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

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SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and reagents

Human breast cancer cell line MCF-7 and human cervical cancer cell line HeLa were cultured in Dulbecco’s modified Eagle medium (Gibco, New York, USA). Mouse melanoma cell line B16-F1, human non-small cell lung cancer (NSCLC) A549, and human ovarian cancer cell line SKOV-3 were cultured in RPMI-1640 medium (Gibco, New York, USA). All the media were supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS, Gibco; New York, USA), penicillin G (100 U/mL), and streptomycin (100 μg/mL). The cells were cultured in an incubator, maintained at 37°C with 5% CO₂. Anti-His-tag (2A8) mouse mAb was purchased from Abmart (Shanghai, China) and anti-PD-1(E-18) antibody was purchased from Santa Cruz, Inc (USA). Anti-MMP2 antibody was purchased from Abcam (Cambridge, UK). Anti-PD-L1 antibody was purchased from Novus (Littleton, USA). Anti-rabbit IgG and Cy3-labeled goat antibody was purchased from Boster (Wuhan, China). Anti-mouse IgG and Cy3-labeled goat antibody was purchased from Abcam (Cambridge, UK). Goat anti-mouse peroxidase-coupled antibody and rabbit anti-goat peroxidase-coupled antibody were purchased from Thermo (Waltham, USA). The plasmid pET30a(+)/dFv-LDP carrying the gene of dFv (anti-gelatinases) was provided by Prof. Yongsu Zhen from Chinese Academy of Medical Sciences and Peking Union Medical
College. *E.coli* DH5α was preserved in our laboratory and *E.coli* Transetta (DE3) was purchased from TransGen Biotech Co., Ltd. (Beijing, China).

**Construction of plasmid pET-30a (+)/dFv-ePD1.**

Mouse spleen cells were isolated from C57BL/6 mice under aseptic conditions using mouse splenic lymphocyte separation solution (Dakewe, Beijing, China). Subsequently, the spleen cells were harvested and total RNA was extracted from the splenic cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA), according to the manufacturer's instructions. The RNA samples were then reverse transcribed into cDNA using a PrimeScript RT Reagent kit (Thermo, Waltham, USA). The cDNA was used as the template for polymerase chain reaction (PCR) analysis. The extracellular coding sequence of PD-1 (ePD1) was amplified from the cDNA template using a pair of primers: P1,

5'-TCGACAAGCTTGAGGTGGAGGCGGTTCAATGTGGGTCCGGCAGGTACCCTGG-3'

and

P2,

5'-CGCACTCGAGTTGAAACCGGCCTTCTGGTTTGGGCGAGGGGCTGGGATA-3'. The restriction recognition sites of *Hind*III and *Xho*I were introduced into the 5' ends of P1 and P2, respectively. PCR was performed as follows: 94°C for 5 min, 94°C for 30 sec, 61°C for 30 sec
and 72°C for 1 min, then held at 72°C for 5 min, and finally a 4°C standby. Subsequently, the PCR product ePD1 and plasmid pET30a(+)/dFv-LDP were double-digested with restriction enzymes HindIII and XhoI, respectively, and then mixed the two digested products followed by ligating at 16°C overnight to generate the recombinant plasmid pET-30a(+)/dFv-ePD1. Finally, DNA sequencing analysis was carried out to verify the correct insertion of the dFv-ePD1 gene.

**Preparation of fusion protein dFv-ePD1**

*E.coli* Transetta (DE3) transformed with pET30a(+)/dFv-ePD1 was cultured in LB medium at 37°C. The fusion protein dFv-ePD1 was induced by 0.2 mmol/L IPTG and 220 rpm shaking for 4 h at 37°C. The cells were pelleted by centrifugation at 3000 ×g for 5 min. The pellets were resuspended in binding buffer I (5 mmol/L imidazole, 0.5 mol/L NaCl and 20 mmol/L Tris-HCl, pH 7.9) and sonicated, and then the cell lysate was centrifuged to gain the inclusion body. The inclusion body was resuspended in binding buffer II (5 mmol/L imidazole, 0.5 mol/L NaCl and 20 mmol/L Tris-HCl, 2 mol/L urea, pH 7.9) and incubated for 2 h, followed by sonicated and centrifuged. The pellets were resuspended in binding buffer III (5 mmol/L imidazole, 0.5 mol/L NaCl and 20 mmol/L Tris-HCl, 6 mol/L urea, pH 7.9) and incubated overnight to resolve the proteins. After centrifugation at 16000 ×g for 30 min, the proteins in the
supernatant was purified by affinity chromatography through a C-terminal 6×His-tag (His Trap HP, GE Healthcare) according to the manufacturer’s protocol. The sample was dialyzed in renaturing buffer I (50 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 200 mmol/L NaCl, 5 mol/L urea) for 12 h. Dialysis was subsequently performed in the same buffer with step-wise reduction of the urea concentration (3, 2, 1, 0.5, and 0 mol/L), and then dialyzed in phosphate buffered saline (PBS) solution (pH 7.4) for twice. After centrifugation at 10000 ×g for 30 min at 4°C, the supernatant was collected. The proteins were condensed using Amicon® Ultra Centrifugal filters (Merck, Darmstadt, Germany) through centrifugation at 12000-14000 ×g and the concentration was determined using BCA protein assay kit (Thermo, Waltham, USA).

**Identification of fusion protein dFv-ePD1**

The proteins were analyzed using SDS-PAGE and Western blotting. Equal amounts of protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 2 h, incubated with respective primary antibody (anti-His-Tag antibody, 1/4000 dilution, and PD-1 antibody 1/3000 dilution) at 4°C overnight, and then incubated with appropriate secondary antibodies (1/5000 dilution).
Protein bands were visualized using a chemiluminescent HRP substrate kit (Millipore, Temecula, USA).

**Reverse Zymography**

The inhibition of fusion protein dFv-ePD1 to gelatinase was analyzed using reverse Zymography. MCF-7 cells were seeded in a cell culture dish and maintained in exponential growth to 90% confluence. After washed gently with serum-free DMEM medium thrice, the cells were incubated for 24 h with serum-free DMEM medium. The medium was harvested and centrifuged at 1000 rpm to remove cellular debris. Then 30μl of medium was mixed with different concentration of proteins (dFv-ePD1 and dFv) and 5× sample buffer (1 mol/L Tris-HCl, pH 6.8, 50% glycerol, 0.1% bromophenol blue). Samples were subjected to non-denaturing polyacrylamide gel through 10% polyacrylamide gels containing 0.1% gelatin. Gels were rinsed in washing buffer (50 mM Tris-HCl, pH 7.6, 2.5% Triton X-100) at room temperature for 1 hour and incubated in incubation buffer (50 mM Tris-HCl, pH 7.6, 10 mM CaCl$_2$, and 150 mM NaCl) at 37°C overnight. Gels were fixed in 25% isopropanol and 10% acetic acid and stained with 0.1% Coomassie blue R250 (Sigma-Aldrich, St. Louis, Missouri, USA). After destained in 5% methanol and 7% acetic acid, proteolytic degradation of gelatin was visualized as clear bands against blue background of stained gelatin.
Proteolytic signals were quantified by densitometry using the EL logic 200 image analysis system (Koda, USA).

**Binding specificity of dFv-ePD1 with B16-F1 cells by immunofluorescence.**

The B16-F1 cells were cultured in 24-well plate and fixed with precool methanol for 30 min at 4°C. Non-specific binding was blocked with 1% BSA in PBS. After washed with PBS including Tween (PBST), the cells were incubated with dFv-ePD1. Following washed with PBS, cells were incubated with anti-His-Tag mAb, and then with Cy3-conjugated goat anti-mouse antibody, following by stained with 4, 6-diamino-2-phenyl indole (DAPI). The fluorescence images were captured using TS100 fluorescence microscope (Nikon, Tokyo, Japan).

**Flow cytometry**

The B16-F1 cells were digested with trypsin and fixed with 80% ethanol for 30 min at 4°C. Than the B16-F1 cell suspensions were blocked with 1% goat serum for 2 h at 37°C. After washed with PBS, the cells were incubated with 0.2 mg/mL dFv-ePD1 and Anti-MMP2 antibody (1:100) at 37°C for 4 h. The cells were then incubated with mouse anti-His-Tag mAb and Cy3-conjugated goat anti-mouse antibody. Isotype matched antibody was used as control. Samples were analyzed on a LSR-II flow
cytometer.

**Immunohistochemistry.**

Human paraffin embedded melanoma tissue microarray (ME482a, Alenabio, China), including 24 cases of melanoma and 24 normal skin tissues, was baked for 60 minutes at 60°C in a dehydration oven and heat-induced epitope was retrieved in the boiling pressure cooker for 3 minutes and then cooled to room temperature in PBS for 5 minutes. Non-specific binding was blocked with 5% BSA in PBS. Next, the array was incubated with dFv-ePD1 in a humid chamber at 4°C overnight. anti-His-Tag mAb (diluted 1:1000) was used as the primary antibody. After three times of PBST washing, array was incubated with horseradish peroxidase conjugated secondary antibodies for 20 min, followed by staining using diaminobenzidine (DAB) substrate. Then the array was counterstained with hematoxylin. Finally, the photographic image results of the entire tissue of each case of the melanoma tissue microarray were captured under the same illumination condition and magnification. Then, the cumulative optical density value (Integrated Optical Density, IOD) of each image was calculated by Image-Pro Plus 6.0 software. And the mean cumulative optical density of melanoma tissues and normal skin tissues were analyzed statistically.
MTT assay.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell proliferation. Cells were seeded in 96-well plates at a density of 3000 cells/well, incubated in 37°C for 24 h and then exposed to different agents for 24 h, 48 h and 72 h. MTT (Sigma, St. Louis, Missouri, America) solution (5 mg/mL) was added to each well and incubated for another 4 h at 37°C. The supernatant was removed and 150 µl DMSO were added to each well. The absorbance at 570 nm was measured by a microplate reader. Growth inhibition was calculated as times of controls in 0 h.

Cell migration and invasion assay.

The migration and invasion of cells was measured using transwell inserts fitted with polycarbonate filter (8 µm available pore size, #3422; Corning Inc., Corning, NY, USA). B16-F1 cells in FBS-free medium (1×10^5 cells/mL) were seeded in the upper compartment while the lower wells contained 20% FBS medium as a chemoattractant. Following incubation with 100 µg/mL protein dFv-ePD1 and dFv for 24 h respectively, the cells in the upper chamber were removed with a cotton swab while other cells, which had passed through the filter on the underside of the membrane, were fixed with methanol, stained with 0.01% crystal violet and then
captured by the camera on invert microscope. Subsequently, the crystal violet was then dissolved with 33% acetic acid and the absorbance was measured at 540 nm by using a microplate reader. In invasion assay, the upper compartments coated with matrigel matrix were put into cell incubator for 4 h before seeding B16-F1 cells. Than B16-F1 cells in FBS-free medium (5×10^5 cells/mL) were seeded in the upper compartment while the lower wells contained 20% FBS medium as a chemoattractant. Following incubation with 100 μg/mL protein dFv-ePD1 and dFv for 48 h respectively, the cells in the upper chamber were removed with a cotton swab while other cells, which had passed through the filter on the underside of the membrane, were fixed with methanol, stained with 0.01% crystal violet and then captured by the camera on invert microscope. Subsequently, the crystal violet was then dissolved with 33% acetic acid and the absorbance was measured at 540 nm by using a microplate reader.

**In vivo imaging of fluorescein-labeled proteins**

For imaging experiment, dFv and dFv-ePD1 were labeled with the fluorescein DyLight 680 according to the manufacturer’s instruction (DyLight 680 Antibody Labeling Kit, Thermo, Rockford, IL, USA). The tumor-targeting capability of dFv and dFv-ePD1 was investigated using B16-F1 melanoma model. B16-F1 melanoma cells were inoculated to the
right flank of C57BL/6 mice (female, 6-8 weeks), which were ordered from Beijing Anikeeper Biotech Co. Ltd. (Beijing, China) after a 7-day period acclimatization. When the tumor size reached a volume of 300-400 mm$^3$, the mice were injected with DyLight 680 labeled dFv (or dFv-ePD1) at a dosage of 20 mg/kg through tail veins. Following, the mice were anesthetized by isofluorane at a series of time intervals and imaged using IVIS system (PerkinElmer Inc., Waltham, MA, USA).

**In vivo therapeutic efficacy**

Animal experiments were conducted using 6-8 weeks male C57BL/6 mice, which were purchased from the Experimental Animal Center, Three Gorges University (Yichang, China). The melanoma B16-F1 model was generated by subcutaneously injecting 1×10$^6$ cells in 100 µl of PBS into the right flank of mice. Tumor volume (mm$^3$) was calculated according to the equation $V = (a \times b^2)/2$, in which $a$ and $b$ indicate the length and width of the tumor. When the average tumor size reached 50-100 mm$^3$, the mice were randomly divided into 3 groups (n=8). Each group was treated by intraperitoneal injection of dFv (20 mg/kg), dFv-ePD1 (20 mg/kg) or PBS. Tumor volumes were measured every day with a caliper.

**Statistical analysis.**

Significant differences between two groups were determined with
Student’s t-test. $p < 0.05$ was considered as statistically significant, and $p < 0.01$ was thought as highly significant.