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PRECLINICAL STUDIES

A novel humanized anti-PD-1 monoclonal antibody potentiates therapy in oral squamous cell carcinoma

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Summary
Currently, immune checkpoint inhibitors have been shown to extend the survival of many cancer patients. However, few studies have focused on immune checkpoint inhibition for the treatment of patients with oral squamous cell carcinoma (OSCC). Here, by screening at an early stage, we obtained a strain of anti-PD-1 monoclonal antibody (mAb) that targets programmed cell death-1 (PD-1) does not contain the C_{H1} and C_L fragment. In this study, the role of our novel mAb was tested in the treatment of OSCC in vitro and in vivo. We found that our novel mAb can significantly augment T cell mediated cytokine secretion, target cellular lytic and apoptotic abilities, and inhibit tumor growth and inflammation in vivo. The PD-L1 blockade was accompanied by the inhibition of AKT and ERK1/2, thus suggesting that the PD-L1/PD-1 signaling pathway may play an important immunopreventive role in the tumorigenic properties of OSCC cells by modulating the AKT and ERK1/2 pathways. Additionally, PD-L1 staining was observed both in human OSCC tissues and normal oral mucous tissue adjacent to the tumor, which occurred at different rates. Taken together, these results indicated that our novel anti-PD-1 mAb may be used as a clinical therapy in human OSCC development and progression.

Keywords Oral squamous cell carcinomas · Monoclonal antibody · PD-1 · PD-L1

Introduction

Oral squamous cell carcinoma (OSCC) is among the ten most common malignant tumors worldwide and is highly immunosuppressive in general [1, 2]. Although recent advancements in oral maxillofacial surgery and diagnostic accuracy have improved the survival rate of patients with OSCC, the 5-year survival rate is still very low [3].

With the development of immunotherapy as a cancer treatment, there is growing awareness that immune escape mechanisms may contribute to therapeutic failure when treating tumors. Currently, immune checkpoint inhibitors and monoclonal antibodies have been shown to extend the survival of many cancer patients [4–6]. Immune checkpoints and their ligands are expressed on a wide range of cell types, which are essential for central and peripheral tolerance because they counteract simultaneous activation signaling via co-stimulatory molecules. As an important immune checkpoint, programmed cell death protein 1 (PD-1) is often expressed in exhausted T cells in patients with chronic viral infections and cancer [7, 8]. PD-1 has two ligands: PD-L1 and PD-L2, which can be found on the surface of antigen-presenting cells and tumor cells [9, 10]. Under physiological conditions, the PD-L1/PD-1 pathway plays an important role in regulating the severity of immune responses during infection and allows for peripheral self-antigen tolerance, preventing autoimmune disease

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[11–15]. However, tumor-associated PD-L1 can induce anergy and apoptosis or downregulate of effector cytotoxic T lymphocytes (CTL) in the tumor microenvironment, which facilitates tumors escape from T cell mediated immune surveillance [16, 17]. Activating the PD-L1/PD-1 pathway by binding to the PD-1 receptor on activated T lymphocytes can attenuate the immune response, suggesting that inhibitory receptor-ligand interactions targeting antitumor T lymphocyte immunity are occurring in the tumor microenvironment, and the expression of PD-1 on effector T cells is a marker of T cell unresponsiveness or exhaustion [18]. Tumor expression of PD-L1 has also been associated with deregulated oncogene signaling [19, 20], tumor hypoxia [21], and the production of local inflammatory signals, including IFN-γ [22].

Blockade of PD-L1/PD-1 immune checkpoints using monoclonal antibodies can alleviate local immunosuppression and augment the T cell response against tumor cells, efficiently reducing tumor growth and improving survival [23–26]. In recent clinical studies, durable tumor regression with blockade of the PD-L1/PD-1 checkpoints has been demonstrated, which lead to the recent registration of an anti-PD-1 antibody for the treatment of advanced melanoma and non-small cell lung cancer [27, 28]. Pembrolizumab and nivolumab are the two immune checkpoint inhibitors approved by the US Food and Drug Administration for treating melanoma and non-small cell lung cancer [29, 30]. These results provide evidence for the potential utility of PD-L1/PD-1 blockade therapy clinically.

OSCC is highly immunosuppressive, and PD-L1 expression has been proposed as a potential mechanism that contributes to the development of this phenotype [31, 32]. Therefore, OSCC patients may benefit from cancer prevention strategies that include immune checkpoint inhibition. The clinical efficacy of an anti-PD-1 monoclonal antibody in OSCC has been observed in phase I studies [33]. However, despite evidence that anti-PD-1/PD-L1 mAb treatment can produce durable responses, it appears to only benefit a small group of patients. There is still a subset of patients that have not benefited from anti-PD-1/PD-L1 mAb treatment, and few studies have used a PD-L1/PD-1 checkpoint inhibitor in the treatment of patients with OSCC. Thus, it is important to understand the mechanisms underlying regulation of the PD-L1/PD-1 signaling pathway in the OSCC microenvironment.

The purpose of this study was to explore PD-L1 expression in OSCC, investigate the efficacy of our novel anti-PD-1 mAb in the treatment of OSCC, and clarify the mechanism of regulation of the PD-L1/PD-1 signaling pathway in the tumor microenvironment in the development of OSCC. We found that our novel anti-PD-1 mAb significantly augmented T cell mediated target cell lytic and apoptotic ability, while inhibiting tumor growth and inflammation in vivo. PD-L1 blockade resulted in the inhibition of AKT and ERK1/2 signaling. Therefore, we uncovered a novel mechanism relevant to future clinical trials of an anti-PD-L1/PD-1 mAb in OSCC.

Materials and methods

Cell lines and cell culture conditions

CAL-27 was obtained from the American Type Culture Collection (ATCC), and HN30 was obtained from the National Institutes of Health (NIH). HIOEC, which is human immortalized oral epithelial cells, was provided by the Laboratory of Oral Oncology, the Ninth People's Hospital affiliated to School of Medicine, Shanghai Jiaotong University, China. HN30 and CAL-27 were cultured in DMEM and supplemented with 10% FBS (GIBCO) and of 1% penicillin/streptomycin (GIBCO). HIOEC cells were maintained in defined keratinocyte-SFM (Gibco, NY, USA) medium. Cells were incubated at a 37 °C humidified incubator containing 5% CO_2.

Reagents and antibodies

The anti-PD-1 mAb was synthesized in our laboratory. Human recombinant IFN-γ and PHA was purchased from GenScript (Nanjing, China). The antibodies used include: PD-L1 mAb (#13684), AKT mAb (#9272), phospho-AKT (Ser473) mAb (#4060), p44/42 MAP kinase mAb (#4696), phospho-p44/42 MAP kinase (Thr202/ Tyr204) mAb (#4376), GAPDH mAb (#2118). Anti-mouse IgG HRP-linked antibody (#7076) was used as secondary antibody. All antibodies were purchased from Cell Signaling Technology, Inc.

Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining

Forty human OSCC specimens were collected from patients who had undergone surgery between September 2009 and September 2010 at the Department of Oral and Maxillofacial Surgery, Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University. The pathological characterization of the OSCC patients included in this study is summarized in Table 1. The IHC as previously described [34]. The IHC results in tissues were scored by two independent investigators based on the level of staining intensity as follows: none (−), 0% of stained cells; weak (+), 1–25% of stained cells; moderate (++), 26–50% of stained cells; strong (+++), >50% of stained cells. Mice liver tissue sections were dewaxed in xylene,

Table 1 The correlation between clinicopathological features and expression of PD-L1

Characteristics	Case. no	PD-L1 positive grade	Nonparametric tests value	*P.* value
Tobacco				
Yes	17	1.52 ± 1.06	Z = −0.961	0.437
No	23	1.04 ± 1.12		
Alcohol				
Yes	18	1.5 ± 0.98	Z = −0.297	0.778
No	22	1.59 ± 1.05		
Sex				
Male	28	1.54 ± 1.03	Z = −0.169	0.873
Female	12	1.58 ± 0.99		
Tumor site				
Oral cavity	21	1.61 ± 0.973	$\chi 2 = 0.653$ d.f. = 3	0.884
Gingiva	6	1.33 ± 1.03		
Mouth floor	7	1.71 ± 1.11		
Other	6	1.5 ± 1.04		
Tumor stage				
T1	17	1.5 ± 0.96	$\chi 2 = 0.778$ d.f. = 3	0.855
T2	12	1.69 ± 1.03		
T3	6	1.5 ± 1.04		
T4	5	1.6 ± 1.14		
Nodal status				
N0	27	1.55 ± 0.97	Z = −0.181	0.857
N1–2	13	1.61 ± 1.04		
Pathological grade				
Well	23	1.17 ± 0.94	$\chi 2 = 8.95$ d.f. = 2	0.011
Moderatedly	13	2.07 ± 0.76		
Poorly	4	2.25 ± 0.96		

d.f. degree of freedom

rehydrated through decreasing concentrations of ethanol, and washed in PBS. Then sections stained with H&E. After staining, sections were dehydrated through increasing concentration of ethanol and xylene.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from each cell line with TriReagent (Sigma-Aldrich) according to the manufacturer's instructions. The primer pairs used were: PD-L1, forward 5'-TGGCATTT GCTGAACGCATTT3', reverse 5'-GTGGTGGTCTTACC A- CTCAGG-3′; GAPDH, forward 5'-CATCTCTG CCCCCTCTGCTGA-3′, reverse 5'-GGATGACCTTGCCC ACAGCCT-3′.

Human PBMC preparation and transplantation

Blood from healthy volunteers was collected in heparinized tubes. For the isolation of PBMCs, blood was diluted 1:1 with RPMI1640 medium (vol/vol) prior to transferring into the leucosep tube. Following centrifugation (20 min, 2000 rpm), the PBMC layer was pooled and transferred into a 15 ml falcon tube. The sample was washed with 10 ml phosphate-buffered saline (PBS) and centrifuged again for 10 min at 1500 rpm. The obtained cell pellet was re-suspended in PBS. A total of 1×10^7 PBMCs per mouse were injected into NOD/SCID mice through tail vein for the reconstitution of immune system.

Elisa

The cytokines levels of IL-2 and IFN-γ (culture supernatants), TNF-α and IL-6 (mice serum) were measured using the specific ELISA kits (R&D Systems) following the manufacturer's instructions.

Effects of mAb on T cell induced lysis and apoptosis of tumor cells

T cell induced lysis and apoptosis of tumor cells were conducted as previously described [34, 35].

Western blotting

Western blot analysis was performed to detect key proteins involved the pathway. The cells were lysed with M-PER® Mammalian Protein Extraction (Pierce) and the protein were tested as previously described [34].

Tumor xenograft study

Approximately 5- to 6-week male NOD/SCID mice (about 20 g) were obtained from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were kept in a specific pathogen-free (SPF) facility. They are absent of T, B lymphocytes, and NK cells. All mice were treated in accordance with international ethics guidelines and the National Institutes of Health Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of Tongji University. CAL-27 cells (5×10^6cells) were subcutaneously injected into the right flank region of mice. After 7 days, allowing the tumors to grow to about 50 mm3, mice were randomized into control and treatment groups. For CAL-27 xenograft, mice were treated as follows: anti-PD-1 mAb and IgG4 (5 mg/kg, twice per week), T cells (1×10^7) were injected through the tail vein during the first and fourth administration of drugs.

Statistical analysis

The statistical analysis between groups was performed using GraphPad Prism 5 software. The unpaired 2-tailed *t* test was used for the comparison and the level of significance was defined when $P < 0.05$.

Results

Structural characteristics and binding properties of the anti-PD-1 mAb

Our anti-PD-1 mAb is an IgG4-based antibody without the C_{H1} and C_L regions that binds with two arms to PD-1 (Fig. 1a). From the DNA encoding the anti-PD-1 mAb, the V_H, V_L and Fc regions of the mAb were individually amplified by PCR. Then, using overlapping PCR, a mAb was constructed. The variable regions were connected with a $(G4S)_3$ linker. For eukaryotic expression, the mAb was cloned into the expression vector pCEP4. Through the above measures, the mAb was obtained after expression and purification procedures with greater than 90% purity, which was confirmed by HPLC analysis (data not shown). The mAb was shown to bind to CHO cells expressing PD-1 with an increasing mean fluorescence intensity (MFI) as dose increased from 10^{-6} μg/mL to 100 μg/mL (Fig. 1b), but did not bind to the parental CHO cell line (data not shown). The curves indicate the hIgG4 and anti-PD-1 mAb treated groups binding to CHO-PD-1 cell. The results of this study demonstrate that the anti-PD-1 mAb exhibits potent binding activity against cells that express the target antigen.

The anti-PD-1 mAb significantly increases T cell mediated cytokine secretion, cytotoxicity, and apoptosis

Cytokine secretion is an important criterion for evaluating T cell function. To assess the preventive effects of CAL-27 cell PD-L1-expressing cells on T cell function, T cells were cocultured with IFN-γ-pretreated with CAL-27 cells at different E:T proportions in the presence of phytohaemagglutinin (PHA) for 48 h and then tested IL-2 production by ELISA. CAL-27 cells cocultured with T cells at an E:T of 5:1 significantly reduced T-cell IL-2 production (Fig. 2a, left). In a co-culture system, CAL-27-associated inhibition of IL-2 secretion was shown to be cell-contact-dependent (Fig. 2a, right, blue histogram). IL-2 levels were significantly inhibited in T-cells cocultured in contact with CAL-27 cells ($P < 0.01$) compared to those in separated coculture (Fig. 2a, right, red histogram). The anti-PD-1 mAb pretreatment can significantly rescue IL-2 secretion in T cells cocultured in contact with CAL-27 cells compared versus IgG4-treated cells ($P < 0.01$). The expression level of IFN-γ was also tested by ELISA (Fig. 2b).

At the same time, we evaluated the effect of the anti-PD-1 mAb on T-cells mediated target cell lytic and apoptosis abilities. T cells were treated with anti-PD-1 mAb or IgG4 (0.5 μM) for 6 h, and then T-cells were cocultured with human CAL-27 cells in the presence of PHA (1 μg/mL) for 8 or 48 h. After 8 h of treatment, the killing efficacy of the T cells was dose-dependent at 5:1 to 10:1 ratio (Fig. 3a, left column), and the anti-PD-1 mAb augmented T cells induced tumor killing efficacy (Fig. 3b, right column). After 48 h of treatment, apoptotic cells were stained with Annexin V and propidium iodide and analyzed by flow cytometry to measure the apoptotic index. It can significantly augment T-cell induced CAL-27 apoptosis when T cells are pretreated with anti-PD-1 mAb (Fig. 3b).

The anti-PD-1 mAb inhibits tumor development and inflammatory infiltration in vivo

The effect of the anti-PD-1 mAb on tumor development was evaluated by heterotopic xenograft analysis in vivo. NOD/SCID mice were subcutaneously injected with CAL-27 cells. After 7 days, the anti-PD-1 mAb and IgG4 were administered twice a week for 3 weeks. T cells were injected through the tail vein during the first and fourth administration of drugs. The results showed that our anti-PD-1 mAb can significantly inhibit CAL-27-induced tumor growth compared to IgG4 treatment. It also reduced the tumor volume by nearly 50%

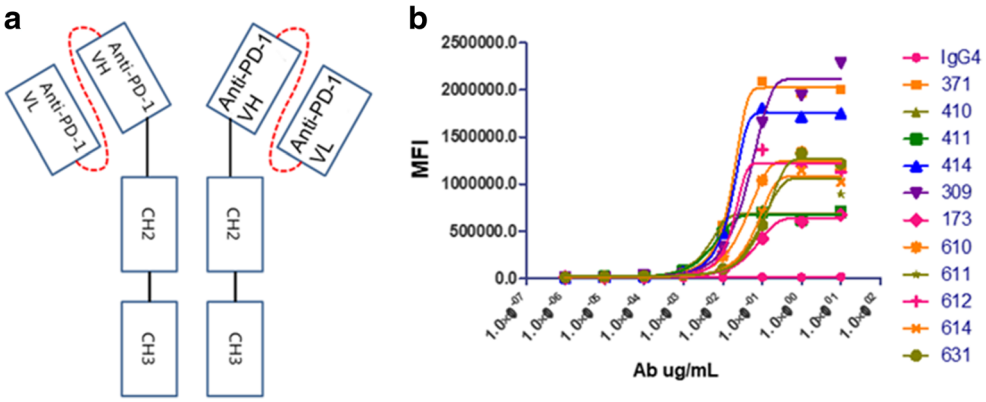

Fig. 1 The structure and binding of the anti-PD-1 mAb. **a** Schematic diagram of the mAb design. The red dashed line indicates the $(G4S)_3$ linker between the V_L region and V_H region; **b** Mean fluorescence intensity detected for the binding of the mAb to CHO-PD-1 cells at various concentrations (EC_{50} of binding 10 ng/mL). The curves indicate the hIgG4 and the anti-PD-1 mAb treated groups

(Fig. 4a) and inhibited tumor weight by approximately 40% (Fig. 4b). There was no significant difference in the total body weight of the mice (data not shown). HE staining showed that there were abundant lymphocytes in the mouse livers of IgG4-treated mice. However, we did not find any lymphocytes in the mouse livers of anti-PD-1 mAb-treated mice (Fig. 4c). In addition, we found that the anti-PD-1 mAb reduced the secretion of inflammatory cytokines in vivo (including IL-6, but not TNF-α) compared with control mice (Fig. 4d), which suggests that the anti-PD-1 mAb treatment can inhibit chronic inflammation in vivo.

PD-L1 blockade inhibits IFN-γ-triggered PD-L1/PD-1 downstream AKT/ERK1/2 signaling

The PD-L1/PD-1 signaling pathway plays a significant role in tumor development and immune escape, often through activation of downstream molecules, including the JAK/STAT3 and PI3K/AKT pathways [36]. In the present study, we found that IFN-γ-mediated upregulation of PD-L1 in OSCC cells following the activation of AKT, which was partly inhibited with anti-PD-L1 mAb treatment. Upregulation of p-ERK1/2 was also inhibited by anti-PD-L1 mAb in response to IFN-γ stimulation (Fig. 5).

Expression of PD-L1 in human OSCC tissues and cell lines

A total of 40 human OSCC specimens and 10 normal mucous tissues adjacent to the tumors were included in this study. The clinicopathological characteristics of patients are listed in Table 1. PD-L1 staining was observed at a different rate in OSCC specimens and normal mucous tissues adjacent to the tumors. PD-L1 was positive in 58% of the tumor samples,

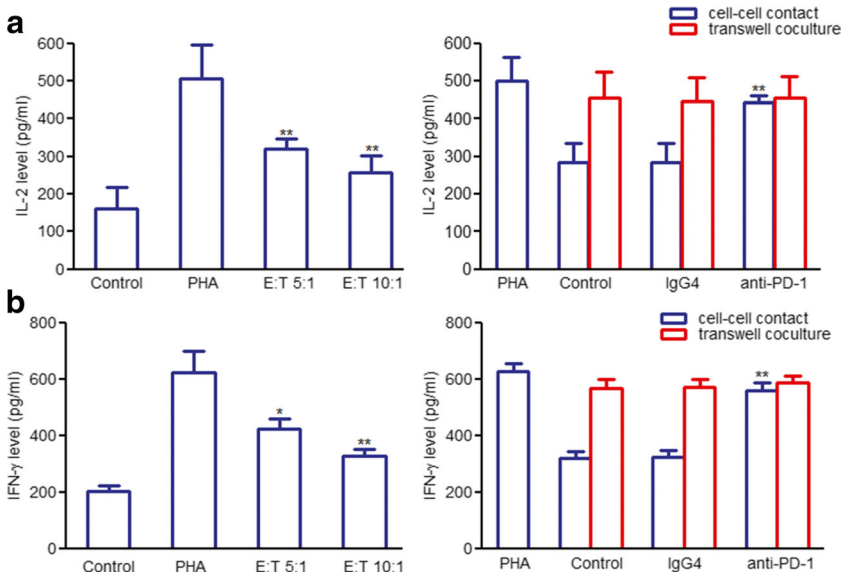

Fig. 2 The anti-PD-1 mAb promotes cytokine production. CAL-27 cells were pretreated with IFN-γ (500 U/mL) for 48 h, washed and cocultured with human T cells, either with cell contact or in transwell coculture, in the presence of PHA (1 μg/mL) for 48 h. IL-2 production was detected by ELISA. **a** Inhibition of IL-2 production by T cells in coculture with CAL-27 cells at different E:T proportions, and restoration of IL-2 production by addition of the mAb to T cell-contact cocultures with CAL-27 cells. **b** Inhibition of IFN-γ production by T cells in coculture with CAL-27 cells at different E:T proportions and restoration of IFN-γ production by addition of the mAb to T cell-contact cocultures with CAL-27 cells. Each experiment was repeated 3 times, and the results were shown as mean ± SD. *: $P < 0.05$, **: $P < 0.01$

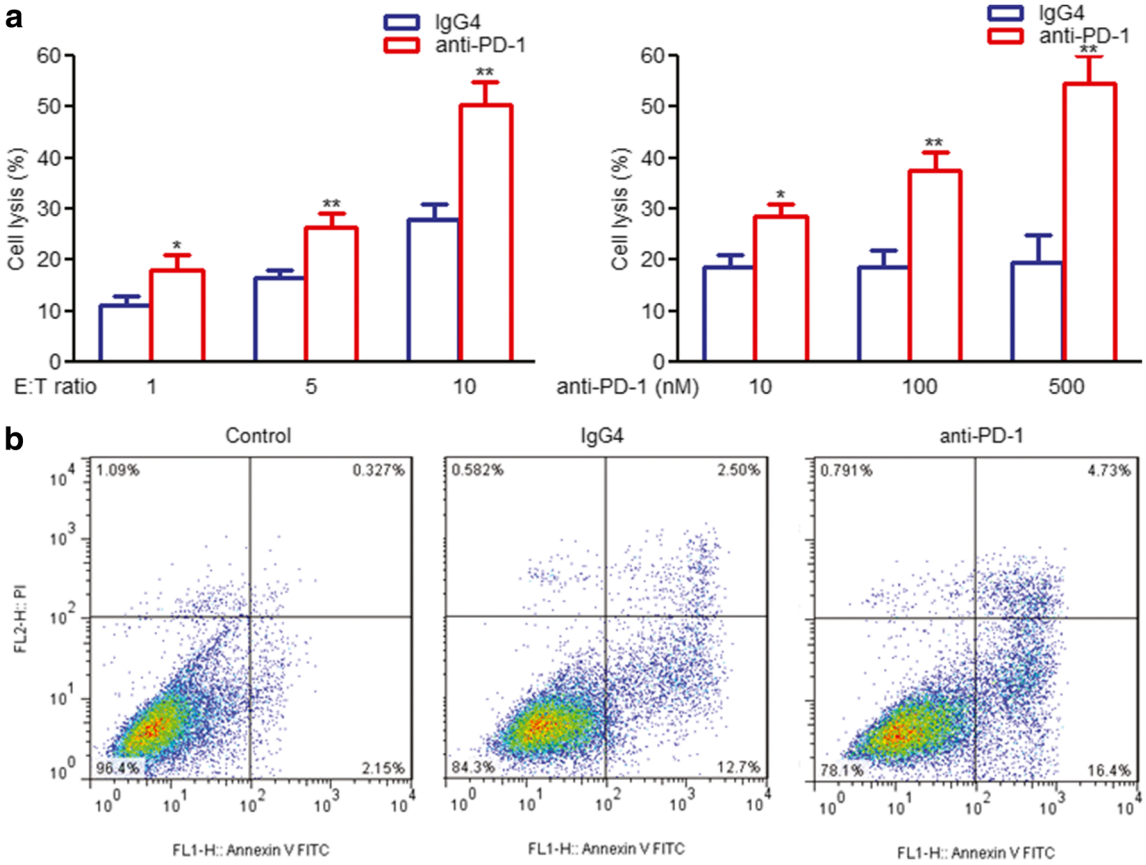

Fig. 3 The anti-PD-1 mAb enhances T cell-induced target cellular lysis and apoptosis. **a** The anti-PD-1 mAb enhances target cells cytotoxicity. T cells were tested for their cytolylic activity at different E:T ratios for 8 h. **b** The anti-PD-1 mAb enhances target cellular apoptosis by human T cells, apoptotic cells were stained with Annexin V and propidium iodide and analyzed by flow cytometry. Control, CAL-27 cells cultured alone. Each experiment was repeated 3 times, and the results were shown as mean ± SD. *: $P < 0.05$, **: $P < 0.01$

while the normal colonic mucosa did not show any staining (Fig. 6a). In the tumor samples, PD-L1 was found to localize to the the cell membrane and cytoplasm and was highly expressed in poorly differentiated tumors. However, PD-L1 was weakly expressed in well-differentiated and moderately-differentiated tumors ($P = 0.011$, Table 1). Additionally, the expression of PD-L1 has been demonstrated in human OSCC cell lines, such as HN30 and CAL-27, and our data also indicated that IFN-γ upregulates the surface expression of PD-L1 in cancer cells (Fig. 6b).

Discussion

Currently, immunotherapy is focused on immune checkpoints that control T cells activation, such as PD-1, CTLA-4, TIM3, and LAG3. Monoclonal antibodies that block these immune checkpoints can unleash antitumor immunity and produce durable clinical responses in a subset of patients with advanced cancers, such as melanoma and non-small-cell lung cancer [37–43]. However, these immunotherapeutics still are constrained by their large molecular size and poor permeability and inability to induce clinical responses in a portion of the patients. In this study, we obtained a strain of monoclonal antibodies by screening at an early stage. Our novel mAb is an IgG4-based monoclonal antibody for targeting of PD-1-expressing T cells, which can block PD-L1/PD-1 signaling to prevent T-cell exhaustion in a PD-L1-expressing solid tumor microenvironment. Our novel mAb is different from previously described scFv or normal monoclonal antibody targeting PD-1, including nivolumab or ipilimumab [44, 45]. Our anti-PD-1 mAb molecule bears several technological features, including (i) bivalent binding to PD-1, (ii) head-to-tail fusion via a flexible linker $(G4S)_3$ of the V_L- and V_H-binding domains, (iii) removal of the C_{H1} and C_L fragment, and (iv) a robust production process based on standard manufacturing steps enabled by genetic recombination [35, 46]. The bivalency of the tumor antigen confers high binding avidity to T cells and retains the Fc fragment. This means that its molecular weight is only three-fourths of a normal antibody, which may have better tumor permeability due to its smaller molecular weight (Fig. 1a). At the same time, it still retains normal antibody functions, such as ADCC, CDC, neutralization, and phagocytosis.

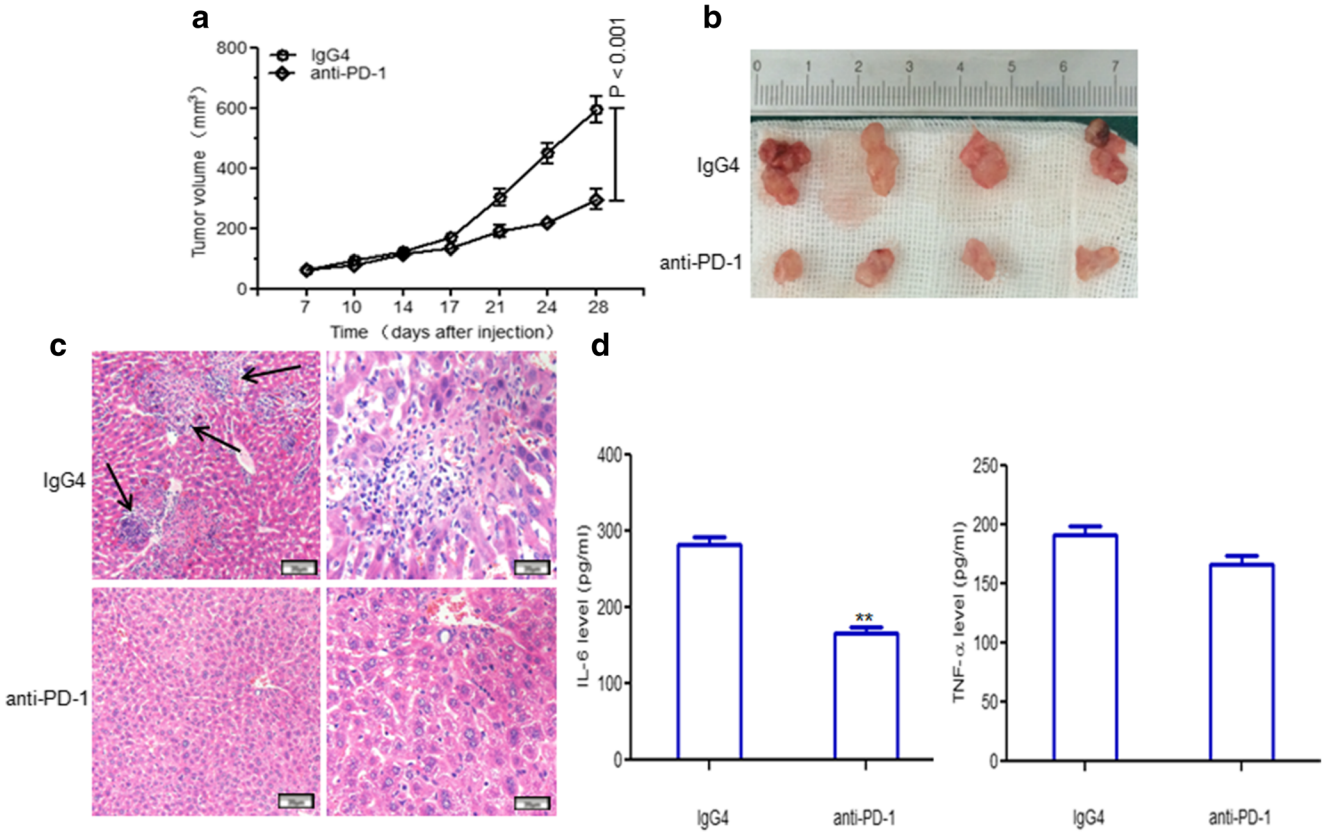

Fig. 4 The anti-PD-1 mAb inhibits tumor development and chronic inflammation in vivo. **a** Xenograft studies were performed using 5–6 week old male NOD/SCID mice ($n = 4$/per group), undergoing subcutaneous injection of CAL-27 cells (5×10^6 cells). Tumor volumes measured on indicated days were plotted for the mAb treatment and control groups. **b** CAL-27 tumor xenografts were stripped 4 days after the last treatment. **c** HE stained the mouse livers. The arrows show lymphocytes (left, magnification ×100; right, ×400). **d** Serum levels of IL-6 and TNF-α were measured by ELISA. The assays were repeated 2 times and each sample has 3 holes data represent the mean ± SD of 4 individual mice per group. **: $P < 0.01$

Binding of the anti-PD-1 mAb to PD-1-expressing T cells leads to T cell crosslinking to PD-L1-expressing tumors, then T cell activation, and secretion of cytotoxic granules, IL-2, IFN-γ, and TNF-α, which ultimately result in tumor cell lysis. The anti-PD-1-mediated tumor lysis is PD-L1-specific and does not occur in the absence of PD-L1 expression or in the absence of simultaneous binding of T cells to PD-L1-expressing tumor cells. We and other studies have highlighted that the PD-L1 expression level in the target organ is a key determinant in the development of toxicity.

PD-L1 upregulation is a common immune escape mechanism of tumor cells [47], and higher PD-L1 expression in tumor patients predicts a poor prognosis in various cancers, including HNSCC [48]. IFN-γ has emerged as the most potent inducer of PD-L1, although many other cytokines have been shown to play a role as well [49, 50]. Following stimulation with IFN-γ, PD-L1 was broadly induced in the lymphoid and nonlymphoid cells of mice and humans [51]. Previous studies have shown that the PD-L1/PD-1 interaction contributes to the functional suppression of T cell

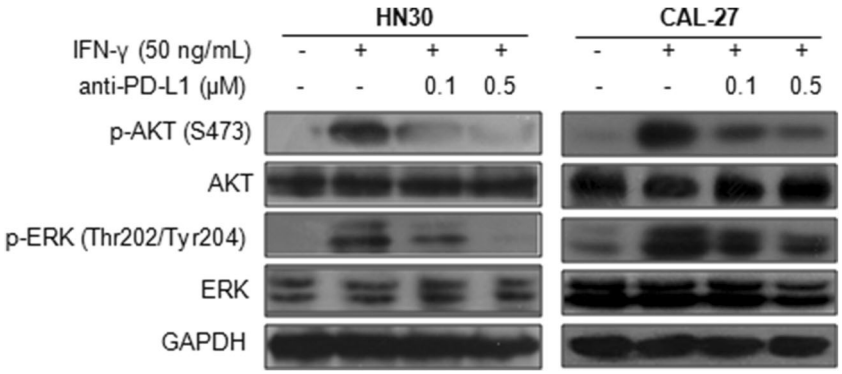

Fig. 5 Anti-PD-L1 mAb inhibits PD-L1/PD-1 downstream molecules. HN30 and CAL-27 cells were treated with the indicated concentrations of Anti-PD-L1 mAb for 8 h and then stimulated with or without IFN-γ (50 ng/mL) for 30 min. Total cell lysates were evaluated by Western blot using specific antibodies. GAPDH expression was used as an internal control

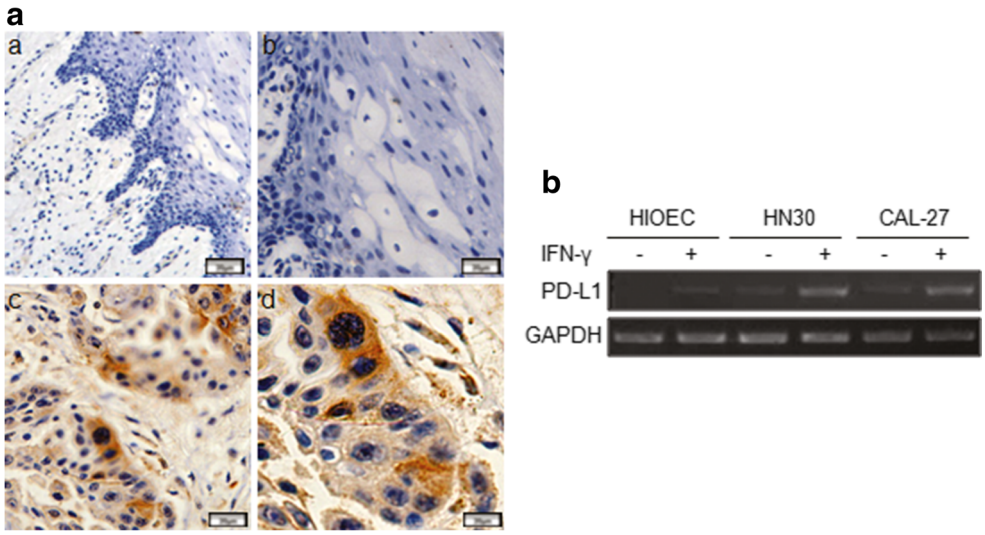

Fig. 6 The expression of PD-L1 in human OSCC tissues and cell lines. **a** Immunohistochemical examination of the expression of PD-L1 in normal mucous tissues adjacent to tumors (a, magnification ×100; b, ×400) and human OSCC tissues (c, magnification ×100; d, ×400). **b** The expression of PD-L1 mRNA was analyzed in HIOEC, HN30, and CAL-27 cell lines by RT-PCR

responses characterized by impaired IL-2 production in Jurkat T cells [52]. Our data show that our anti-PD-1 mAb can bind to CHO cells expressing PD-1 with an increasing mean fluorescence intensity (MFI) as concentration increases (Fig. 1b), but does not bind to the parental CHO cell line (data not shown). Cytokine secretion is an important criterion for evaluating T cell function. To assess the preventive effects of PD-L1-expressing CAL-27 cells on T cells function, T cells were cocultured with IFN-γ-pretreated CAL-27 cells at different E:T proportions in the presence of PHA for 48 h. CAL-27 cells cocultured with T cells significantly reduced T-cells IL-2 and IFN-γ production (Fig. 2, left). Our anti-PD-1 mAb pretreatment can significantly rescue IL-2 and IFN-γ secretion in T cells cocultured in contact with CAL-27 cells ($P < 0.01$) (Fig. 2, right, red histogram). At the same time, we evaluated the effect of the mAb on T-cells mediated target cell killing efficacy and apoptosis. We found that T cells pretreated with the anti-PD-1 mAb significantly augmented T-cells induced CAL-27 lytic (Fig. 3a) and apoptosis abilities (Fig. 3b). In the oral cavity, OSCC cells might encounter IFN-γ secreted by activated T cells in the tumor's local microenvironment and thus could respond by upregulating PD-L1 on the cell membrane and inhibit T-cells production IL-2 and other inflammatory cytokines, which may promote the immune escape of the tumor cells. Given the success of anti-PD-1 pathway agents in activating T cells, we could seek the means of harnessing their potency to produce more durable immunological responses.

It was reported that PD-1 inhibition accompanied the production of the T cells effector, granzyme B, in infiltrating cells and the induction of apoptosis in the epithelial cells of oral lesions, suggesting that T cells activation mediates the immunopreventive effects of anti-PD-1. Anti-PD-1 treatment significantly reduced the number of oral lesions in mice and prevented malignant progression. Low-grade dysplastic lesions responded to PD-1 blockade with a significant increase in the recruitment of $CD8^+$ and $CD4^+$ T cells with their microenvironment [53]. The effect of our anti-PD-1 mAb on tumor development was evaluated by heterotopic xenograft analysis in vivo. NOD/SCID mice were subcutaneously injected with CAL-27 cells. The results showed that our anti-PD-1 mAb significantly inhibited CAL-27-induced tumor growth compared to IgG4, which reduced the tumor volume by nearly 50% (Fig. 4a) and inhibited tumor weight by approximately 40% (Fig. 4b). However, there was no significant difference in mouse body weight (data not shown). HE staining showed that there were abundant lymphocytes in the livers of IgG4 treated mice. However, we did not find any lymphocytes in the livers of anti-PD-1 mAb treated mice (Fig. 4c). In addition to tumor growth inhibition, we also found that our anti-PD-1 mAb reduced the secretion of inflammatory cytokines in vivo (including IL-6, but not TNF-α) compared to the control group (Fig. 4d), which further suggests that anti-PD-1 mAb treatment can inhibit chronic inflammation in vivo.

PD-L1/PD-1 binding leads to recruitment of the tyrosine phosphatases SHP-1 and SHP-2 [53]. Recruiting SHP-2 produces an inhibitory signal, blocking the downstream effects of PI3K and AKT [54]. PD-L1/PD-1 inhibition was accompanied by PI3K-AKT, JAK-STAT, and AKT-mTOR pathway activation in gastric and non-small cell lung cancers [55–58]. However, the molecular mechanism by which PD-L1 expression in OSCC induced by IFN-γ is not clear. In the present study, we also found that IFN-γ-mediated upregulation of PD-L1 in CAL-27 cells (Fig. 6b), following the activation of AKT, which was partly inhibited by anti-PD-L1 mAb treatment. Upregulation of p-ERK1/2 was also inhibited by the anti-PD-L1 mAb in response to IFN-γ stimulation

(Fig. 5). The AKT-ERK1/2 signal pathway was involved in the expression of PD-L1 induced by IFN-γ. In addition to augmenting T cells killing, our study has identified a novel mechanism of immune escape mediated by IFN-γ in the oral tumor microenvironment, which may be secreted by T-cells in response to the anti-PD-1 mAb and mediates immune escape, which also reflects the complexity, diversity, and intrinsic link to local immune regulation in the oral tumor microenvironment.

Many cells located in the tumor microenvironment function to protect or to promote tumor cells escape from immune surveillance [59]. PD-L1, a key protein located in the tumor microenvironment, may promote the immune escape of tumor cells. In this study, we investigated OSCC tissues and found that PD-L1 staining both in OSCC tissues and normal mucous tissues adjacent to tumors, which occurred at a different rate (Fig. 6a). In the tumor samples, PD-L1 was found to localize to the cell membrane and cytoplasm and was highly expressed in poorly differentiated tumors. However, PD-L1 was weakly expressed in well-differentiated and moderately-differentiated tumors ($P = 0.011$, Table 1). Our data indicate that IFN-γ may induce cancer immunoresistance by upregulation of PD-L1 cell surface expression in weakly-differentiated OSCC cells. It is conceivable that cancer cells are exposed to IFN-γ in the tumor microenvironment, which can result in harsh conditions for cancer cells. The cells can escape immune surveillance by suppressing cancer-specific CTLs through upregulation of a coinhibitory molecule, PD-L1, on the surface. Combined with previous results, this suggests that induction of PD-L1 surface expression by IFN-γ in cancer cells is a common effect. Therefore, we should be extra cautious when IFN-γ is applied for cancer treatment.

This study has several limitations. First, there were abundant lymphocytes in mouse liver tissues, but we did not further identify the T cell type, such as $CD8^+$, $CD4^+$, and Foxp3. Second, PD-L1/PD-1 inhibition was accompanied by the activation of numerous pathways in the solid tumor, including the PI3K-AKT, JAK-STAT, and AKT-mTOR pathways. In this study, we only focused on the AKT-ERK1/2 signaling pathway, involving in the expression PD-L1, which was induced by IFN-γ. Finally, assessment of PD-L1 status in OSCC tissues was limited by the small sample size.

Taken together, the current data show that our anti-PD-1 mAb is a novel T cell targeted mAb for the treatment of OSCC with promising antitumor activity and the ability to modify the OSCC microenvironment. Our anti-PD-1 mAb may have better tumor permeability due to its smaller molecular weight (versus normal mAbs) and may be more efficacious in poorly-differentiated OSCCs with PD-L1 higher expression. Future studies will focus on unleashing the full potential of anti-PD-1-mediated T cell activity against OSCCs. Phase I clinical trials with our anti-PD-1 mAb will be carried out as soon as possible.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animal were followed. All procedures performed in studies involving human participants received ethics approval from the independent Ethics Committee of the Shanghai Ninth People's Hospital Affliated to Shanghai Jiaotong University School of Medicine (No. 200926). All experimental procedures were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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