Cancer Nanotechnology

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Novel nanotherapeutics for cancer immunotherapy by albumin nanoparticles functionalized with PD-1 and PD-L1 aptamers

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Abstract

Background: PD-1/PD-L1 blockade plays a crucial role in cancer immunotherapy. Exploration of new technologies to further enhance the efficacy of PD-1/PD-L1 blockade is therefore of potential medical importance. Nanotherapeutics can accumulate in tumor tissues due to enhanced permeability and retention (EPR) effects. In this study, a novel nanotherapeutic for cancer immunotherapy was implemented with albumin nanoparticles functionalized by both PD-1 and PD-L1 aptamers.

Results: Albumin nanoparticles (NP) were functionalized with either PD-1 aptamers (PD1-NP), PD-L1 aptamers (PDL1-NP), or both types of aptamers (PD1-NP-PDL1). Average sizes of PD1-NP, PDL1-NP, and PD1-NP-PDL1 were 141.8 nm, 141.8 nm, and 164.2 nm, respectively. PD1-NP had good affinity for activated T cells that expresses PD-1. Similarly, PDL1-NP could bind with MDA-MB-231 or CT26 tumor cells that express PD-L1. Moreover, the bispecific PD1-NP-PDL1 could bind with both the activated T cells and the PD-L1-expressing tumor cells, and tether the two type of cells together. Functionally, aptamer-modified nanoparticles exhibited stronger immune-stimulating effects vs. free aptamers. Specifically, PD1-NP or PDL1-NP induced stronger lymphocyte-mediated cytotoxicity against PD-L1-expressing tumor cells in vitro vs. free PD-1 or PD-L1 aptamers. Animal studies also showed that PD1-NP or PDL1-NP significantly improved antitumor efficacy against CT26 colon cancer in vivo vs. free PD-1 or PD-L1 aptamers. Importantly, the bispecific PD1-NP-PDL1 further boosted the in vivo antitumor efficacy compared with PD1-NP or PDL1-NP, without raising systemic toxicity.

Conclusion: The results suggest that the bispecific PD1-NP-PDL1 is a promising nanotherapeutic to improve the efficacy of PD-1/PD-L1 blockade, and may have application potential in colon cancer treatment.

Keywords: Aptamers, Albumin, Nanoparticles, Immunotherapy, PD-1, PD-L1, Immune checkpoint blockade, Bispecific, Colon cancer

Background

Colorectal cancer (CRC) is the third most commonly diagnosed cancer that makes a great impact on human health in the world, contributing to approximately 1.93 million deaths in 2020 (Fan et al. 2021; Fabregas et al. 2022) and representing 10% of all new

cancer cases (Sung et al. 2021). Although screening by colonoscopy has reduced colon cancer mortality, about 25% CRC patients are still at the advanced disease stage upon diagnosis. Moreover, patients at the early stage have a 25–50% possibility to have progressive diseases after surgery (Pinsky and Doroudi 2016; Sargent et al. 2009). Therefore, it is important to explore effective strategies to treat metastatic CRC. Conventional treatments of colorectal cancer primarily include surgery and chemotherapy. Surgery is indicated for early-stage malignancies, while late-stage diseases are often unresectable. Chemotherapy is regularly used to treat advanced metastatic diseases to reduce the tumor load (Wu et al. 2012; Bregni et al. 2020). Oxaliplatin and 5-Fluorouracil (5-Fu) are the first-line agents for CRC chemotherapy (André et al. 2004; Haller et al. 2011). However, these chemotherapeutics act on the whole body and are associated with severe adverse effects, including acute kidney injury, anorexia, gastrointestinal toxicity, and bone marrow suppression (Zhang et al. 2008; Hsu et al. 2018). Hence, treatment modalities other than surgery and chemotherapy are warranted for colon cancer treatment.

Immune checkpoint blockade (ICB) has emerged as an effective approach to treat CRC with promising clinical results (Wang et al. 2020; Sahin et al. 2019). The PD-1/PD-L1 axis is a major pathway involved in tumor immune evasions (Bylicki et al. 2018). PD-1 is expressed on activated T cells, dendritic cells (DCs), monocytes, B cells, regulatory T cells (Tregs), and natural killer T cells (NKT) (Keir et al. 2008; Sharpe and Pauken 2018), while PD-L1 is expressed on many types of cancer cells (Kornepati et al. 2022; Gato-Cañas et al. 2017). PD-L1 is vital in regulating the antitumor function of T cells. Tumor cells can upregulate the expression of PD-L1 protein that binds to PD-1 on the surface of T cells, thereby inhibiting T cell functions to achieve immune escapes (Li, et al. 2016). Hence, blocking the PD-1/PD-L1 interaction may augment the immune response against colon cancer. In 2015, a clinical study on pembrolizumab, a PD-1-blocking agent, revealed a progression-free survival rate of 78% at 20 weeks in metastatic CRC with trait of deficient mismatch repair (dMMR) (Le et al. 2015). In 2017, the US FDA approved Pembrolizumab for treatment of metastatic CRC with dMMR trait (Oliveira et al. 2019). Currently, it is widely recognized that blocking PD-1/PD-L1 interaction is a promising and efficacious cancer treatment strategy, which is well worthy of further research and development. Therefore, it is important to explore novel technology to implement PD-1/PD-L1 blockade that may further improve the outcome of colon cancer treatment.

In addition to antibodies, aptamers can also be used for the PD-1/PD-L1 blockade. Aptamers are short oligonucleotides that can form complicated 3D structures for binding to molecular targets with high affinity and specificity (Zhang et al. 2019). Compared with antibodies, aptamers have certain advantages for biomedical applications, including low production cost, easy chemical modification, better tumor penetration, and low immunogenicity. Moreover, aptamers also hold great expectations to become a valid and universal therapeutic tool (Zhao et al. 2015). In fact, FDA has approved Macugen®, an aptamer drug, for the treatment of age-related macular degeneration in 2004. Since then, more researchers have launched many studies and clinical investigations on using aptamers to treat various diseases (Byun 2021). Of note, PD-1 and PD-L1 aptamers have also been developed with remarkable efficacy against colon cancer. Gao et al. has selected a PD-1 aptamer that has good binding affinity for human or murine PD-1 proteins (Gao and Pei 2020). Moreover, this aptamer promotes T cell activation,

increases the secretion of IL-2, and inhibits the growth of CT26 colon cancer in vivo (Gao and Pei 2020). Lai et al. has developed a PD-L1 aptamer, which binds to human and murine PD-L1 proteins with K_d of 4.7 nM and 72 nM, respectively. Moreover, this PD-L1 aptamer can suppress the growth of CT26 colon cancer and LL/2 lung cancer in murine tumor models (Lai et al. 2016a, b). Furthermore, Li and An et al. have confirmed that this PD-L1 aptamer can be efficacious in colon cancer treatment in vivo (An et al. 2022). These results indicate that the PD-1 and PD-L1 aptamers have promising potential in ICB therapy against colon cancer.

In this study, a novel nanotherapeutic is designed to further enhance the anticancer efficacy of the PD-1 and the PD-L1 aptamers. Specifically, the above two aptamers are jointly conjugated to an albumin nanoparticle (NP), forming PD1-NP-PDL1, which is a bispecific nanostructure capable of binding with both PD-1 and PD-L1. This nanotherapeutic has several potential advantages. First, the aptamer-modified NPs have the benefits to enrich in tumor tissues via the enhanced permeability and retention (EPR) effects, because tumor blood vessels are usually more permeable and allow proper-sized nanoparticles to accumulate in tumor tissues (Li et al. 2021). Second, each nanoparticle has multiple aptamers attached and thus may generate multivalent bindings with better affinity vs. the free aptamers. Third, free aptamers usually have very small sizes and tend to be eliminated quickly from the body via renal filtration, while the designed nanotherapeutics have sizes far above the renal filtration threshold. Fourth, PD1-NP-PDL1 is a bispecific nanostructure that can bind with both the PD-1-expressing T cells and the PD-L1-expressing tumor cells, facilitating the formation of immunological synapses between T lymphocytes and tumor cells to further enhance immune responses.

To date, no study in literature has investigated the therapeutic efficacy of nanoparticles concomitantly modified by PD-1 and PD-L1 aptamers for treatment of colon cancer. We now report that PD1-NP-PDL1 significantly enhances the therapeutic efficacy against colon cancer in vivo.

Materials and methods

Cell lines and cell culture

CT26 (murine colon cancer cell line), A549 (hypotriploid alveolar basal epithelial cell line), and MDA-MB-231 (human triple-negative breast cancer cell line) were purchased from National Infrastructure of Cell Line Resource (Beijing, China). The peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers' blood using a lymphocyte separation medium (TBD, Tianjin, China). CT26 and MDA-MB-231 cells were cultured in DMEM medium (Gibco) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/mL streptomycin. PBMC and A549 cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin, at 37 °C with 5% CO_2. All volunteers have signed an informed consent. Moreover, the experimental protocol was approved by the Ethics Committee of Chinese Academy of Medical Sciences and Peking Union Medical College. All methods were conducted in accordance with the Declaration of Helsinki.

Animals

BALB/c female mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing). All mice were fed with standard diet and water. All animal study and experiments were performed in accordance with guidelines approved by the Ethics Committee of Peking Union Medical College and the Chinese Academy of Medical Sciences (ACUC-A02-2018–029).

Reagents

The PD-L1 aptamer had a 5′ poly C linker and 5′-SH modification, with the sequence of 5′-SH-CCCCCCCCCC-ACGGGCCACATCAACTCATTGATAGACAATGCGTCC ACTGCCCGT-3′. The PD-1 aptamer had a 5′ poly G linker, 5′-SH modification, and 3′-phosphorothioate modification, with the sequence of 5′-SH-GGGGGGGGGG-CGC ACTATGTTTTACGAGCCGTTTCCTCGGCAGATAGTAAGTGCG-3′. Both aptamers were synthesized by Invitrogen (Shanghai, China). The bovine serum albumin (BSA) was purchased from TBD Science Bio-engineering Co., Ltd. (Tianjin, China). Tris (2-carboxyethyl) phosphine (TCEP) was purchased from Sigma-Aldrich (Shanghai, China). Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Aladdin Chemical Co., Ltd. (Shanghai, China).

Conjugation of aptamers to albumin

Sulfo-SMCC was commonly utilized to connect the thiol-modified aptamers to albumin (Hu et al. 2018). Briefly, 1 mg BSA and 1.44 mg sulfo-SMCC were mixed in 2 ml PBS (pH 7.2) and reacted for 3 h at room temperature. Excess sulfo-SMCC was removed by a filtration device with 30 kDa cut-off threshold, and the processed albumin was resuspended in PBS. After that, 51 ug thiol-modified PD-L1 aptamer or 85 ug thiol-modified PD1 aptamer powder was separately dissolved in 160 μL PBS, and mixed with 40 μL of 800 mM TCEP solution for 1 h to expose the sulfhydryl group. The aptamer solutions were next mixed with the SMCC-treated BSA solution and reacted at room temperature overnight. Subsequently, cysteine was added to the mixtures to seal off superfluous SMCC groups on albumin. Finally, the product (BSA-Apt) was purified by a filtration device with 30 kDa cut-off, and resuspended in 9%Nacl.

Evaluation of conjugation of DNA to albumin

Agarose gel electrophoresis was applied to assess if the aptamers were linked to the SMCC-treated albumin. Tris–borate-EDTA 0.5 x (TBE) buffer solution with 2% (w/v) agarose containing the GelRed DNA dye (Invitrogen, Shanghai, China) was used to prepare the gel. Free aptamers and aptamer-modified albumin were loaded into the gel and subjected to 110 V for 20 min. The DNA was visualized by exposing the gel to UV light by an imaging documentation device (Alliance, London, UK).

Preparation of nanoparticles

PD1-NP was fabricated using a modified self-assembling method that was described previously (Qu et al. 2016; Qu et al. 2017). Briefly, 0.65 mL 9% NaCl was mixed with 1 mL pure ethanol. Next, 19.7 mg BSA and 0.13 mg Apt-BSA at BSA-equivalent weight were dissolved in 100ul 9% NaCl. The above two solutions were heated at 65 °C for

10 min, and mixed on a rotating mixer for 20 min at room temperature. Subsequently, the mixture was poured into 10.5 mL Milli-Q water preheated to 65 °C under rapid stirring for 20 min. Finally, the solution was put into an ice bath for 10 min. The preparation protocols for PDL1-NP and were similar to that for PD1-NP. In preparation of PD1-NP-PDL1, the ratio of albumins modified by PD1 aptamer to albumins modified by PDL1 aptamer was adjusted to 10:6. Coumarin6-containing NPs, including PD1-NP-Cou6, PDL1-NP-Cou6 and PD1-NP-PDL1-Cou6, were prepared using a similar method. Coumarin6 was dissolved in pure ethanol to make a coumarin6 ethanol solution of 7.8 μg/mL. This coumarin6 ethanol solution replaced the pure ethanol in the above described protocol, when preparing PD1-NP-Cou6, PDL1-NP-Cou6 or PD1-NP-PDL1-Cou6. All types of nanoparticles were washed with PBS four times using a cross-flow filtration device with a cut-off threshold of 100 kDa.

Characterization of aptamer-modified albumin and nanoparticles

The average particle size and zeta potential of BSA-Apt or nanoparticles were evaluated by dynamic light scattering (DLS) at 25 ℃, using Zeta Sizer Nano ZS90 (Malvern Instruments, Malvern, UK).

Binding of the nanoparticles to cells

Cellular binding capacity of the nanoparticles was evaluated by flow cytometry. CT26, A549 and MDA-MB-231 cells (1×10^5 cells/well) were cultured in 48-well plates for 12 h. Next, NP-Cou6, PDL1-NP-Cou6, or PD1-NP-PDL1-Cou6 were mixed with CT26, A549, or MDA-MB-231 cells, and incubated for 30 min at 37 ℃. In the meantime, PBMCs were activated via 500 ng/ml anti-CD3 (OKT3) and 2000 IU/ml IL-2. Activated or fresh PBMCs were incubated with NP-Cou6, PD1-NP-Cou6 and PD1-NP-PDL1-Cou6 for 30 min at 37 ℃. The cells were washed twice with PBS and resuspended in 300 uL PBS. Cellular fluorescent signals were analyzed by Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Confocal imaging studies

A549, activated PBMCs, fresh PBMCs and MDA-MB-231 cells (3×10^4 cells/well) were cultivated in Lab-Tek Chamber Slide System (ThermoFisher Scientific, Waltham, MA, USA). After 24 h, A549 and MDA-MB-231 cells were incubated with 30μL of NP-Cou6, PDL1 -NP-Cou6 or PD1-NP-PDL1-Cou6 (Cou6 concentration: 0.06 μg/mL). Activated and fresh PBMCs were incubated with NP-Cou6, PD1-NP-Cou6 and PD1-NP-PDL1-Cou6. The cells were further cultured for 30 min and washed thrice in PBS. Afterwards, 4% formaldehyde polymer was added to fix cells for 10 min. After being washed twice with PBS, the cell nuclei were counterstained by Hoechst 33342 (2 mg/mL; ApexBio Technology, Boston, MA, USA). Thirty minutes later, the cells were washed with PBS thrice and analyzed by a confocal laser-scanning microscope (Perkin Elmer Ultraview, Perkin, Waltham, MA, USA).

Phase contrast microscopy studies

A549 and MDA-MB-231 cells were grown in 6-well plates (6×10^4 cells/well) for 12 h. Activated PBMCs were added to tumor cells at an effector:target ratio of 5:1. The

mixtures were incubated at 37 ℃ for 30 min with NP or PD1-NP-PDL1 containing 100 pmol ssDNA, with gentle shaking. All cells were washed with 1000μL PBS thrice and resuspended in PBS. Phase contrast microscopy was applied to evaluate the spatial relations between tumor cells and immunocytes.

In vitro cytotoxicity studies

MDA-MB-231 tumor cells (1×10^4 cells/well) were cultivated in 96-well plates and mixed with PBMC at an E:T ratio of 5:1. Free PDL1 aptamers, free PD1 aptamers, PDL1-NP, PD1-NP, or PD1-NP-PDL1 were separately added to the cells that were incubated at 37 ℃. The dosages of the NPs were adjusted in such a way so that each treatment group contains 0.1 μM of PD-1 aptamer and/or 0.06 μM of PD-L1 aptamer. Seventy-two hours later, the cells were washed thrice with PBS to remove PBMC and dead cells. MTS assay was applied to evaluate the cell viability according to the standard protocol as outlined by the manufacturer (Promega, Madison, WI, USA).

In vivo antitumor studies

To set up the murine colon cancer model, a CT26 cell suspension (2×10^5 cells in 100μL PBS) was injected subcutaneously on the right rear flank of BALB/c mice. When tumor diameter reached approximately 5 mm, tumor-bearing mice were divided into 7 groups at random. The mice were treated with PBS, free PD-L1 aptamers, free PD-1 aptamers, PDL1-NP, PD1-NP, a combination of PDL1-NP and PD1-NP, PD1-NP-PDL1, via intraperitoneal injection every three days for a total of four times. The dosages of PD1 aptamers and PDL1 aptamers were 3.0 mg/kg and 1.8 mg/kg of ssDNA per animal. Tumor size and body weight were recorded every two days. Tumor volume was calculated according to the formula $(a \times b^2)/2$, where a and b are the largest diameter and the smallest diameter, respectively.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and two-tailed Student's t-test. All experimental data were presented as the mean value with its standard error (mean ± SEM). $P < 0.05$ was considered statistically significant. Statistical analysis was conducted using GraphPad Prism 5 software (La Jolla, CA, USA).

Results

Preparation of aptamer-modified nanoparticles

In this study, the PD-1 and/or PD-L1 aptamers were attached to albumin NPs to construct the immune-modulating nanotherapeutics, which might accumulate in tumor tissues and boost antitumor immunity (Fig. 1). The thiol-modified aptamers were first conjugated to albumin via standard sulfo-SMCC chemistry (Qu et al. 2017). The aptamer-modified albumin (Apt-BSA) was mixed with plain albumin to fabricate the functional nanoparticles. To evaluate whether the aptamers were conjugated to albumin, agarose gel electrophoresis was performed. As shown in Fig. 2A, Apt-BSA moved more slowly vs. free aptamer in the gel, indicating that some aptamers were indeed attached to albumin. To further validate the result, the average zeta-potentials of aptamer-modified albumin or unmodified albumin were also measured. The data

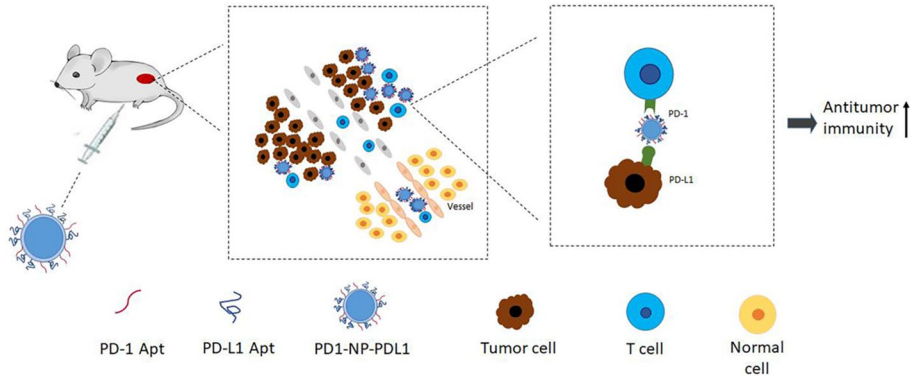

Fig. 1 Schematic illustration of PD1-NP-PDL1 designed for immunotherapy of colon cancer. Thio-modified PD-1 and PD-L1 aptamers were jointly conjugated to albumin nanoparticles to construct PD1-NP-PDL1, which could bind with PD-1 expressed on T cells and PD-L1 expressed on tumor cells

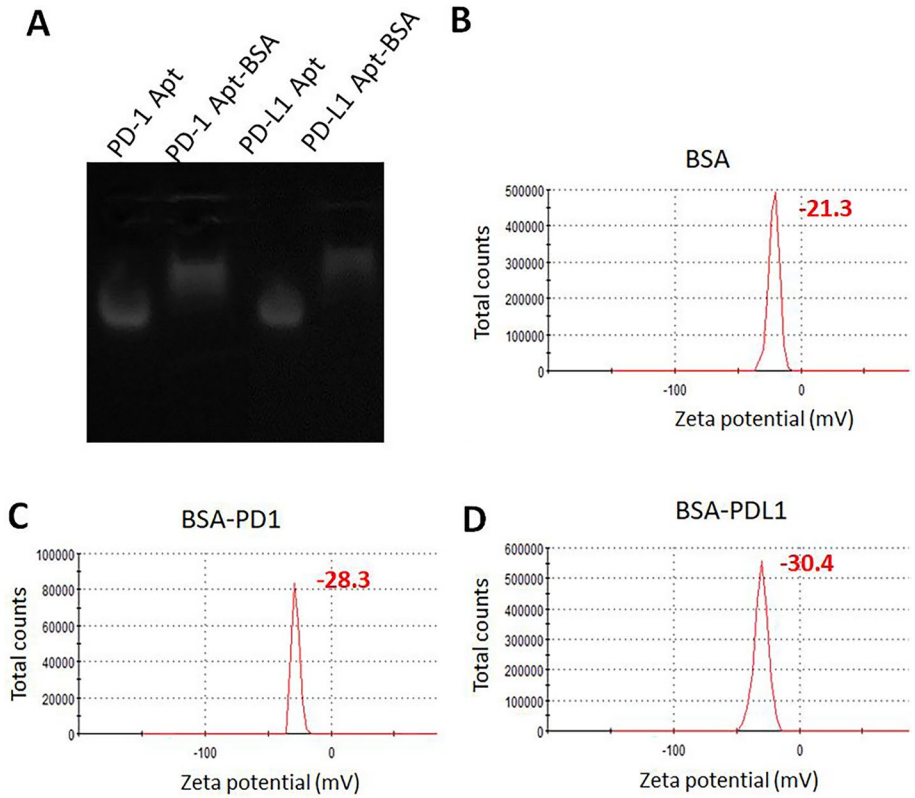

Fig. 2 Evaluation of the conjugation of DNA aptamers to albumin (BSA). **A** Evaluation of Apt-BSA conjugation by electrophoresis in agarose gel, which was stained for DNA by GelRed and visualized under UV light. Zeta potential of **B** albumin (BSA), **C** BSA conjugated with PD-1 aptamer, and **D** BSA conjugated with PD-L1 aptamer by DLS. Aptamer-modified BSA tended to be more negatively charged

showed that aptamer-modified albumin had more negative charges than plain albumin (Fig. 2B, C, and D), again indicating that the negatively charged DNA aptamers were conjugated to albumin as expected.

Characterization of aptamer-modified nanoparticles

The size of nanoparticle plays a critical role in therapeutic and EPR effects. If the size of a nanoparticle is less than 10 nm, the particle tends to be quickly removed from the body via renal filtration. If the size exceeds 200 nm, the particle tends to be devoured by the reticular endothelial system (liver and spleen) of the body (Kobayashi et al. 2013). In this study, we made four types of nanoparticles, plain albumin nanoparticles (NP), PD-1 aptamer-modified NP (PD1-NP), PD-L1 aptamer-modified NP (PDL1-NP), and nanoparticles modified by both types of aptamers (PD1-NP-PDL1). To characterize the four types of nanoparticles, their average sizes and zetapotentials were evaluated by DLS. The results revealed that albumin NP had an average size of 122.4 nm, while PD1-NP, PDL1-NP, and PD1-NP-PDL1 had average size of 141.8 nm, 141.8 nm, and 164.2 nm, respectively (Fig. 3). The polydispersity indices (PDIs) for NP, PD1-NP, PDL1-NP, and PD1-NP-PDL1 were 0.29, 0.24, 0.21, and 0.27, respectively. The results indicated that

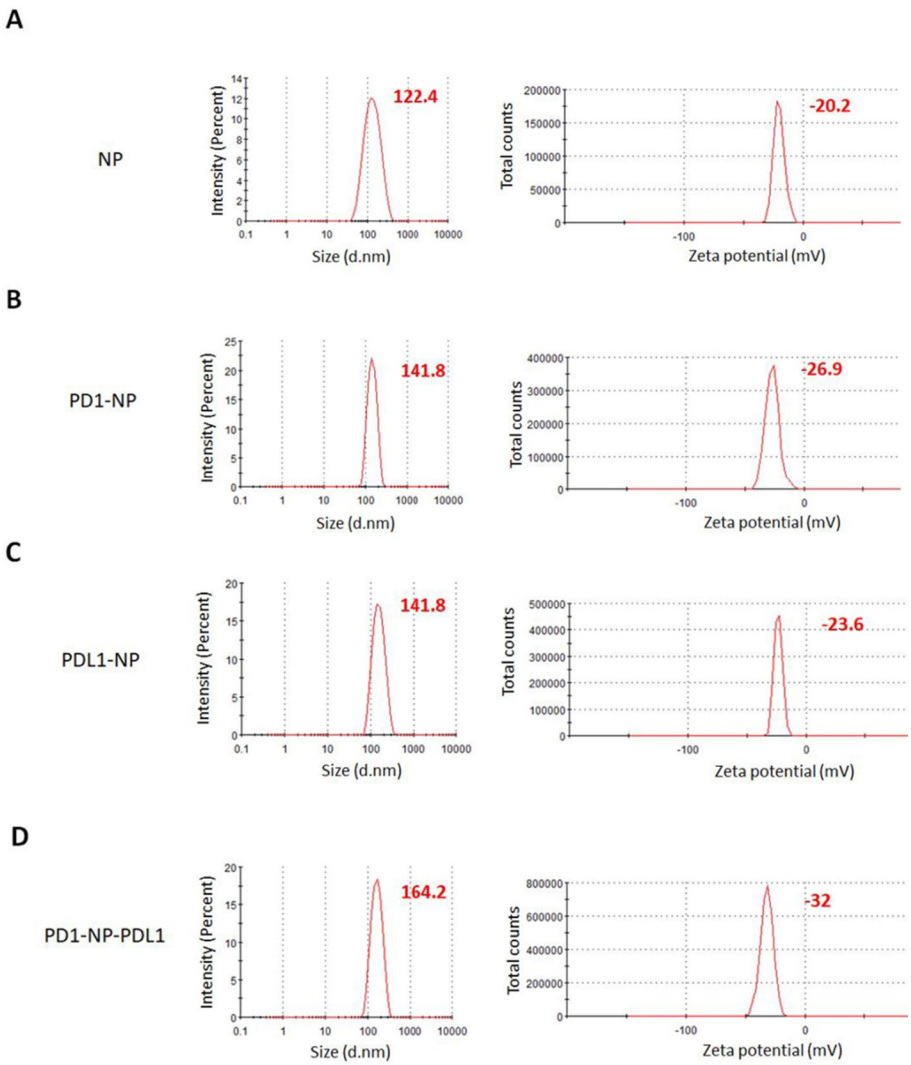

Fig. 3 Characterization of the nanoparticles. The average size (left panel) and zeta potential (right panel) of the NPs: **A** plain NP, **B** PD1-NP, **C** PDL1-NP, and **D** PD1-NP-PDL1

PD1-NP and PDL1-NP were larger than NP, and that PD1-NP-PDL1 were slightly larger than PD1-NP or PDL1-NP. Importantly, sizes of all four nanoparticles were appropriate to circulate in the blood and minimize the capture by the reticular endothelial system. Moreover, the average zeta-potentials of NP, PD1-NP, PDL1-NP, and PD1-NP-PDL1 were − 20.2 mV, − 26.9 mV, − 23.6 mV, and − 32 mV, respectively (Fig. 3). The three aptamer-modified nanoparticles had more negative charges vs. plain NP, presumably because they were conjugated to DNA aptamers that were also negatively charged.

Cellular binding capacity of the nanoparticles

Some cancer cell lines, including CT26 and MDA-MB-231 cells, express plenty of PD-L1 protein in cell membrane (Chatterjee et al. 2016), while others, such as A549 cells, have minimal expression of PD-L1 protein (Lai et al. 2016a, b). PD-L1-aptamer modification of the nanoparticles might change the affinity of the particles to certain cancer cells. However, it was unknown whether the PD-L1 aptamers could still bind with its target after being attached to nanoparticles. To address this issue, we compared the binding of NP, PDL1-NP and PD1-NP-PDL1 to CT26, MDA-MB-231, and A549 cells. Specifically, a fluorescent dye (Coumarin-6) was encapsulated by either plain albumin NPs (NP-Cou6) or aptamer-modified NPs (PDL1-NP-Cou6, PD1-NP-PDL1-Cou6). These particles were incubated briefly with the cells, which were subsequently evaluated by flow cytometry. As shown in Fig. 4A, B, stronger fluorescent signals were observed in CT26 and MDA-MB-231 cells treated with PDL1-NP-Cou6 or PD1-NP-PDL1-Cou6 vs. NP-Cou6, indicating that PD-L1 aptamer-modification of the nanoparticles enhanced the binding

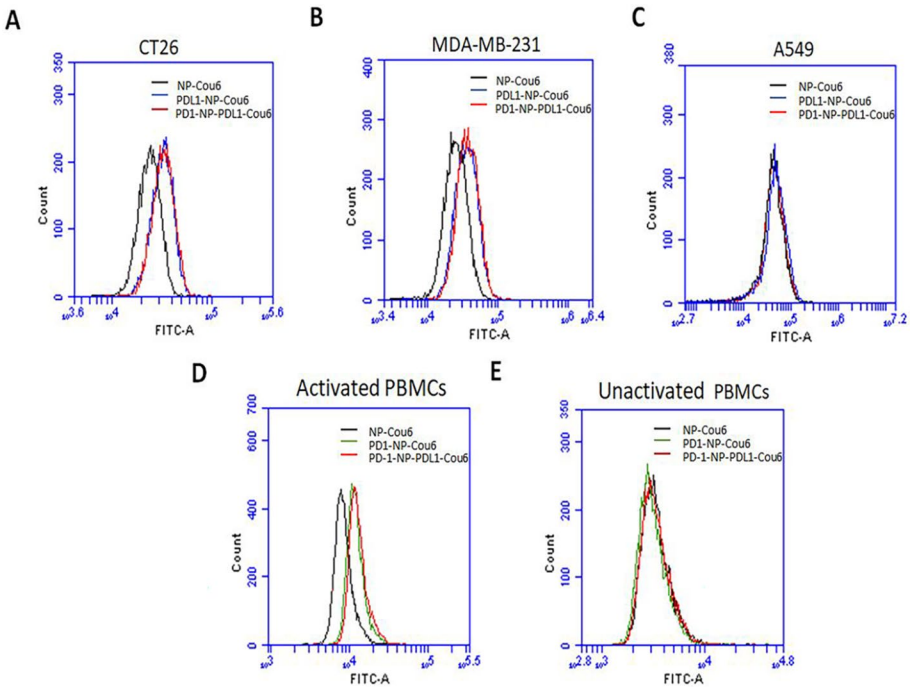

Fig. 4 Flow cytometry evaluation of the bindings of coumarin-containing nanoparticles to various cells. **A** PD-L1-positive CT26 cells, **B** PD-L1-positive MDA-MB-231 cells, **C** PD-L1-negative A549 cells, **D** Activated PBMCs with high PD-1 expression, and **E** Fresh PBMCs with low PD-1 expression

of the particles to PD-L1-expressing cells. In contrast, PDL1-NP-Cou6 or PD1-NP-PDL1-Cou6 did not increase the binding to the PD-L1-negative A549 cells vs. NP-Cou6 (Fig. 4C), suggesting that PD-L1 expression in cell membrane was a necessary prerequisite for the enhanced cellular binding by PD-L1 aptamer-modified nanoparticles.

It has been reported that PD-1 is highly expressed on activated T cells, but minimally expressed in unactivated lymphocytes (Keir et al. 2008). To evaluate whether PD-1 aptamer-modification of the nanoparticles would affect the binding of the particles to lymphocytes, we compared the bindings of PD1-NP-Cou6 or PD1-NP-PDL1-Cou6 vs. NP-Cou6 to immunocytes, including activated PBMC (with high expression of PD-1) and unactivated fresh PBMC (with low PD-1 expression). As shown in Fig. 4D, E, both PD1-NP-Cou6 and PD1-NP-PDL1-Cou6 had stronger bindings to activated PBMC vs. NP-Cou6, but no such difference was observed in fresh PBMC. The results indicated that PD-1 aptamer-modification increased the binding of the particles to PD-1-expressing lymphocytes.

To further validate the above results, confocal microscopy was also employed to evaluate the binding of the nanoparticles to various target cells. As shown in Fig. 5A, B, both PDL1-NP-Cou6 and PD1-NP-PDL1-Cou6 generated stronger fluorescence vs. NP-Cou6 in PD-L1-expressing MDA-MB-231 cells, but not in PD-L1-negative A549 cells. The results again demonstrated that PD-L1 aptamer-modification of the nanoparticles boosted the binding of the particles to the PD-L1-expressing target cells. Similarly, as shown in Fig. 5C, D, PD1-NP-Cou6 and PD1-NP-PDL1-Cou6 had a targeting preference

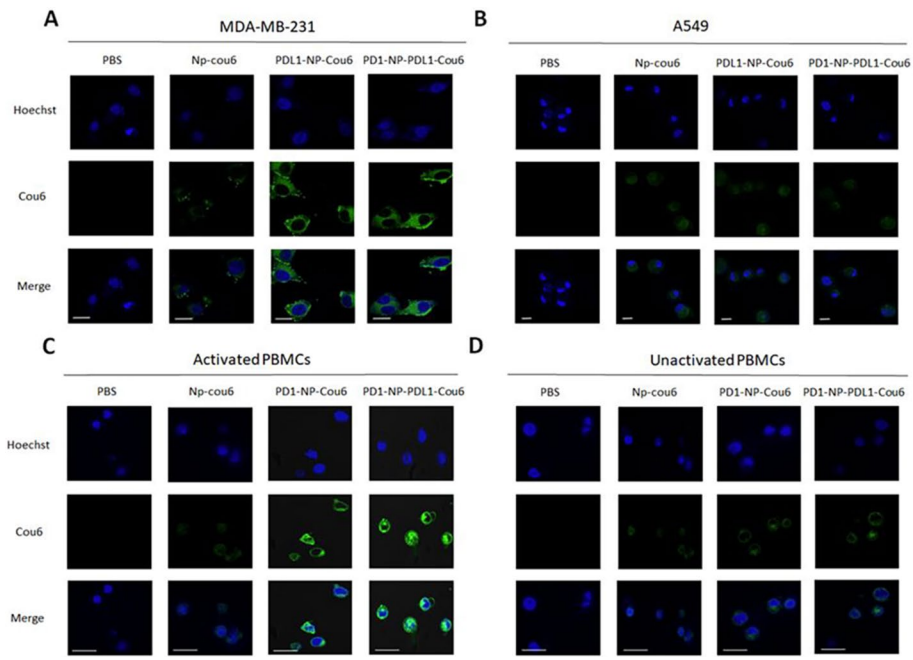

Fig. 5 Confocal microscopy images of various target cells treated by nanoparticles. Green fluorescent signals were generated by coumarin-6 (Cou6) loaded in the nanoparticles. The nuclei were stained blue with Hoechst. **A** PD-L1-expressing MDA-MB-231 cells or **B** PD-L1-negative A549 cells were treated with PBS, NP-Cou6, PDL1-NP-Cou6, or PD1-NP-PDL1-Cou6. **C** Activated PBMCs with high PD-1 expression or **D** Unactivated PBMCs with low PD-1 expression were treated with PBS, PD1-NP-Cou6, or PD1-NP-PDL1-Cou6. Scale bar represents 20 μm

for activated PBMC vs. unactivated PBMC, indicating that PD-1 aptamer-modification of the nanoparticles improved the binding of the particles to PD-1-expressing lymphocytes.

Recruitment of immunocytes to tumor cells by bispecific PD1-NP-PDL1

PD1-NP-PDL1 was designed as a bispecific nanoparticle. As shown in the above experiments, this nanoparticle could bind with both the PD-L1-expressing tumor cells and the PD-1-expressing lymphocytes. Theoretically, PD1-NP-PDL1 might tether the two types of cells together and recruit more immunocytes to the target tumor cells. To test this hypothesis, PDL1-positive MDA-MB-231 cells and PDL1-negative A549 cells were coincubated with activated PBMCs in the presence or absence of PD1-NP-PDL1, washed gently to remove the unattached PBMCs, and analyzed by phase contrast microscope, which was an appropriate tool to observe live cells. As is shown in Fig. 6A, in the presence of PD1-NP-PDL1, there were more lymphocytes gathered around the MDA-MB-231 tumor cells, indicating that PD1-NP-PDL1 recruited more lymphocytes to the vicinity of the PDL1-expressing tumor cells. However, neither PD1-NP-PDL1 nor NP could gather lymphocytes around the PDL1-negative A549 cells (Fig. 6B), presumably because these nanoparticles lack the targeting ligand for A549 cells.

Modulation of in vitro immune cytotoxicity by the nanoparticles

PD-1 and PD-L1 aptamers can enhance anticancer immunity. To evaluate whether nanoparticles functionalized by these aptamers could also enhance the antitumor immune reactions, PDL1-positive MDA-MB-231 cells were cultured with PBMC, and treated by free PD1 aptamers, free PDL1 aptamers, PD1-NP, PDL1-NP, or PD1-NP-PDL1. As shown in Fig. 7, PD1-NP significantly decreased the viability of tumor cells vs. free PD-1

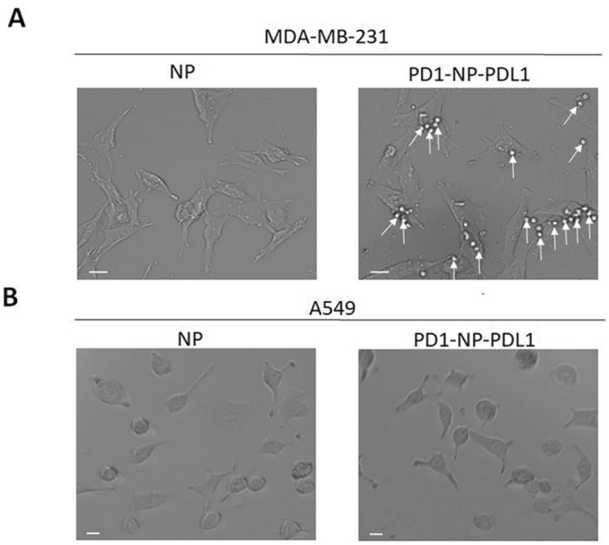

Fig. 6 Recruitment of immunocytes to target cells in the presence or absence of PD1-NP-PDL1. Live **A** PD-L1-positive MDA-MB-231 cells or **B** PD-L1-negative A549 cells were mixed with activated PBMC in presence of either NPs or PD1-NP-PDL1, and gently washed thrice by PBS. The remaining cells were observed by phase contrast microscope. The white arrows pointed to lymphocytes in the image. Scale bar represents 20 μm

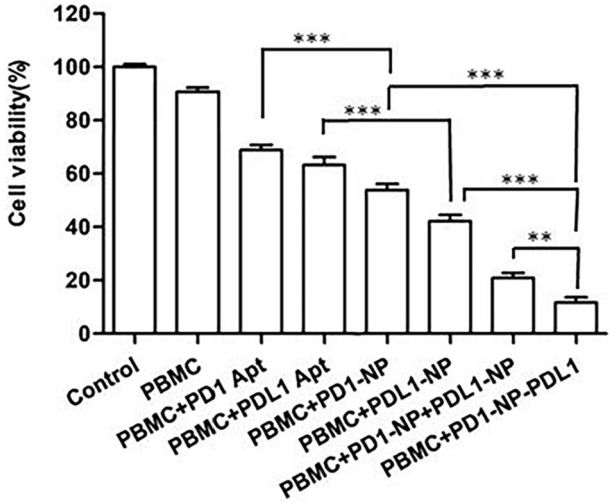

Fig. 7 Immune cytotoxicity against PDL1-expressing MDA-MB-231 cells with various treatments. MDA-MB-231 cells were co-cultured with PBMC in vitro, and treated with free PD1 aptamers, free PDL1 aptamers, PD1-NP, PDL1-NP, a combination of PD1-NP and PDL1-NP, or PD1-NP-PDL1. Seventy-two hours later, the cells were washed thrice with PBS and the viability of tumor cells was evaluated by MTS assay. Untreated MDA-MB-231 cells served as the control ($n = 6$, mean ± SEM)

aptamers, and PDL1-NP induced evidently stronger cytotoxicity vs. free PD-L1 aptamers. Moreover, PD1-NP-PDL1 further improved PBMC-mediated antitumor cytotoxicity vs. either PD1-NP, PDL1-NP, or the combination of PD1-NP and PDL1-NP. The results suggested that conjugation of PD1 or PDL1 aptamers to nanoparticles could improve the PBMC-mediated antitumor cytotoxicity vs. the free aptamers, and that PD1-NP-PDL1 had the potential to further boost lymphocyte-mediated antitumor immunity.

In vivo tumor inhibition study

Previously published studies have shown that the PD-1 and the PD-L1 aptamers could inhibit CT26 colon cancer in vivo (Lai et al. 2016a, b; Gao and Pei 2020). To investigate if aptamer-modified nanoparticles could generate stronger in vivo antitumor efficacy against colon cancer vs. the free PD-1 or PD-L1 aptamers, animal studies were conducted. Specifically, CT26-bearing mice were treated with PBS, free PD-1 aptamers, free PD-L1 aptamers, PD1-NP, or PDL1-NP via systemic administration every three days, for a total of four injections. PD1-NP or PDL1-NP treatment had aptamer molar concentration equal to that of the corresponding free aptamer. As illustrated in Fig. 8A, although free PD-1 aptamers could inhibit the tumor growth vs. the control (PBS), PD1-NP had a stronger antitumor efficacy vs. free PD-1 aptamers. Similarly, PDL1-NP also generated stronger tumor inhibition vs. free PD-L1 aptamers. The results indicated that aptamer-modified nanoparticles enhanced the antitumor efficacy in vivo. To evaluate whether the bispecifc nanotherapeutics could further suppress tumor growth, CT26-bearing mice were also treated by PD1-NP-PDL1, or a combination of PD1-NP and PDL1-NP. As shown in Fig. 8A, PD1-NP-PDL1 had superior therapeutic efficacy vs. either PD1-NP or PDL1-NP, or the combination of the two. The results indicated that the bispecific PD1-NP-PDL1 had the potential to further boost the antitumor efficacy against colon cancer in vivo. To evaluate the toxicity of the nanoparticles, the body weights of all treatment

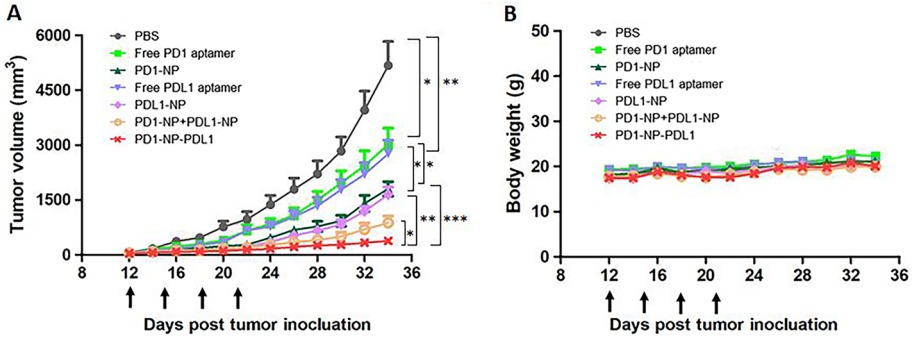

Fig. 8 In vivo antitumor efficacy by various treatments in mice bearing CT26 colon cancer. **A** Tumor growth curve in mice treated with PBS, free PD-1 aptamers, free PD-L1 aptamers, PD1-NP, PDL1-NP, a combination of PD1-NP and PDL1-NP, or PD1-NP-PDL1. **B** Body weights recorded for various groups. Vertical arrows indicated treatment administration ($n = 6$, mean ± SEM)

groups were also recorded. There was no significant difference among body weights of various treatment groups, suggesting no extra systemic toxicity was generated by a particular treatment (Fig. 8B).

Discussion

PD-1/PD-L1 blockade plays a major role in modern cancer immunotherapy. It is therefore important to explore novel technological approaches that may further boost the therapeutic efficacy of PD-1/PD-L1 blockade. In this study, we designed and constructed novel nanotherapeutics for cancer immunotherapy by conjugating PD-1 and/or PD-L1 aptamers to albumin nanoparticles. The average sizes of PD1-NP, PDL1-NP and PD1-NP-PDL1 were between 140 and 170 nm, suitable for circulating in blood and the EPR effects (Fig. 3). The aptamers attached to albumin nanoparticles were still capable of binding with their targets (Figs. 4, 5). Moreover, the bispecific PD1-NP-PDL1 recruited more lymphocytes around the PD-L1-expressing tumor cells (Fig. 6). Compared with free PD-1 or PD-L1 aptamers, PD1-NP and PDL1-NP boosted anticancer immune responses (Figs. 7, 8). Moreover, the bispecific PD1-NP-PDL1 further enhanced the therapeutic efficacy in comparison with PD1-NP or PDL1-NP (Figs. 7, 8). These results suggest that the bispecific PD1-NP-PDL1 represents a novel strategy for enhancing the therapeutic efficacy of PD-1/PD-L1 blockade.

Currently, antibodies are the mainstream technology for realizing PD-1/PD-L1 blockade in clinical application. In this study, aptamers were employed for ICB due to their technical advantages. Aptamers can bind to targets with high affinity and specificity (Tuerk and Gold 1990; Ellington and Szostak 1990). The small size of aptamers allows each nanoparticle to conjugate with multiple aptamers (Mayer 2009). Technically, aptamers can be chemically synthesized and modified easily, ensuring batch-to-batch consistency and facilitating optimization of aptamer properties such as stability and functionality (Zhou and Rossi 2017). Aptamers also have a relatively low production cost compared with antibodies (Rusconi et al. 2004; Oney et al. 2009). In addition, aptamers have good biocompatibility and low toxicity, reducing the risk of adverse immune reactions upon administration (Nimjee et al. 2005). Therefore, aptamers have good potential in biomedical applications.

As shown in Fig. 8, aptamer-modified nanoparticles tend to have better immune-stimulating function than free PD-1 or PD-L1 aptamers. Several factors may have contributed to this phenomenon. First, free aptamers are very small and tend to filtrate through glomeruli and be cleared quickly from the body via kidney. Generally, the kidney can remove particles smaller than 10 nm very efficiently (Liu et al. 2020). Aptamer-modified nanoparticles in this study have sizes between 140 and 170 nm, which are far above the renal clearance threshold, and thus have prolonged circulating time. Second, the sizes of PD1-NP and PDL1-NP are suitable for EPR effects, allowing these nanoparticles to accumulate in tumor tissues and exhibit stronger anticancer effects (Li et al. 2021). Third, because each NP has multiple aptamers attached to it, PD1-NP or PDL1-NP may have multivalent binding with the target cells, improving the binding affinity and the blocking effect. Taken together, these factors may allow PD1-NP or PDL1-NP to generate stronger antitumor efficacy vs. free PD-1 or PD-L1 aptamers.

It should be noted that the bispecific PD1-NP-PDL1 further improved the therapeutic efficacy compared with either PD1-NP or PDL1-NP (Figs. 7, 8). The hypothetical explanation for this finding involves several aspects. First, PD1-NP-PDL1 are capable of blocking both PD-1 and PD-L1, and thus may have superior efficacy vs. nanoparticles that can block only one of these two targets. Second, PD1-NP-PDL1 can bind with both the activated T cells and the PD-L1-expressing tumor cells, with the potential to tether them together (Figs. 5, 6). As a result, the bispecific nanoparticle may recruit more T cells to tumor tissues, facilitating the formation of immunological synapses for anticancer immune reactions. Third, PD1-NP-PDL1 can also accumulate in tumor tissues via EPR effects. Collectively, these factors may enable PD1-NP-PDL1 to achieve stronger therapeutic efficacy vs. PD1-NP or PDL1-NP (Figs. 7, 8), with better application potential for anticancer immunotherapy.

Although numerous materials can be used to make nanostructures (Amreddy et al. 2018), here in this study, we choose albumin as the constructing block to fabricate our nanoparticles for the following reasons. First, albumin has minimal toxicity, low immunogenicity, and good biocompatibility and biodegradability. In fact, albumin represents approximately half of the total proteins in plasma and is well tolerated by the body (He and Carter 1992). Second, each albumin molecule has 103 amino groups that sulfo-SMCC can interact with, making it easy to conjugate with aptamers using chemical linkers (Pereira and Lai 2008). Third, the production of albumin nanoparticles usually does not involve toxic organic solvents, improving the chance for clinical translation (Weber et al. 2000). Fourth, the transcytosis of albumin via Gp60 receptor on endothelial cells allows albumin nanoparticles to be transported into tumor tissues against the efflux induced by the interstitial fluid pressure of solid tumors (Larsen et al. 2016; Merlot et al. 2014). Additionally, albumin is a readily available excipient with relatively low price, making it possible for mass production of albumin nanoparticles with controlled cost (Prajapati and Somoza 2021). Taken together, it is reasonable to use albumin as the building block of the nanoparticles for in vivo applications.

To facilitate the chance of clinical applications, ideally all the components of the nanotherapeutics should be potentially approvable by regulatory agencies for human use. An effort has been made towards this goal in this study. First, the nanoparticle is made of albumin, which is highly biodegradable and biocompatible, with low toxicity and

immunogenicity. Actually, the approval of Abraxane® (paclitaxel albumin-bound NP) has led to further efforts in development of albumin-based nanotherapeutic products (Yardley 2013). Second, aptamers also have relatively low toxicity, with good biodegradability and biocompatibility. In fact, an aptamer drug Macugen® has been approved by FDA for the treatment of age-related macular degeneration (Byun 2021). Third, the SMCC linker used in this study was also approved for human use by FDA. Specifically, in T-DM1®, a HER2-targeting ADC, the same chemical linker was employed to conjugate the anticancer drug to the antibody (Cortés et al. 2022). Taken together, PD1-NP-PDL1 has potential for clinical applications because it is made of biocompatible components potentially approvable by FDA.

Conclusion

In conclusion, ICB is a proven strategy for cancer immunotherapy. By exploiting nanotechnology to further boost the efficacy of ICB therapy, here in this study, a bispecific nanotherapeutic PD1-NP-PDL1 was constructed with albumin nanoparticles functionalized by both PD-1 and PD-L1 aptamers. This bispecific nanotherapeutic significantly improved the in vivo antitumor efficacy vs. free PD-1 or PD-L1 aptamers, and may have application potential in ICB therapy against colon cancer.

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Author contributions
XDY and QPJ designed the research. FJY, YCA, XLL and ZY carried out the experiments and collected data. XDL provided technical expertise and support. QPJ wrote the original manuscript. XDY revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
All experiments in this study were carried out in accordance with the institutional animal use guidelines, and approved by the Ethics Committee of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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