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Novel bispecific aptamer targeting PD-1 and nucleolin for cancer immunotherapy

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Abstract

Background: Immune checkpoint blockade (ICB) is a promising strategy for cancer treatment and has achieved remarkable clinical results. Further improvement of ICB efficacy may advance cancer immunotherapy and has evident medical importance. Here in this study, a PD-1 aptamer was functionalized with a tumor-homing nucleolin aptamer (AS1411) to build a novel bispecific agent (BiApt) for boosting the efficacy of ICB therapy.

Results: The two aptamers were coupled together via sticky ends to form BiApt, which had an average size of 11.70 nm. Flow cytometry revealed that BiApt could bind with both the activated T cells and the nucleolin-expressing tumor cells. In addition, BiApt could recruit more T cells to the vicinity of nucleolin-positive tumor cells. Functionally, BiApt enhanced the PBMC-mediated anticancer cytotoxicity in vitro compared with free PD-1 aptamer. Moreover, in an animal model of CT26 colon cancer, BiApt significantly boosted the antitumor efficacy vs. free PD-1 aptamer.

Conclusion: The results suggest that bispecific agent combining ICB and tumor-homing functions has potential to improve the efficacy of ICB immunotherapy.

Keywords: Immune checkpoint, PD-1, AS1411, Nucleolin, Aptamer, Bispecific, Cancer immunotherapy

Background

Cancer is a major public health issue and one of the most common causes of mortality worldwide (Siegel et al. 2022). Over the last decade, anticancer treatment has experienced encouraging changes with the development of immunotherapy. One promising strategy of cancer immunotherapy is immune checkpoint blockade (ICB), which aims at reducing tumor immune evasion and stimulating immune system by blocking immune inhibitory receptors such as CTLA-4, PD-1, or PD-L1 (Petitprez et al. 2020). ICB is now the first-line therapy for multiple malignancies due to its clinical efficacy, especially for patients with advanced diseases (Bagchi et al. 2021). PD-1 antibodies, such as Nivolumab and Pembrolizumab, have exhibited potent and durable antitumor activity with limited immune toxicity in various malignancies, including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, and colorectal cancer (Brahmer et al. 2015; Garon et al. 2015; Patnaik et al. 2015; Robert et al. 2015; Topalian et al. 2014). Among patients

with advanced squamous-cell NSCLC, overall survival, response rate, and progression-free survival were significantly better with PD-1 antibody vs. conventional chemotherapy (Brahmer et al. 2015). In metastatic melanoma patient treated with PD-1 antibody, about 16% achieved complete response (CR) (Robert et al. 2018). At present, ICB is generally considered a promising and efficacious treatment for multiple malignancies. Consequently, it is necessary and meaningful to explore new technologies to further improve the efficacy of ICB therapy.

Currently, most ICB therapeutics are antibodies. In addition to antibodies, aptamers can also block immune checkpoints with certain technical advantages. Aptamers are short single-stranded RNA or DNA oligonucleotides with unique tertiary structures that can bind to target molecules with high affinity and specificity (Bie et al. 2022). Compared with antibodies, aptamers have the characteristics of low immunogenicity, good biocompatibility, lower molecular weight, rapid tissue penetration, and low production cost (Wan et al. 2019). Moreover, aptamers have also been developed as therapeutics, which aim at modulating the biological pathways for the intervention of multiple diseases, including cancer, infectious diseases, and cardiovascular diseases (Zhu and Chen 2018). For instance, Macugen was the first aptamer drug approved by FDA for treatment of wet age-related macular degeneration (Parashar 2016). In addition, aptamers can also be used as targeting ligands for drug delivery. Yu et al. designed a targeted drug delivery system guided by tumor-targeting aptamer that significantly improved antitumor response in tumor-bearing mice (Yu et al. 2020). Yao et al. designed a DNA nanostructure loaded with doxorubicin and functionalized by four AS1411 aptamers for targeted treatment of colon cancer (Yao et al. 2020). Compared with free doxorubicin, this system markedly enhanced antitumor efficacy in animal studies, without raising systemic toxicity (Yao et al. 2020). Aptamers have also been developed for blocking immune checkpoints. Lai et al. developed a PD-L1 aptamer, which could block the binding between PD-1 and PD-L1, and exhibited similar tumor inhibition power as PD-L1 antibody (Lai et al. 2016). These results suggest that, in addition to antibodies, aptamers also have the potential to function as therapeutics in clinical applications.

In order to further enhance antitumor immunity of ICB, here in this study, we designed an aptamer-based bispecific agent (BiApt) that could not only block immune checkpoint, but also pull together T lymphocytes and tumor cells. The BiApt was constructed with a PD-1 aptamer and a tumor-targeting AS1411 aptamer, which were coupled together by sticky ends. The PD-1 aptamer can bind to both human and murine PD-1 proteins expressed on activated T cells with decent specificity and affinity. Moreover, the PD-1 aptamer can raise the function of lymphocytes by blocking the PD-L1/PD-1 interaction and significantly suppress tumor growth in animal studies (Gao and Pei 2020). AS1411 is a 26-nt DNA aptamer discovered by Bates et al. It can recognize and bind with nucleolin (Bates et al. 2009), which is highly expressed on the surface of multiple cancer cells (Yazdian-Robati et al. 2020), and thus considered a valuable molecular target for cancer therapy. Hence in this study, both AS1411 and the PD-1 aptamers were utilized to construct the BiApt, in order to recruit lymphocytes to tumor tissue.

To date, cancer immunotherapy with bispecific agent targeting both PD-1 and nucleolin has not been reported in literature. Here in the study, a PD-1 aptamer and a nucleolin aptamer (AS1411) were assembled together to build the BiApt. We now report that

BiApt could significantly enhance the antitumor efficacy in vivo compared with regular PD-1 aptamer.

Materials and methods

Cell lines

CT26 (mouse colon cancer cell line), MDA-MB-231 (human triple-negative breast cancer cell line), and CHO (Chinese hamster ovary cell line) were purchased from the Cell Center of Chinese Academy of Medical Sciences (Beijing, China), and cultured in DMEM High Sugar Medium (Livning, Beijing, China). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers by density gradient centrifugation using a lymphocyte separation medium (TBD, Tianjin, China), and cultured in RPMI Medium 1640 (Livning, Beijing, China). All donors provided written informed consent before blood collection. All experiments in this study were carried out in accordance with Declaration of Helsinki and approved by the Ethics Committee of Chinese Academy of Medical Sciences and Peking Union Medical College. Both DMEM medium and RPMI-1640 medium were supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). All cells were grown in an incubator at 37 °C with 5% CO_2. PBMCs were activated with 0.1 μg/mL of anti-human CD3 mAb (Sino Biological) and 1000U hIL-2 (Immunotools) for 7 days. Cultures were re-fed with hIL-2 (1000 U) at days 3, and 5. At day 7, stimulated cells were harvested for flow cytometry analysis.

Synthesis of DNA

All DNAs used in this study were synthesized by Invitrogen (Shanghai, China). The first nucleotide at the 5' end of DNA was phosphorothioate-modified. All the aptamers and control DNA used in this study were equally modified. The DNA sequences were shown in Table 1.

Preparation of BiApt

To form BiApt, S1 and S2 were dissolved in a 0.9% NaCl solution that contained $MgCl_2$ at the concentration of 10 mM. The final concentration of S1 or S2 was 20 μM. The mixture was incubated at 95 °C for 5 min, and slowly annealed to 25 at a fixed rate of 0.1 °C/s.

Characterization of BiApt

Agarose gel electrophoresis made of TBE buffer with 2.5% (w/v) agarose and GelStain (TransGen Biotech, Beijing, China) was utilized to evaluate the formation of BiApt.

Table 1 The DNA sequences used in this study (from 5′ to 3′)

PD-1 aptamer	CGCACTATGTTTTACGAGCCGTTTCCTCGGCAGATAGTAAGTGCG
AS1411 aptamer	GGTGGTGGTGGTTGTGGTGGTGGTGG
S1	AAAAAAAAAAAAAAAAAAAAAAAAA GGTGGTGGTGGTTGTGGTGGTGGTGG
S2	TTTTTTTTTTTTTTTTTTTGGGGG CGCACTATGTTTTACGAGCCGTTTCCTCGGCAGATAGTAAGTGCGA

The samples mixed with DNA loading buffer (TransGen Biotech, Beijing, China) were loaded onto the gel and ran for 30 min at 120 V. The result was analyzed under UV light. Dynamic light scattering (Malvern Zetasizer Nano ZS, UK) was used to measure the size and zeta-potential of BiApt.

Serum stability of BiApt

The stability in serum of phosphorothioate-modified and non-modified BiApt was evaluated by agarose gel electrophoresis. DNA samples were prepared at the concentration of 10 μM, and incubated with 50% fetal bovine serum (FBS) at 37 °C. At the designated time point, the DNA samples were heated at 95 °C for 5 min. Thereafter, 2.5% agarose gel electrophoresis was used to evaluate the degree of BiApt degradation. The gray-scale value was analyzed by ImageJ software.

Cellular binding capacity

CT26, MDA-MB-231, and CHO cells in the logarithmic growth phase were immerged in PBS containing 0.02% EDTA for 30 s, gently scraped, and washed with PBS twice. Activated and non-activated PBMCs were harvested and washed with PBS twice gently. Subsequently, 2×10^5 cells were incubated with 60 pmol of FAM-labeled PD-1 aptamer, AS1411 aptamer, BiApt, or random DNA in 300 μL PBS for 30 min with gentle shaking at room temperature, and washed thrice with PBS. Thereafter, the cells were resuspended in 300 μL PBS and analyzed by flow cytometry (AccuriC6 Flow Cytometer, BD, San Jose, CA, USA).

Confocal imaging studies

MDA-MB-231 tumor cells (1×10^4) were seeded in Lab-Tek Chamber Slide System (ThermoFisher, Waltham, MA, USA) for 24 h and washed gently with PBS twice. Tumor cells were stained with 10 uM CFSE (DOJINDO, Shanghai) for 15 min with gentle shaking at 37 °C. After two gentle washes with PBS, activated PBMCs (5×10^4) were stained with 10 μM eFluro670 for 10 min (Thermo Fisher, Waltham, MA, USA). Both tumor cells and activated PBMCs were washed thrice and resuspended in PBS. Next, activated PBMCs were added to the tumor cell chambers, followed by 20 pmol of BiApt, random DNA, PD-1 aptamer, or AS1411 aptamer. The mixtures were co-incubated at 37 °C for 30 min with gentle shaking, and washed thrice with PBS to remove the unattached PBMCs. At last, the fluorescence image was taken by a confocal laser-scanning microscope (Perkin Elmer Ultraview, Perkin, Waltham, MA, USA).

In vitro *cytotoxicity assays*

MDA-MB-231 tumor cells (1×10^4) were seeded in 96-well plates and cultured in DMEM for 24 h. PBMCs (6×10^4) were added to the tumor cells at an effector: target ratio (E: T) of 6:1. The mixture was treated with 0.2 μM of PD-1 aptamer, AS1411 aptamer, or BiApt. After incubation at 37 °C for 72 h, the cell mixture was washed gently thrice with PBS, and the cell viability was measured by MTS assay (Promega, Madison, WI, USA) following the standard procedure recommended by the manufacturer.

In vivo *antitumor study*

BALB/c mice of 6–8 weeks, with an average weight of 20 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments 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. CT26 tumor cells (2.5×10^5) were inoculated into BALB/c mice to establish murine colon cancer model. When the tumors were about 5 mm in diameter, the mice were randomly assigned to various treatment groups. The mice were injected intraperitoneally with PBS, PD-1 aptamer, AS1411 aptamer, PD1-polyA, AS1411-polyT, or BiApt every three days for a total of four injections (4000 pmol per injection). The tumor volume and body weight of the mice were measured every two days from the time of drug administration. Tumor volume was calculated with the formula $(a \times b^2)/2$, where a and b represent the length and width of the tumor, respectively.

Statistical analysis

All the data were analyzed by GraphPad Prism 8 software and one way ANOVA with Dunnett's multiple comparison test. A statistically significant difference was defined as $P < 0.05$.

Results

Preparation and Characterization of BiApt

In this study, we designed a bispecific aptamer (BiApt) consisting of a PD-1 aptamer and an AS1411 aptamer (Fig. 1A). Previous studies have shown that the PD-1 aptamer can enhance antitumor immunity of T cells (Gao and Pei 2020), and that AS1411 aptamer can target the nucleolin protein expressed on tumor cells (Carvalho et al. 2019). The purpose of the BiApt is to further enhance the efficacy of ICB and recruit immunocytes to tumor cells. Theoretically, the BiApt should tether PD-1-positive T cells and nucleolin-positive tumor cells together, thereby enhancing the antitumor efficacy generated by T lymphocytes (Fig. 1B).

The BiApt was made of two single-stranded DNA chains, S1 and S2. S1 chain was 51-nt long, and had an AS1411 aptamer at the 3' end, with a 25-nt polyA sequence at the 5' end. S2 chain was 70-nt long, and had a PD-1 aptamer at the 3' end, a 20-nt polyT

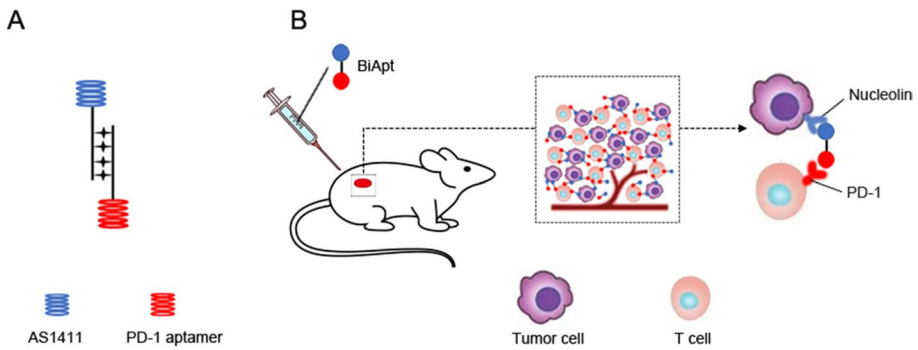

Fig. 1 Schematic illustration of the BiApt design. **A** The structure of BiApt, which was made of an AS1411 aptamer and a PD-1 aptamer coupled together by sticky ends. **B** Illustration of the proposed mechanism by which BiApt recruits PD-1-positive T cells to nucleolin-positive tumor cells

sequence at the 5' end, with a 5-nt polyG spacer in between. The detailed sequences were shown in Table 1. Theoretically, polyA and polyT sequences would hybridize with each other and form a DNA nanostructure (BiApt). To assess whether the two strands indeed coupled together, agarose gel electrophoresis was performed. As shown in Fig. 2, the mixture of S1 and S2 generated a complex with larger molecular weight, indicating that the BiApt was formed as expected.

Numerous studies have established that the size of a nanoparticle plays a key role in determining whether it will be eliminated from the body via renal clearance (An et al. 2022; Li et al. 2021; Yao et al. 2020). Nanoparticles with a size smaller than 10 nm are rapidly cleared from the body by the kidneys (Tang et al. 2014). To characterize the BiApt, its size and zeta-potential were measured by dynamic light scattering (DLS). As illustrated in Fig. 3, free PD-1 aptamer and free AS1411 aptamer had average sizes of 6.50 nm and 3.12 nm, respectively, whereas BiApt had an average size of 11.70 nm, which was above the renal clearance threshold. The results suggested that BiApt might circulate in the body longer than free PD-1 aptamer. Moreover, BiApt had an average zeta-potential of − 21.2 mV, which might maintain its stability and prevent agglomeration (An et al. 2022).

Serum stability of BiApt

To be durable and stable in vivo, the ability to avoid rapid degradation by nucleases in serum is needed for DNA nanostructures. It has been demonstrated that chemical modifications can enhance the resistance of DNA nanostructures to enzymatic digestion (Morita et al. 2018). Among them, phosphorothioate modification of DNA backbone has been proved to be a useful option, which can strengthen the resistance against nuclease (Gao et al. 2021; Li et al. 2021). Here in this study, the first nucleotide at the 5′ end of each ssDNA for BiApt was modified with phosphorothioate, in order to reduce the digestion by exonucleases. To assess the serum stability of BiApt with or without phosphorothioate modification, they were incubated in PBS

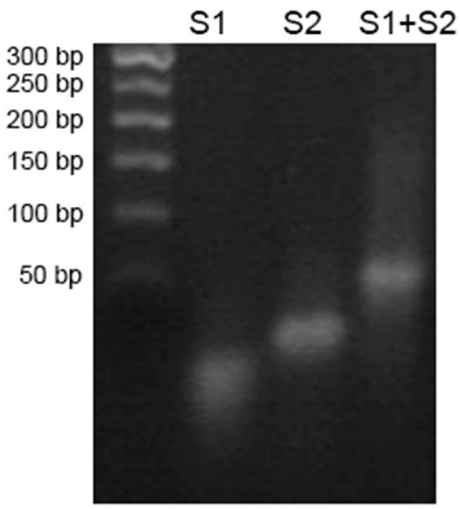

Fig. 2 Agarose gel electrophoresis for verifying the formation of BiApt. The lane on the far left is a 50 bp DNA ladder. Lanes 1–3 represent images of S1 chain, S2 chain, and the mixture of S1 and S2 chains, respectively

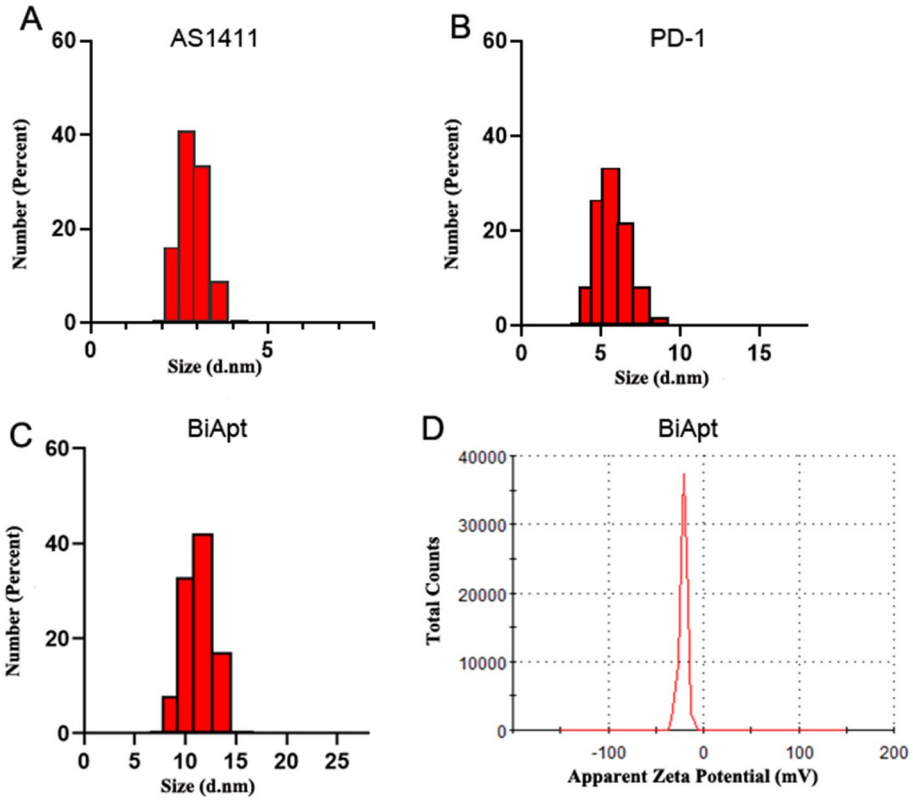

Fig. 3 Characterization of BiApt. Size distributions of free AS1411 aptamer (**A**), free PD-1 aptamer (**B**), and BiApt (**C**). Zeta potential distribution of BiApt (**D**)

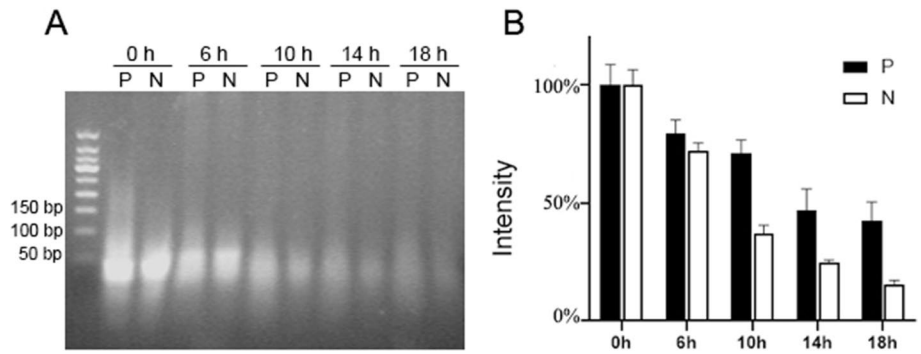

Fig. 4 Serum stability of BiApt with or without phosphorothioate modification. **A** Electrophoresis of BiApt after incubation in PBS supplemented with 50% FBS for various times (N: unmodified BiApt; P: phosphorothioate-modified BiApt). **B** Quantitative gray-scale values of the DNA complexes were obtained by Image J software (n = 3, mean + SEM)

supplemented with 50% FBS for various times, and evaluated by electrophoresis. As shown in Fig. 4, modified BiApt had lower degradation vs. non-modified BiApt. The results indicated that the chemical modification of phosphorothioate could improve the serum stability of BiApt in vivo.

Affinity of BiApt to target cells

When two aptamers are mixed together, entirely new DNA structure may form, and it is possible that each aptamer may lose its target-binding capability. Although free PD-1 and AS1411 aptamers could bind with PD-1-expressing and nucleolin-positive cells, respectively (Bates et al. 2009; Gao and Pei 2020), it was unknown whether the BiApt made of the two aptamers could retain the capability to bind with their targets. To address this issue, nucleolin-positive CT26 and MDA-MB-231 cells, or nucleolin-negative CHO cells were incubated with FAM-labeled BiApt or FAM-labeled AS1411 aptamer and evaluated by flow cytometry. Multiple studies have confirmed that CT26 and MDA-MB-231 cells have high expression of nucleolin in cell membrane, whereas CHO cells have almost no nucleolin expression (Kang and Nguyen 2017; Soundararajan et al. 2008; Yao et al. 2020; Yu et al. 2020). As shown in Fig. 5, both BiApt and AS1411 aptamer could bind with the nucleolin-positive CT26 and MDA-MB-231 cells, but not the nucleolin-negative CHO cells. To evaluate whether the BiApt could still bind with target cells expressing PD-1, similar experiments were conducted using PBMCs. Previous studies have shown that PD-1 is highly expressed on activated T cells (Aksoylar et al. 2020; Gao and Pei 2020). In this study, PBMCs were stimulated with agonistic CD3 mAb and IL-2 to induce activated T cells, incubated with FAM-labeled BiApt or PD-1 aptamer, and evaluated by flow cytometry. As shown in Fig. 5D and E, activated PBMCs could bind with both BiApt and PD-1 aptamer, while naive PBMCs bound with neither. Taken together, the above results indicated that the BiApt

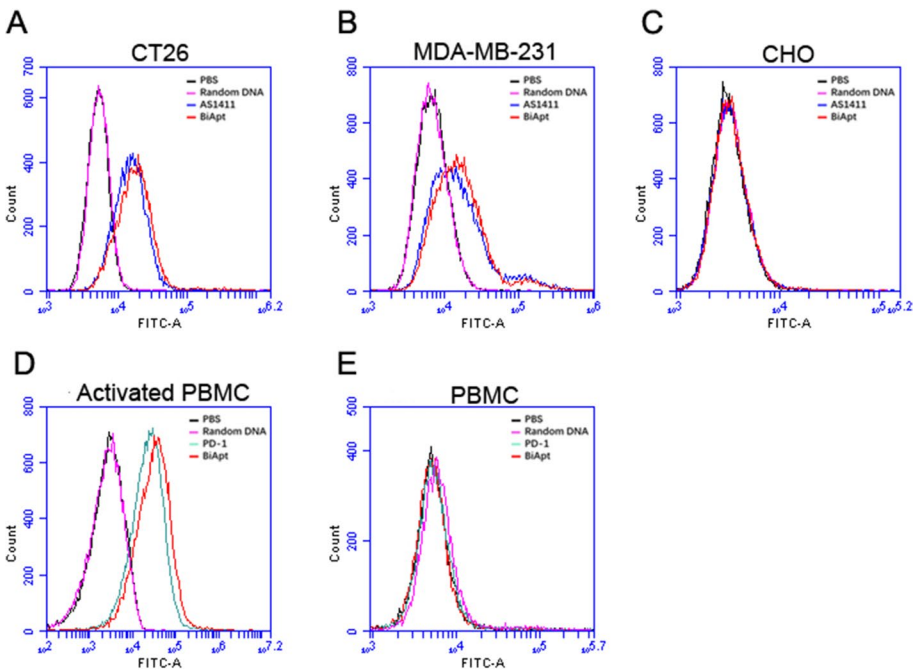

Fig. 5 Bindings of BiApt and free PD-1 or AS1411 aptamer to target or control cells. Nucleolin-positive CT26 cells (**A**), MDA-MB-231 cells (**B**), and nucleolin-negative CHO cells (**C**) were incubated with FAM-labeled free AS1411 aptamer (blue), BiApt (red), random DNA (pink), and PBS (black), respectively. PD-1-overexpressing activated PBMCs (**D**) and non-activated PBMCs (**E**) were incubated with FAM-labeled free PD-1 aptamer (green), BiApt (red), random DNA (pink), and PBS (black), respectively. FAM-labeled random DNA served as the control. All the cells were washed with PBS and analyzed by flow cytometry. All experiments were repeated at least three times

retained the capability of AS1411 and PD-1 aptamers, and could bind with either nucleolin-expressing or PD-1-expressing target cells.

BiApt recruited activated T cells around nucleolin-positive tumor cells

The BiApt was designed to pull together the PD-1-positive T cells and the nucleolin-positive tumor cells, facilitating the T cells attack of the tumor cells. Although BiApt showed good affinity for both nucleolin-positive MDA-MB-231 cells and activated PBMCs, it was still unknown whether it could facilitate the interaction between the two types of cells. To study this issue, the two types of cells were mixed together in the presence of BiApt, random DNA sequence, PD-1 aptamer, or AS1411 aptamer. To remove unattached PBMCs, the cell mixture was washed gently for three times, and imaged by confocal microscopy. As presented in Fig. 6, in the absence of BiApt, few PBMCs were found near MDA-MB-231 cells. Conversely, in the presence of BiApt, more PBMCs were observed around the nucleolin-positive MDA-MB-231 cells. These data suggested that BiApt could recruit more lymphocytes to the vicinity of target tumor cells.

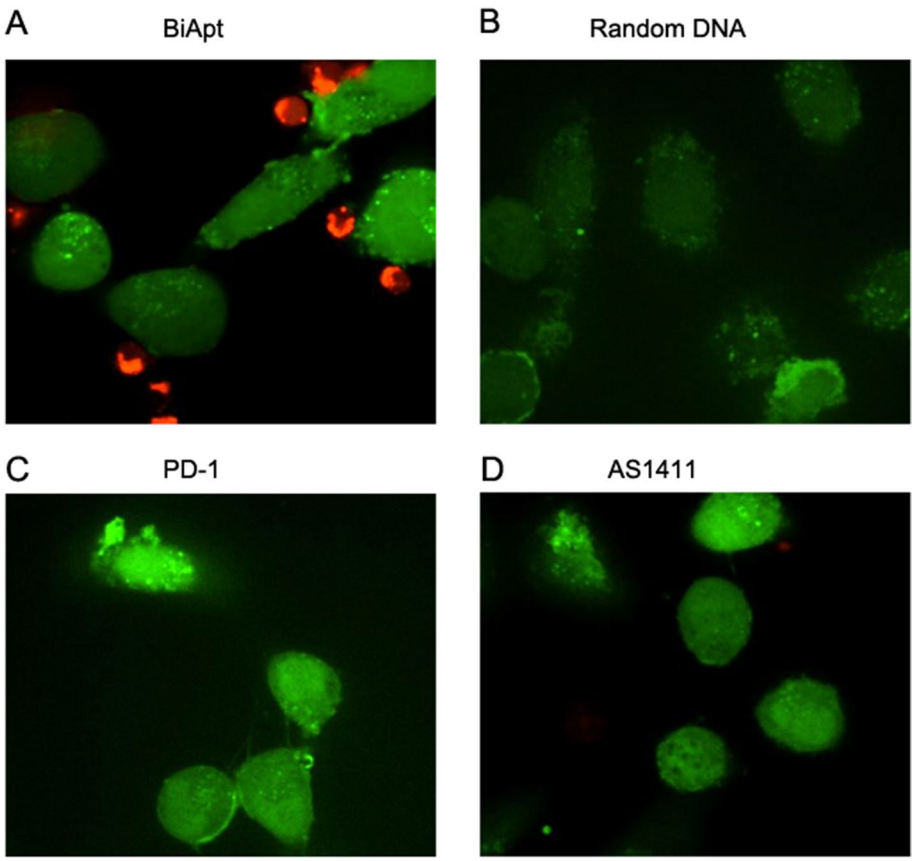

Fig. 6 Recruitment of immunocytes to target cells with or without BiApt. Live nucleolin-positive MDA-MB-231 cells were stained green with CFSE, while activated PBMCs were stained red with eFluro670. Tumor cells and activated PBMCs were incubated together in the presence of BiApt (**A**), random DNA sequence (**B**), PD-1 aptamer (**C**), or AS1411 aptamer (**D**). The mixture was gently washed trice to remove unattached PBMCs, and the remaining cells were imaged by confocal microscopy

BiApt enhanced cytotoxicity of PBMCs against tumor cells in vitro

Although BiApt appeared capable of recruiting more lymphocytes to nucleolin-positive tumor cells, it was still unclear whether BiApt could boost antitumor immunity compared with free PD-1 aptamer. To address the issue, nucleolin-positive MDA-MB-231 cells and PBMCs were co-cultured for 72 h and treated with PBS, free PD-1 aptamer, free AS1411 aptamer, or BiApt. The viability of tumor cells was measured by standard MTS assay. As shown in Fig. 7, in the presence of BiApt, PBMC-mediated cytotoxicity was significantly enhanced compared with free PD-1 aptamer ($p < 0.05$). The result suggested that BiApt could further boost immune response of PBMCs to nucleolin-positive tumor cells in vitro.

BiApt generated superior antitumor efficacy in vivo

The main purpose of this study was to compare the antitumor response generated by BiApt vs. free PD-1 aptamer. However, BiApt had a larger size than PD-1 aptamer (Fig. 3). It has been demonstrated that the size of a nanostructure influences the rate at which it will be cleared from the body via renal filtration (Li et al. 2021). For fair comparison of the in vivo functions of PD-1 aptamer vs. BiApt, the PD-1 aptamer was attached to a DNA structure to form PD1-polyA, which had a similar molecular weight as BiApt. Specifically, PD1-polyA was a DNA nanostructure based on BiApt, in which the AS1411 aptamer was replaced by a nonfunctional DNA sequence of polyA, but the PD-1 aptamer remained unchanged. Prior study has shown that the PD-1 aptamer could suppress tumor growth in CT26-bearing mice (Gao and Pei 2020). To evaluate whether PD1-polyA could also suppress tumor growth, we compared the in vivo antitumor efficacy of free PD-1 aptamer vs. PD1-polyA. Mice bearing CT26 tumors were treated with PBS, free PD-1 aptamer, or PD1-polyA every 3 days for a total of 4 injections, at a dose of 4000 pmol per injection. As shown in Fig. 8A, both free PD-1 aptamer and PD1-polyA inhibited tumor growth ($p < 0.05$). Although PD1-polyA appeared to inhibit the tumor slightly more than free PD-1

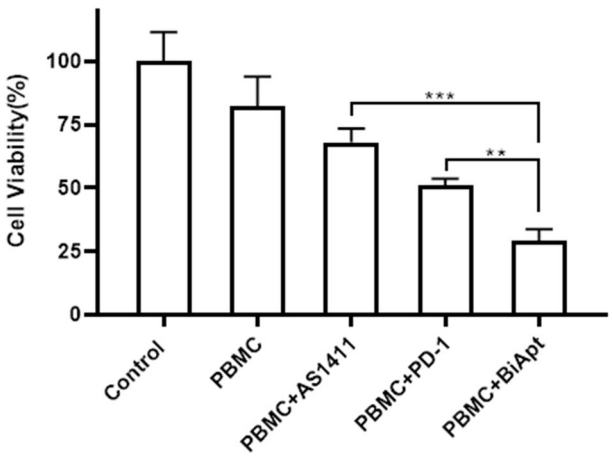

Fig. 7 PBMC-mediated cytotoxicity against nucleolin-positive MDA-MB-231 cells in vitro. Tumor cells and PBMCs were cocultured and treated with PBS, free PD-1 aptamer, free AS1411 aptamer, or BiApt in the medium. After 72 h, unattached PBMCs were washed off, and tumor cells were evaluated for viability with standard MTS assay ($n = 7$, mean + SEM). Star indicates statistically significant differences ($p < 0.05$)

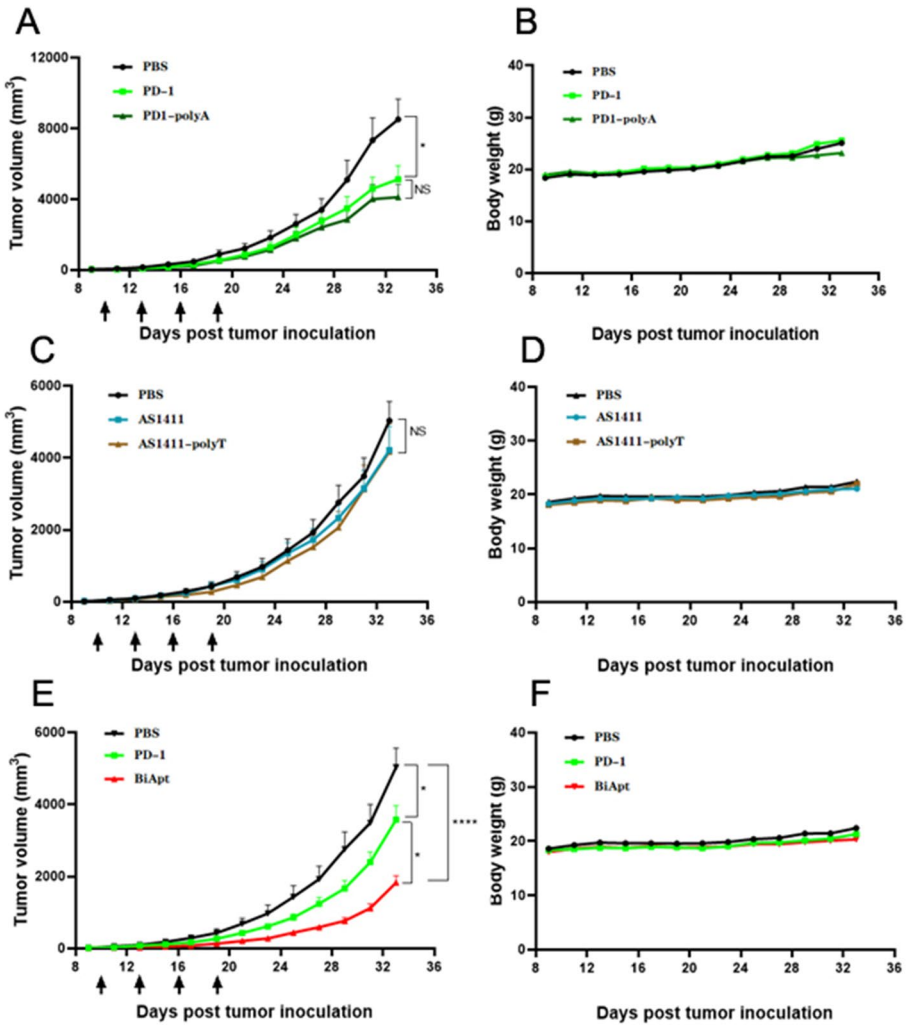

Fig. 8 In vivo antitumor study with CT26-bearing BALB/c mice. CT26 tumor-bearing mice were intraperitoneally injected with PBS, free PD-1 aptamer, PD1-polyA, free AS1411 aptamer, AS1411-polyT, or BiApt every 3d for a total of 4 injections (arrows). Tumor volume and body weight were recorded and shown (n = 8, mean + SEM). Star indicates statistically significant differences ($p < 0.05$), and "ns" indicates differences that are not statistically significant

aptamer, the difference between the two treatment groups was not statistically significant ($p = 0.71$). Furthermore, there was no difference in body weight among the treatment groups (Fig. 8B).

The BiApt evaluated in this study was made of a PD-1 aptamer and an AS1411 aptamer. Previous studies have shown that the G-rich aptamer AS1411 can inhibit the growth of a broad range of cancer cells (Bates et al. 1999; Yazdian-Robati et al. 2020). To evaluate the in vivo effect of AS1411 aptamer at the dosage used in this study, CT26 tumor-bearing mice were treated with PBS, free AS1411 aptamer, or AS1411-polyT (a DNA nanostructure based on BiApt, in which the PD-1 aptamer was replaced by polyT, but the AS1411 part remained unchanged, with a molecular weight similar to that of BiApt). As shown in Fig. 8C, at the designated dose used in this study (4000 pmol per injection), neither AS1411 nor AS1411-polyT generated

obvious tumor inhibition ($p = 0.66$ and $p = 0.63$, respectively), presumably because the AS1411 dosage used here was insufficient to suppress tumor growth in vivo.

Although BiApt enhanced immune cytotoxicity against the target tumor cells in vitro (Fig. 7), it was still unclear whether it could generate superior antitumor efficacy in vivo. To address this issue, CT26 tumor-bearing mice were treated with PBS, PD-1 aptamer, or BiApt. As shown in Fig. 8E, free PD-1 aptamer again generated marked tumor inhibition ($p < 0.05$). Of note, BiApt further enhanced the antitumor efficacy vs. free PD-1 aptamer ($p < 0.05$). Meanwhile, there was no difference in mice body weight among groups (Fig. 8F). The results indicated that BiApt could further enhance antitumor immunity in vivo, without raising systemic toxicity.

Discussion

ICB is an important strategy for cancer immunotherapy. To further improve the efficacy of ICB therapy, it appears advantageous to construct a tumor-targeting bispecific agent that can also realize the function of ICB. Bispecific agent can recruit T lymphocytes to tumor tissue, thereby enhancing antitumor immunity (Li et al. 2019; Suurs et al. 2019). Both ICB and bispecific agent can boost anticancer response and have generated remarkable clinical results (Brahmer et al. 2015; Topp et al. 2014). It is reasonable to hypothesize that implementing the two strategies with one nanostructure may further improve the therapeutic efficacy. On the one hand, such a design can recruit more lymphocytes to the vicinity of cancer cells, forming immune synapses and promoting T cell-mediated lysis of cancer cells (Fucà et al. 2021). On the other hand, targeted delivery of ICB agent to tumor tissue may facilitate anticancer immunity while mitigating the undesirable side effects of ICB therapy. Hence, a bispecific agent made of an ICB agent and a tumor-homing ligand appears a promising strategy worthy of further exploration.

In this study, we designed a novel bispecific agent (BiApt) based on a PD-1 aptamer and a nucleolin aptamer (AS1411). The BiApt had an average diameter of 11.7 nm, which was above the threshold of renal clearance (Fig. 3). Phosphorothioate modification of BiApt increased its serum stability (Fig. 4). The BiApt maintained good affinity to both PD-1 expressing T cells and nucleolin-positive tumor cells (Fig. 5). Moreover, in the presence of BiApt, more T cells were recruited to the vicinity of nucleolin-positive cancer cells (Fig. 6). Furthermore, compared with free PD-1 aptamers, BiApt significantly enhanced the PBMC-mediated antitumor cytotoxicity in vitro (Fig. 7). Importantly, in tumor-bearing mice, BiApt also significantly improved the anticancer efficacy vs. PD-1 aptamer (Fig. 8).

Although most ICB agents currently in clinical use are antibodies, aptamers can also fulfill such function with certain technical fortes. First, aptamers for a certain molecular target can be cost-effectively screened in vitro. Second, aptamers can be easily synthesized and manufactured in large-scale (Thomas et al. 2022; Zhu and Chen 2018). Third, the stable structure of aptamers affords them a long storage period and a relatively high resistance to heat, for their tertiary structures can be quickly restored following heat denaturation (Morita et al. 2018). Another advantageous property of aptamer is that it can be easily modified site-specifically and conjugate with various functional agents for biomedical applications (Thomas et al. 2022; Zhao et al. 2015). Because of these technical advantages, aptamers have also

been developed for cancer immunotherapy. For example, the PD-1 aptamer used in this study could bind with both human and murine PD-1 proteins, and inhibited tumor growth in CT26-bearing mice (Gao and Pei 2020). Huang et al. selected a CTLA-4-antagonizing DNA aptamer, which could promote lymphocyte proliferation and suppress tumor growth in animal models (Huang et al. 2017). These studies demonstrate that, aside from antibodies, aptamers can also be used for cancer immunotherapy with potential for clinical applications.

Aptamers have also been employed to construct bispecific agents for boosting anticancer immune response. Pastor et al. showed a bispecific agent with a bivalent 4-1BB agonistic aptamer and a PMSA-binding tumor-targeting aptamer, which could deliver the 4-1BB agonist to PSMA-expressing cancer cells in situ and improve antitumor immunity (Pastor et al. 2011). Soldevilla et al. engineered a MRP1-CD28 bispecific aptamer, which could target CD28 co-stimulation to drug-resistant MRP1-positive melanoma stem cells and thereby boost immune response (Soldevilla et al. 2016). Boltz et al. designed a bispecific agent based on CD16 aptamer and tumor-homing c-Met aptamer, which could specifically recruit CD16-expressing NK cells to c-Met-overexpressing tumors, and induce ADCC-mediated tumor lysis (Boltz et al. 2011). Li et al. constructed a bispecific agent with two CD16 aptamers and two MUC1 aptamers, which enhanced the immune cytotoxicity against MUC1-expressing tumor cells by CD16-positive immunocytes (Li et al. 2019). These developments indicate that aptamer-based bispecific agents have application potential in cancer immunotherapy.

So far, however, no bispecific aptamer that combines a tumor-homing ligand with a PD-1 aptamer has been reported in literature. Since PD-1 protein is expressed on activated T cells (Aksoylar and Boussiotis 2020), a bispecific agent made of PD-1 aptamer can not only block inhibitory immune checkpoints, but also recruit T cells into tumor tissue. We speculated that such a design could further improve the anticancer efficacy of PD-1 blockade, and constructed a BiApt based on a PD-1 aptamer and a tumor-homing AS1411 aptamer in this study. As shown in Fig. 8, BiApt indeed significantly enhanced antitumor efficacy in CT-26 bearing mice.

The mechanisms by which BiApt further improves therapeutic efficacy may involve several aspects. First, conjugating PD-1 aptamer with tumor-homing AS1411 aptamer may enrich the BiApt in tumor tissue, thereby strengthening the ICB effect around the tumor cells. Second, in addition to blocking PD-1 pathway, BiApt can also recruit activated T cells to nucleolin-expressing tumor cells, form a bridge between the two, and facilitate T cell-mediated antitumor response. Third, although AS1411 aptamer is mostly used as a tumor-targeting ligand, the aptamer also has a moderate tumor inhibitory effect (Soundararajan et al. 2008; Yazdian-Robati et al. 2020). However, at the dosage used in this study, the antitumor effect by AS1411 was probably quite weak (Fig. 8C). Nevertheless, the tumor inhibitory effect of AS1411 may have contributed to the overall therapeutic efficacy generated by BiApt. Fourth, a free aptamer generally has a very small size and is rapidly eliminated via renal filtration, whereas BiApt has a size of 11.7 nm, which is above the renal clearance threshold, allowing a longer circulation time and prolonged functions. Taken together, all these mechanisms may collectively contribute to the enhanced antitumor efficacy of BiApt.

Conclusion

In conclusion, a BiApt consisting of a PD-1 aptamer and an AS1411 aptamer improved antitumor response in vivo. By replacing AS1411 with alternative tumor-targeting aptamers, this design may also have potential to treat other malignancies. Our results suggest that a bispecific agent made of a PD-1 inhibitor and a tumor-homing ligand represents a promising strategy for cancer immunotherapy.

Abbreviations

ICB	Immune checkpoint blockade
BiApt	Bispecific aptamer
NSCLC	Non-small cell lung cancer
CR	Complete response

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Author contributions
JF: conceptualization, methodology, investigation, data curation, writing—original draft. FY: methodology, investigation. YA: methodology, investigation. XL: methodology, investigation. WW: investigation, resources, project administration. XY: validation, investigation, resources, project administration, funding acquisition. 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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