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An engineered PD-1-based and MMP-2/9-oriented fusion protein exerts potent antitumor effects against melanoma

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Recent studies showed that the PD-1/PD-L1 checkpoint blockade is a dramatic therapy for melanoma by enhancing antitumor immune activity. Currently, major strategies for the PD-1/PD-L1 blockade have mainly focused on the use of antibodies and compounds. Seeking an alternative approach, others employ endogenous proteins as blocking agents. The extracellular domain of PD-1 (ePD1) includes the binding site with PD-L1. Accordingly, we constructed a PD-1-based recombinantly tailored fusion protein (dFv-ePD1) that consists of bivalent variable fragments (dFv) of an MMP-2/9-targeted antibody and ePD1. The melanoma-binding intensity and antitumor activity were also investigated. We found the intense and selective binding capability of the protein dFv-ePD1 to human melanoma specimens was confirmed by a tissue microarray. In addition, dFv-ePD1 significantly suppressed the migration and invasion of mouse melanoma B16-F1 cells, and displayed cytotoxicity to cancer cells *in vitro*. Notably, dFv-ePD1 significantly inhibited the growth of mouse melanoma B16-F1 tumor cells in mice and *in vivo* fluorescence imaging showed that dFv-ePD was gradually accumulated into the B16-F1 tumor. Also the B16-F1 tumor fluorescence intensity at the tumor site was stronger than that of dFv. This study indicates that the recombinant protein dFv-ePD1 has an intensive melanoma-binding capability and exerts potent therapeutic efficacy against melanoma. The novel format of the PD-L1-blocked agent may play an active role in antitumor immunotherapy. [BMB Reports 2018; 51(11): 572-577]

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INTRODUCTION

A dynamic interplay exists between hosts and tumors, and the ability of the tumor to evade immune recognition often determines the clinical course of the disease. Tumors express antigens that can be recognized by host T cells, but full clearance of tumors by the immune system alone is rare. Part of this failure is due to immune suppression in the tumor microenvironment. An important role of the tumor's microenvironment is covered by the programmed cell death-1 (PD-1)/programmed cell death-ligand 1 (PD-L1) axis (1). PD-1 is an immunosuppression receptor mainly expressed on the surface of activated T cells, as well as other immune cells (2, 3). Recent research shows PD-L1, the ligand of PD-1, is expressed on a wide variety of solid tumors, including breast, lung, colon, ovarian cancer, and melanoma (4-8). Binding of PD-1 with PD-L1 can inhibit a T cell-mediated immune response and contribute to tumor immune evasion (9, 10). This potential mechanism of immune resistance in tumor tissues suggests that the treatment directed at blocking the interaction between PD-1 and PD-L1 might enhance endogenous antitumor immunity.

Currently, immunomodulatory agents as a new immuno-therapeutic strategy are used for cancer therapy in many recent studies (11). The anti-PD-1 or anti-PD-L1 strategy can be effective in several solid tumors such as renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC), and melanoma (12-14). Research shows that the expression of PD-L1 is increased on BRAF-mutated melanoma cells as cells resistant to BRAF or MEK inhibitors. This contributes to more aggressive behavior of melanoma (15). The abnormally high expression of PD-L1 in melanoma tissues is not only used in the prognosis of cancer patients, but also is becoming an effective molecular target of cancer therapy (16, 17). A variety of drugs targeting the PD-1/PD-L1 signaling pathway were developed for preclinical research and clinical treatment of tumors. Compared with investigator-choice chemotherapy, the anti-PD-1 antibodies Nivolumab and Pembrolizumab increased patients' objective response and overall survival significantly in clinical trials (18, 19). However, the inability to target leading to toxicity and immune-related toxicities of the PD-1/PD-L1-

blocked agents limit their clinical application (20).

The matrix metalloproteinase (MMP) family as a type of specific proteolytic enzyme is of great concern in recent years. It is abundantly expressed and secreted in various tumor tissues and plays an important role in the degradation of the tumor extracellular matrix (21). Type IV collagenase (also known as gelatinase) is a type of matrix metalloproteinase, including MMP-2 and MMP-9. The abundant expression of gelatinases in destroying the integrity of the basement membrane and extracellular matrix can promote tumor growth, invasion and metastasis, and participate in tumor angiogenesis (22, 23). Furthermore, the expression of gelatinases is considered as an important biomarker and correlates highly with the poor outcome of cancer therapy (24-27). Therefore, it is feasible to employ anti-gelatinase agents as a targeted drug carrier. It is known that single-chain variable fragments (scFv) are considered the most frequently used and the most favored fragment to deliver protein-based drugs to cancer cells because of their moderate molecular size and faster tissue penetration (28). A monoclonal antibody (mAb) 3G11 was produced using gelatinases as the antigen, and employed as a carrier for targeted delivery of Lidamycin (29-31). Moreover, the bivalent variable fragment (dFv) of 3G11 showed the ideal targeting effect and tumor suppression compared to the monovalent format (scFv) (32, 33). The high expression of MMP-2/9 is closely related to tumor formation, development, invasion, metastasis, and poor prognosis. So, MMP-2/9-targeted inhibition is becoming a new approach to treat metastatic tumors (34, 35).

In the present study, we generated the fusion protein dFv-ePD1, which combined an MMP-2/9-targeted dFv and an extracellular domain of PD-1 (ePD1). Exogenous ePD1 including the binding site with PD-L1 can be used to bind PD-L1 on the surface of cancer cells and block the interaction with immune cells. Melanoma cells and tissues were used to evaluate the affinity of dFv-ePD1 fusion protein and its ability to inhibit tumor invasion and metastasis. The therapeutic efficacy of dFv-ePD1 was evaluated using mouse melanoma B16-F1 cells as a model. The study provides evidence that dFv-ePD1 shows potent antitumor efficacy *in vitro* and *in vivo*.

RESULTS

Construction of the dFv-ePD1 and dFv pro35 karyotic expression plasmid

As shown in Fig. 1A, the recombinant plasmid pET30a(+)/dFv-ePD1 was obtained by genetic engineering. The DNA fragment encoding for protein dFv was cloned from Plasmid pET30a(+)/dFv-LDP and inserted into the *Nde*I/*Hind*III restriction sites of the expression plasmid pET30a(+) to generate the plasmid pET-30a(+)/dFv. The DNA fragment (501 bp) encoding for ePD1 was copied from cDNA of mice spleen cells (Fig. 1B). Subsequently, ePD1 was inserted into the *Hind*III/*Xho*I restriction sites of expression plasmid

pET30a(+)/dFv to generate the plasmid pET-30a(+)/dFv-ePD1 (Fig. 1A). The recombinant plasmid was verified using enzyme digestion (Fig. 1C). And DNA sequencing confirmed that the correct DNA fragments were inserted.

Preparation and identification of the fusion protein

The engineered protein dFv-ePD1 was successfully expressed after the plasmid pET30a(+)/dFv-ePD1 was transfected into *E.*
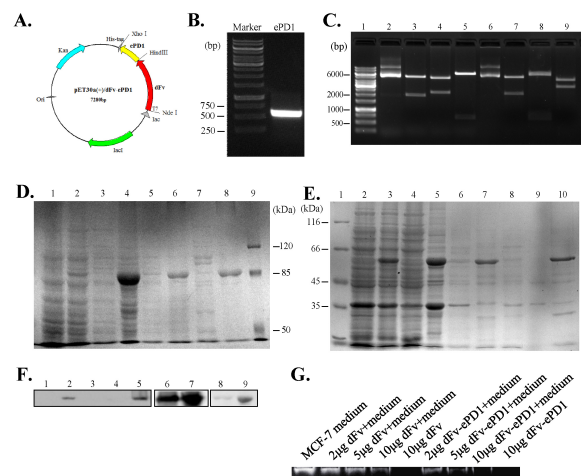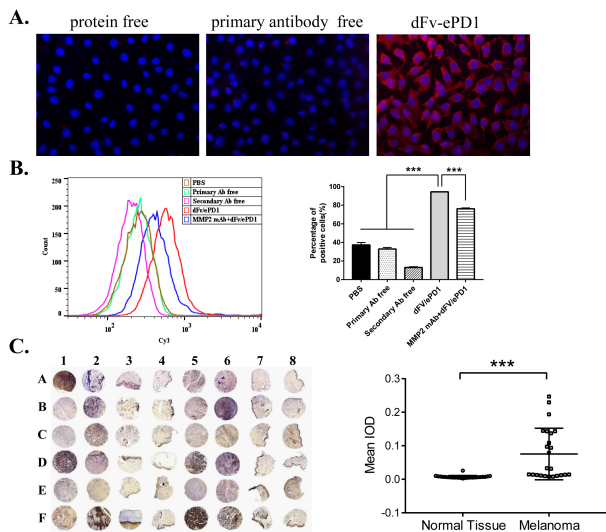

Fig. 1. Construction and expression of fusion gene dFv-ePD1 and dFv. (A) Sketch map of the pET30a(+)/dFv-ePD1 recombinant plasmid. (B) ePD1 fragment was amplified by RT-PCR. (C) Verification of plasmids by enzyme digestion. 1, DNA marker; 2, recombinant plasmid pET30a(+)/dFv-ePD1; 3, 4 and 5, pET30a(+)/dFv-ePD1 plasmid digested with *Nde*I/*Xho*I, *Hind*III/*Mlu*I and *Nde*I/*Eco*RI; Lane 6, recombinant plasmid pET30a(+)/dFv; 7, 8 and 9, pET30a(+)/dFv recombinant plasmid digested with *Nde*I/*Hind*III, *Eco*RI/*Hind*III and *Hind*III/*Mlu*I, respectively. (D) Expression analysis of fusion protein dFv-ePD1. 1), total proteins before IPTG induction; 2), total proteins after IPTG induction; 3), the soluble portion of the cell lysate in binding buffer I; 4), the inclusion bodies; 5), the soluble portion of the cell lysate in binding buffer II; 6), the soluble portion of the cell lysate in binding buffer III; 7), proteins unbound with an Ni^{2+} column; 8), the purified dFv-ePD1 using an Ni^{2+} column; 9), protein marker (kDa); (E) Expression analysis of fusion protein dFv. 1), protein marker (kDa); 2), total proteins before IPTG induction; 3), total proteins after IPTG induction; 4), the soluble portion of the cell lysate in binding buffer I; 5), the inclusion bodies; 6), the soluble portion of the cell lysate in binding buffer II; 7), the soluble portion of the cell lysate in binding buffer III; 8), proteins unbound with an Ni^{2+} column; 9), proteins washed with a washing buffer; 10), the purified dFv using an Ni^{2+} column ; (F) Characterization of dFv-ePD1 by Western blotting. 1), total proteins before IPTG induction; 2), total proteins after IPTG induction; 3), cultural supernatants; 4), the soluble portion of the cell lysate in binding buffer I; 5), the inclusion bodies; 6) and 7), the purified dFv-ePD1 and the renatured dFv-ePD1 detected with anti-His tag mAb, respectively; 8) and 9), the purified dFv-ePD1 and the renatured dFv-ePD1 detected with anti-PD-1 antibody, respectively. (G) The inhibitory effect of different concentrations of dFv and dFv-ePD1 on gelatinase using Reverse Zymography analysis.

coli Transetta (DE3). As shown in Fig. 1D, the total proteins after IPTG induction (Lane 2) have the band of the fusion protein dFv-ePD1 (85 kDa) compared to the ones before IPTG induction (Lane 1). The fusion proteins were accumulated in intracellular inclusion bodies (Lane 4). The band of protein dFv was showed at the site of 55 kDa (Fig. 1E). The inclusion bodies were treated with 2 mol/L and 6 mol/L urea. As shown in Lane 6 of Fig. 1D, the fusion protein was more soluble in a binding buffer III (6 mol/L urea). As shown in the results of an SDS-PAGE and a Western blotting, the purity of dFv-ePD1 treated with a Ni^{2+} column was over 90% (Fig. 1D, 1F). The reverse Zymography assay was used to measure the activity of the renatured fusion protein. The results showed that both the renatured proteins dFv and dFv-ePD1 had an inhibitory effect on gelatinase depending on their concentration (Fig. 1G). The results indicated that the dFv-ePD1 and dFv were successfully purified and renatured.

Binding affinity of dFv-ePD1 with cancer cells and tumor tissue

The binding capability of dFv-ePD1 to B16-F1 cells was examined by immunofluorescence (Fig. 2A) and flow cytometry (Fig. 2B). The results showed that dFv-ePD1 could bind to the B16-F1 cells, and this effect could be blocked by an anti-MMP2 specific antibody (Fig. 2B). Tissue microarray of human melanoma and normal skin tissue was applied to further confirm the that dFv-ePD1 could bind to human melanoma specimens. The scanning pattern in the image of the immunohistochemistry results in each case of the microarray is presented in Fig. 2C. The mean cumulative value of the optical density of the human melanoma tissue group was significantly higher than that of the normal skin tissue group (P < 0.001). Evidently, this indicates that the fusion protein dFv-ePD1 prefers to bind to human melanoma tissue compared to normal skin tissue.

Inhibitory effect of dFv-ePD1 on migration, invasion and cell growth of melanoma B16-F1 cells *in vitro*

We observed the role of dFv-ePD1 in B16-F1 cancer cell migration and invasion. As shown in Fig. 3A and 3B, both dFv-ePD1 and dFv inhibited the migration and invasion of B16-F1 cells. Furthermore, the inhibitory effects of fusion protein dFv-ePD1 were more than that of dFv. Using the MTT assay, fusion protein dFv-ePD1 significantly inhibited the proliferation of mouse melanoma cells B16-F1 and human non-small cell lung cancer cells A549. Fused protein dFv-ePD1 showed moderate inhibition of the proliferation of human cervical cancer cells HeLa and human ovarian cancer cells SKOV-3 cells. Protein dFv did not show significant inhibition to proliferation of tumor cells *in vitro* (Fig. 3C).
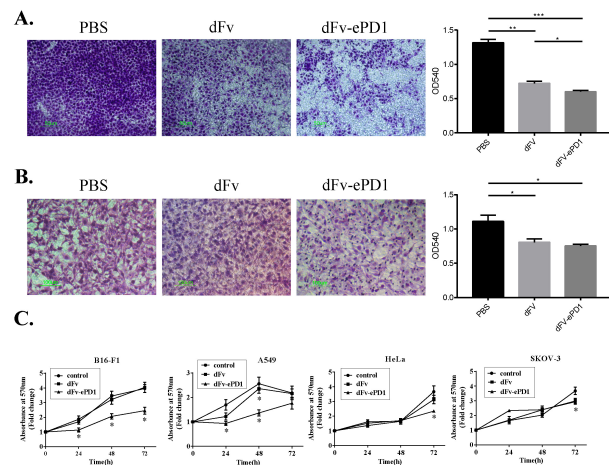

Fig. 2. Fusion proteins dFv-ePD1 have potent binding abilities to binding with B16-F1 cells and human melanoma tissue. (A) The binding affinity of 200 μg/ml dFv-ePD1 protein with B16-F1 cells analyzed by immunofluorescence staining (×40). The red color represents dFv-ePD1; blue color indicates the nuclei. (B) Binding affinity of 200 μg/ml dFv-ePD1 with the B16-F1 cells analyzed by flow cytometry. (C) The binding ability of 80 μg/ml dFv-ePD1 to human melanoma tissue. Columns 1, 2, 5, and 6 are human melanoma tissues and columns 3, 4, 7, and 8 are human normal skin tissues. The right figure is the mean cumulative optical density value of human melanoma and human normal skin tissue, ***P < 0.001.

Fig. 3. Fusion protein dFv-ePD1 inhibited the growth, migration and invasion of B16-F1 cells *in vitro*. (A) The effect of 100 μg/ml dFv-ePD1 on the migration of B16-F1 cells was measured by a transwell migration assay. (B) The invasive abilities of B16-F1 cells were determined in a Boyden chamber assay after treatment with 100 μg/ml protein dFv-ePD1 and dFv, respectively. The right figures indicate absorbance at 540 nm. *P < 0.05 **P < 0.01 ***P < 0.001. (C) Proliferation inhibition of B16-F1, A549, HeLa, and SKOV-3 cancer cells by dFv-ePD1(50 μg/ml) determined by the MTT assay at 24, 48, and 72 hours, respectively. *P < 0.05.
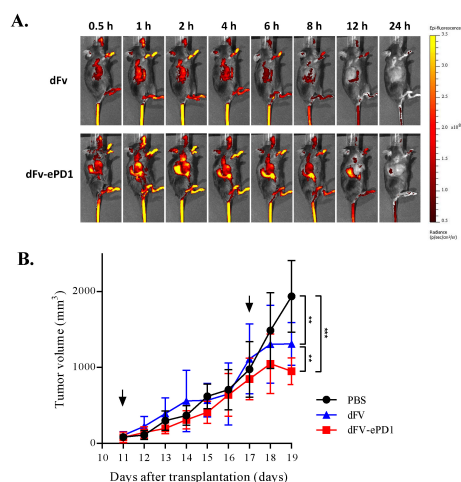
In vivo imaging of fusion proteins

C57BL/6J mice bearing B16 melanoma models were used for the study of tumor accumulation of DyLight 680 labeled dFv and dFv-ePD1 (Fig. 4A) (36). The variations in fluorescence intensity at the tumor region were monitored for 24 hours. As shown in Fig. 5, the fluorescence intensity of both proteins in the tumor location increased gradually with time during the period 2 hours after the injections. The dFv-ePD1 showed stronger fluorescence intensity compared to dFv at the same observation times. This observation provides evidence that dFv-ePD1 can accumulate in B16 melanoma and have better or quicker targeting activity than dFv.

In vivo therapeutic efficacy of fusion protein dFv-ePD1

Mouse melanoma B16-F1 cells were transplanted sub-cutaneously into the flank of C57BL/6 mice at day 0. At day 11, the experimental mice were divided into 3 groups. The three groups were PBS, dFv and dFv-ePD1. At day 11 and day 17, PBS, 20 mg/kg dFv and 20 mg/kg dFv-ePD1 were injected intraperitoneally, respectively. The results showed that both dFv and dFv-ePD1 inhibited the growth of the tumor (Fig. 4B), and the prepared dFv-ePD1 exhibited significantly enhanced therapeutic effects on B16-F1 xenograft as compared with dFv (50.9% versus 32.4%, P < 0.01).

DISCUSSION

Currently, the development of immune-modulating agents has

Fig. 4. *In vivo* tumor targeting and therapeutic efficacy of fusion protein. (A) *In vivo* optical imaging in B16 melanoma-bearing C57BL/6J mice using DyLight680-labeled dFv and dFv-ePD1. Representative *in vivo* fluorescence images at a series of points in time observed after tail vein injection of 20 mg/kg dFv or dFv-ePD1. Color scale represents photons/sec/cm^2/steradian. (B) The growth inhibition of dFv and dFv-ePD1 on mouse melanoma B16-F1. **P < 0.01 ***P < 0.001.

significantly improved the survival for patients with malignancy. Clinical trials with anti-CTLA-4, anti-PD-1, and anti-PD-L1 agents have shown objective clinical activity in melanoma, NSCLC, bladder, and other cancers. Research with anti PD-1/PD-L1 agents have shown higher response rates than that using anti-CTLA-4 (37). Tumor cells exploit the immune-checkpoint PD-1/PD-L1 pathway as a mechanism to evade detection and inhibit the immune response. The deactivated T cells are kept inhibited by interacting with tumor cells in the tumor microenvironment, which possibly provides a way to reactivate them to kill tumor cells. Due to expression on the tumor cells, targeting PD-L1 may theoretically offer benefits above those of PD-1.

As the ligand of PD-1, PD-L1 can bind to particularly to the extracellular portion of PD-1. Through binding to PD-L1, PD-1 transmits an inhibitory signal to the T cell, which reduces cytokine production and suppresses T-cell proliferation. If the binding site of PD-L1 is occupied by a free extracellular portion of PD-1, it cannot bind to the PD-1 portion expressed on T cells. Therefore, we choose the ePD-1 as a PD-L1-blocked agent to check its effect on tumor growth. There were several recent papers that reported that cell-intrinsic PD-L1 proteins regulate the cell growth through PI3K/AKT pathway activation (38-40). Our fusion protein containing a PD-1 extracellular domain could bind to the cell membrane of PD-L1 to block a PD-L1/PD-1 interaction. It could then inactivate the intrinsic cell growth signal of cancer cells. However, PD-L1 is expressed not only in cancer cells, but also in a variety of normal hematopoietic and non-hematopoietic cells. The non-targeting and immune-related toxicities were reported in many studies for PD-L1-blocked agents. Tumor-targeted delivery of a PD-L1-blocked agent is a way to reduce the effects of interactions with non-targeted molecules.

Gelatinase (including MMP-2/9) plays an important role in tumor growth and metastasis; overexpression of these molecules is strongly correlated with a poor prognosis in a variety of cancers. MMP-2/9 is a tumor antigen whose expression is critical for tumor growth or invasiveness in melanoma (41, 42). MMP-2-specific CD4$^+$ T lymphocytes display an inflammatory T$_H$2 profile, and MMP-2-conditioned dendritic cells prime T$_H$2 responses against several melanoma-associated antigens (41). Therefore, gelatinases are a possible target for cancer therapy and immunotherapy. In this article, we prepared and identified a gelatinases-targeting tandem scFv-based fusion protein (dFv-ePD1) successfully, as one that targets the PD-L1-blocked agent. The dFv-ePD1 shows potent binding capability to B16-F1 cells and human melanoma tissue microarray via the interaction of the dFv moiety with gelatinases and the ePD1 binds to PD-L1 as well. The fusion protein dFv-ePD1 can gather to the tumor site in the mouse model because of the targeting ability of dFv. Meanwhile, dFv-ePD1 shows more accumulation to tumor tissues than dFv. We speculate that ePD1 also has the effect of targeting

the tumor due to the binding ability of PD-L1, which is overexpressed by melanoma.

Our results demonstrate that the fusion protein dFv-ePD1 retains the activity of dFv that likely inhibits the activity of gelatinase. Meanwhile, we observed that the fusion protein dFv-ePD1 displayed more potent inhibitory effects on the migration and invasion of B16-F1 cells compared with dFv. The fusion protein dFv-ePD1 can inhibit the proliferation and growth of mouse melanoma B16-F1 cells significantly *in vitro* and *in vivo*.

Taken together, we generated a tandem scFv-based and PD-1 extracellular domain integrated fusion protein that targets gelatinases. Compared to Fv or ePD-1 alone, this fusion protein could target the tumor tissue through the gelatinases-targeting binding activity of dFv to disrupt the tumor microenvironment. And the fusion protein could also block the immunosuppressive signal PD-1/PD-L1 through ePD-1, which is delivered by dFv in tumors to enhance the immune response. The fusion proteins are highly relevant to cancer invasion, metastasis and cancer cell-stromal cell interactions. The fusion protein has the activity of targeting melanoma cells *in vitro* and *in vivo*, and inhibiting the proliferation, migration, and invasion of melanoma cells; with potential clinical applications in the treatment of melanoma.

MATERIALS AND METHODS

Detailed information is in the Supplementary Material.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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