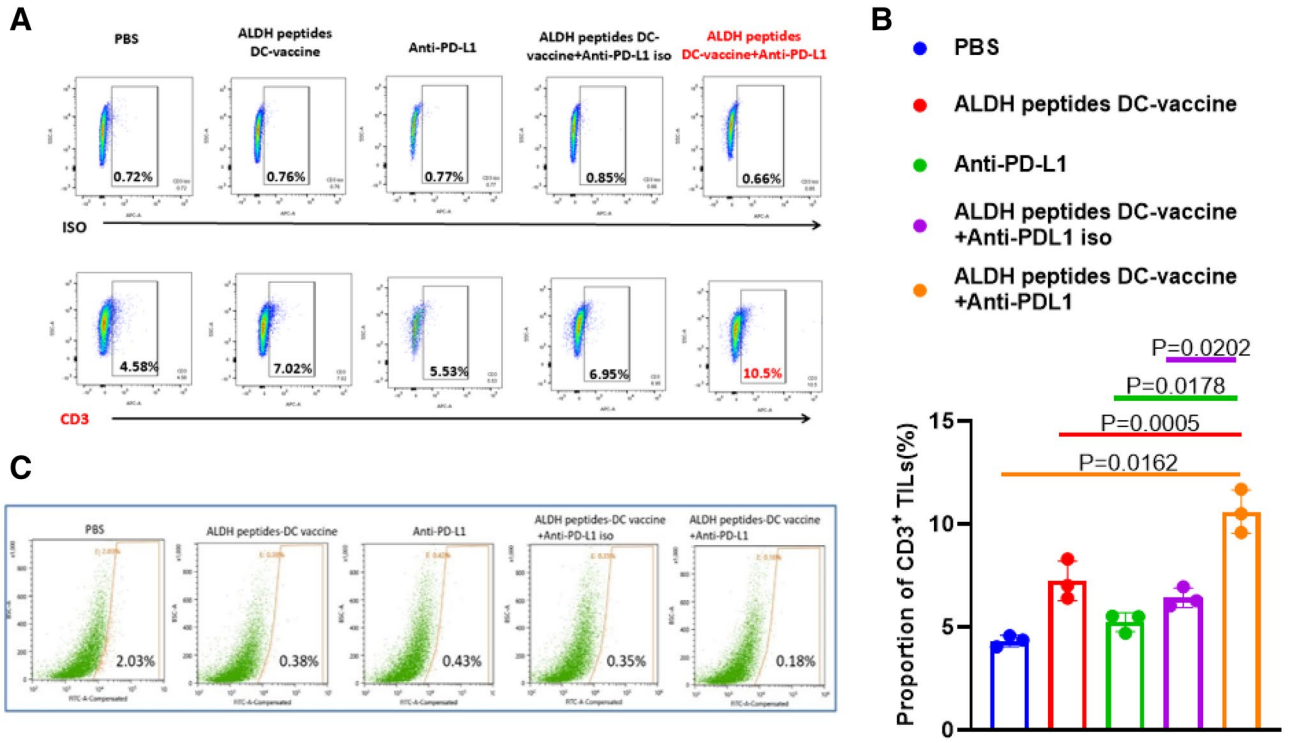

Fig. 8 Anti-PD-L1 enhanced the effect of ALDH peptides-DC vaccine via recruiting more $CD3^+$ TILs and reducing the $ALDH^{high}$ CSCs. **a**: Anti-PD-L1 administration in addition to ALDH dual peptides-DC vaccination increased the $CD3^+$ TILs as shown by flow cytometry. The single tumor cell suspension was made from residual D5 tumors harvested from the mice subjected to treatment as indicated. **b**: Bar graph showing the statistical difference of $CD3^+$ TILs post-treatment as indicated. The experiment was repeated twice. **c**: ALDH dual peptides-DC vaccine with anti-PD-L1 decreased the $ALDH^{high}$ D5 cells in residual tumors. Flow cytometry showed the representative percentages of $ALDH^{high}$ D5 cells in residual tumors harvested from the mice post-treatment as indicated. Five mice were used each group

$ALDH1A1_{88–96}$ peptide-specific $CD8^+$ T cells could recognize and eliminate $ALDH^{high}$ CSCs in vitro. The adoptive transfer of this ALDH1A1 peptide-specific $CD8^+$ T cells inhibited tumor growth and reduced lung metastases in HNSCC and breast cancer in vivo [11]. However, Marcato found that CSC activity in patient with breast cancer correlated with the expression of isoform ALDH1A3 [30]. In other studies, both ALDH1A1 and ALDH1A3 have been identified as important contributors to CSC function [31, 32].

Our previous studies (2–4) demonstrated that ALDH may represent an ideal marker for CSC targeting. To eliminate the requirement to obtain intact CSCs as a source of antigen for CSC-targeted DC vaccine, we tested two peptides derived from ALDH1A1 (LLYKLADLI) and 1A3 (LLHQLADLV) as antigens to generate ALDH peptide-DC vaccines in this study. We found that ALDH 1A1 and 1A3 peptide-DC vaccines, respectively, induced T cell proliferation and anti-$ALDH^{high}$ CSC activity in vitro. CTL primed by these vaccines specific killed the $ALDH^{high}$ D5 CSCs. Importantly, we observed that an ALDH 1A1 + 1A3 dual peptides-DC vaccine demonstrated additive effect *vs.* each single peptide-DC vaccine. We further examined the efficacy of the ALDH peptide-DC vaccine in vivo in both the prevention and minimal disease therapy settings. ALDH1A1 and/or 1A3 peptide(s)-DC vaccine demonstrated significant protective immunity by inhibiting D5 tumor growth after challenge. In the minimal disease setting (day 1 tumor), ALDH peptide(s)-DC vaccination was efficacious in suppressing tumor growth that was enhanced by the co-administration of anti-PD-L1. In a double-blinded, randomized phase II trial in newly-diagnosed glioblastoma (GBM) patients, Wen et al. have recently evaluated an autologous DC vaccine pulsed with six synthetic peptide epitopes targeting GBM tumor/stem cell-associated antigens MAGE-1, HER-2, AIM-2, TRP-2, gp100 and IL-13Rα2 (ICT-107). The vaccine was well tolerated and increased progression-free survival by 2.2 months [33]. However, the primary endpoint of overall survival was not statistically increased. This study indicates that autologous DC vaccine targeting tumor/stem cell-associated antigens may not be sufficient, and the addition of anti-PD-L1 may increase its efficacy.

Immune monitoring in our study revealed that ALDH dual peptides-DC vaccination elicited strong T cell and

antibody immunity targeting ALDH^{high} CSCs, and this targeting was significantly elevated by co-administration of anti-PD-L1. As a result, the percentage of ALDH^{high} D5 CSCs was significantly reduced by 90% after ALDH dual peptides-DC vaccination with anti-PD-L1 administration. Furthermore, we found that ALDH dual peptides-DC vaccination resulted in the recruitment of CD3^{+} TILs, which was also enhanced by anti-PD-L1 administration. Identification of the subsets of these CD3^{+} TILs and characterization of their function, e.g., T effectors versus Treg, warrant further investigation.

The study of interaction between CSCs and the immune system is of great interest, as CSCs may not only evade the immune system's surveillance, but also use the immune system to promote their expansion and tumorigenesis [34]. The innate immune responses mediated by natural killer cells and macrophages have been reported to regulate the stemness of CSCs [35, 36]. Most notably, PD-L1 expression on CSCs was significantly elevated in various cancers, which has been suggested to facilitate CSC immune evasion [37, 38]. In a previous publication, we have demonstrated that a reduction in PD-L1 expression on tumor cells was strongly associated with ALDH^{high} CSC-DC vaccine treatment [4]. On the other hand, PD-1/PD-L1 blockade together with vaccine therapy facilitated effector T cell infiltration into pancreatic tumors [39]. Another study confirmed that PD-1/PD-L1 blockade enhanced the efficacy of SA-GM-CSF surface-modified tumor vaccine in prostate cancer [40]. More recently, we have reported that anti-PD-L1 antibody significantly strengthened the therapeutic efficacy of an integrin β4 (ITGB4)-DC vaccine in both the 4T1 and SCC7 models [41]. ITGB4 has been shown to play an important role in the regulation of CSCs.

This work represents an addition to the initial work targeting ALDH peptide via passive (T cell) immunotherapy [8, 11], but with an alternative approach, e.g., active (vaccine-based) immunotherapy. The potential molecular and immune mechanisms underlining the anti-CSC effect of the vaccine need to be further clarified. Exploring the potential effect of the ALDH peptide(s)-DC vaccine combined with traditional cancer therapies, e.g., surgery, chemotherapy and radiation therapy, needs to be further explored to optimize the potential of this vaccine approach. Technically, ALDH peptide(s)-based vaccines may allow for the development of an "off-the-shelf" product as an immunologic approach for more broadly applicable clinical translation. Another important issue concerns the selectivity of this approach at generating anti-CSC responses while sparing normal tissue stem cells that also express ALDH. In our immunocompetent mouse models, we did not observe any systemic toxicity associated with vaccination. Although the mechanism of selective toxicity of CSCs *vs* normal tissue stem cells remains unknown, we have preliminary evidence for differential distribution of ALDH isoforms in these cells. Additional work will be required to further explore the mechanism(s) of this selective toxicity of CSCs vs normal tissue stem cells to understand the potential toxicity of these ALDH peptide(s)-based vaccines in clinical translation. It is critical to show the ALDH isoform expression on human melanoma tumors and identify HLA-A2 epitopes of these regions for human translation. This warranties further investigation.

Another area to explore is the identification of other CSC-associated antigens that could be utilized in combination with ALDH peptides. To identify other potential CSC antigens, we have begun to examine genes involved in reprogramming adult cells toward a more undifferentiated, pluripotent state including Sox2, Oct4 and Nanog, transcription factors capable of reprograming normal fibroblasts into induced pluripotent stem (iPS) cells [42]. The majority of CSCs express one or more of these reprogramming transcription factors. This concept is supported by recent evidence that a DC vaccine generated against iPS cells generates anti-tumor immunity across a spectrum of tumor types [43, 44] with no apparent systemic toxicity. Another recent report has demonstrated that pharmacologically modified pluripotent stem cells administered as a vaccine have anti-tumor effects in a murine breast cancer model [45]. Since these antigens are widely expressed in CSCs across a variety of tumor types, this approach may have wide applicability.

Author contributions FL, ZJ and YYH performed the experiments, analyzed the data, prepared the figures and drafted the manuscript. QL and AEC designed the project and finalized the manuscript. Other authors provided assistance with partial experiments and data analysis. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The author(s) declare that they have no conflict of interest.

Data availability Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethical approval All the procedures used in the animal studies were approved by the Animal Care and Use Committee of the University of Michigan.

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