Self-Assembly of a Multifunction DNA Tetrahedron for Effective Delivery of Aptamer PL1 and Pcsk9 siRNA Potentiate Immune Checkpoint Therapy for Colorectal Cancer

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ABSTRACT: Compared with the traditional single therapy, nanomedicine has promoted a multimodal combination treatment for various carcinomas, especially the development of corresponding intelligent multifunctional biomaterials based on advanced DNA nanotechnology has great potential in cancer combination therapy. Herein, we describe a strategy to “backpack” aptamer PL1, which specifically binds to PD-L1 and Pcsk9 siRNA on well-defined DNA tetrahedral nanoparticles (TDNs) via DNA hybridization, which collectively contributes to the effective therapy for colorectal cancer (CRC). In addition, we designed a targeted TDN upon folic acid (FA) recognition, limiting its release to the sites of tumors where folic acid receptor (FAR) is encountered. Our results demonstrated that the TDN-FA/PL1/Pcsk9-siRNA could free immune cells to target CRC cells and attenuate 83.48% tumor growth in mouse models of CT26 CRC. Mechanically, the cancer-targeting FA guided TDN-FA/PL1/Pcsk9-siRNA into tumor cells, thereby ensuring that the aptamer PL1 could choke the mutual effects between PD-1 and PD-L1, followed by a 1.69-fold increase in T cell number and a 1.9-fold suppression of T cell activity by the PD-1/PD-L1 pathway, while Pcsk9 siRNA decreased Pcsk9 expression averagely to the extent of 65.13% and then facilitated intratumoral infiltration of cytotoxic T cells robustly with IFN-γ and Granzyme B expression. Our results reveal that the multifunctional TND-FA/PL1/Pcsk9-siRNA is effective and safe for CRC therapy, thereby expanding the application of DNA nanotechnology for innovative therapies of various cancers.

KEYWORDS: DNA tetrahedron nanoassembly, aptamer PL1, PCSK9, colorectal cancer, immunocombination therapy

1. INTRODUCTION
Tumor immune evasion contributes to the escaping of cancer cells from being killed by immune cells, which results in low survival and unfavorable prognosis of cancer patients.1,2 Immune checkpoint treatment has started a fresh era in cancer treatment. Programmed death 1 (PD-1) exists in activated T cells and delivers inhibitory signaling via T cell receptors,3 while programmed death ligand 1 (PD-L1) exists in lymphocytes infiltrated in tumors as well as cancer cells.4 The PD-1/PD-L1 pathway contributes to the functional inhibition of T cells and leads to tumor immune escape.5 The PD-1/PD-L1 pathway blockade mediated through monoclonal antibody could induce sustained tumor suppression and favorable treatment stabilization in refractory cancers, such as colorectal cancer (CRC), non-small-cell lung carcinoma, head and neck squamous cancer, etc.6–8

Aptamers are essentially single-stranded oligonucleotides, which have demonstrated excellent tissue penetration efficiency.9 Due to the capability to form three-dimensional construction, low immunogenicity, and outstanding specific coupling apteney, aptamers are regarded as the potent antibody surrogate.10 As reported, aptamers are synthesized chemically, and the structure could be modified easily.11,12 Therefore, aptamers with controllable variations between batches can be produced in large amounts and then be used to create customized modifications for specific applications of therapy.12–14 Gao et al. isolated an aptamer named PL1, which effectively conduced to the blockade between PD-1/PD-L1 interaction in addition to restoring the T cell multiplication and rescuing the secreted IFN-γ suppressed by the PD-1/PD-L1 pathway.15 Moreover, aside from administration alone,

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many efforts are underway to couple aptamers with materials for controllable and sustainable release in targeted areas to improve the treatment effects of aptamers.\textsuperscript{16,17}

The proprotein convertase subtilisin-kexin type 9 (PCSK9) is known as a secretory serine endoprotease, which could be synthesized from several organisms, and is crucial in regulating cholesterol metabolism.\textsuperscript{16,18} Deleting the \textit{Pcsk9} gene or inhibiting the PCSK9 protein could achieve obvious synergies with anti-PD-1 treatment by restraining tumor growth in animal studies. Mechanistically, PCSK9 could associate with the major histocompatibility protein class I (MHC I) proteins physically by which conducing to its following relocation and degradation in the lysosome and thus disrupting its recycling back to the surface of tumor cells, which results in robustly improved infiltration of cytotoxic T cells in tumors.\textsuperscript{20} Small interfering RNAs (siRNAs) provide a promising treatment for the disease by effectively inhibiting pathogenic gene expression with relatively low effective doses, excellent specificity, and favorable simple process of drug development. Either liposome nanoparticle formulated siRNA\textsuperscript{21} or preclinically triantennary N-acetylgalactosamine in conjugated siRNA\textsuperscript{22} could significantly block the PCSK9 compound by targeting \textit{Pcsk9} mRNA specifically. However, owing to the shortage of tissue-targeted specificity and possible toxicity resulting from heterogeneous components, size, and chemical characteristics, delivering siRNA into the tumor safely and effectively is still challenging.\textsuperscript{23,24}

DNA nanotechnology delivery approaches provide potent clues to overcome the mentioned challenges.\textsuperscript{25} Among them, tetrahedral DNA nanoparticle (TDN) is a classical three-dimensional framework with six edges and four vertexes, which are always focused on developing an ideal platform to make multiple decorations.\textsuperscript{26,27} It is considered as an ideal drug carrier because of the advantages of design ability, predictability, and the ability to form unique morphological characteristics.\textsuperscript{28} Folic acid receptor (FAR) has been widely overexpressed in many types of cancers, including CRC, making it an attractive therapeutic target.\textsuperscript{29,30} In this study, we constructed a FAR-targeted effective aptamer PL1 and \textit{Pcsk9} siRNA nanodelivery approach and evaluated the therapeutic effect on the BALB/c mice model of colon carcinoma and studied the underlying mechanism.

2. EXPERIMENTAL SECTION

2.1. Self-Assembly of Multifunctional Tetrahedron DNA Nanoparticles (TDN-FA/PL1/Pcsk9 siRNA). FA was conjugated to single-stranded 1 (S1) and S2 mediated by a scaffold consisting of hydroxyprolinol, where the prolinol amino agent was conjugated to the COOH of FA with an extended aminocaproic acid tether first.\textsuperscript{28} Then, the associated oligonucleotide components (structural DNA, aptamer DNA, and siRNA were purchased from Sangon Biotech, Shanghai, China) were stoichiometrically mixed in TM buffer (5 mM MgCl\textsubscript{2}, 10 mM Tris), followed by incubation at 95 °C for 2 min and quickly cooled down to 4 °C. The hydrodynamic diameter and ζ-potential of TDN particles were determined in phosphate buffer solution (PBS) (Brookhaven Instruments, Holtsville, NY).

2.2. Atomic Force Microscopy Imaging. TDN was prepared in TM buffer at a concentration of 10 nM in a 10 μL volume and covered with the freshly cleaned mica for 15 min and then imaged in an aqueous buffer using a three-dimensional (3D) atomic force microscope (Bruker, Germany). The images were analyzed by nanoscope analysis 3.0.

2.3. Cell Lines. Mouse colon carcinoma cells, MC38 and CT26, and hamster ovary cells, CHO, were purchased from Cell Resource Center. MC38 and CHO cell lines were incubated with Dulbecco’s modified Eagle’s medium (DMEM, Gibco), and the CT26 cell line was incubated with Roswell Park Memorial Institute (RPMI1640, Gibco); the two media had the following as additive, fetal bovine serum (FBS, Gibco) at a concentration of 10% and penicillin and streptomycin at a concentration of 100,000 units per liter and 100 μg/mL, respectively.

2.4. Cytotoxicity Assay. Mouse colon carcinoma cells CT26 and MC38 (1 × 10\textsuperscript{6} cells per well) in a 100 μL volume were seeded to a 96-well plate and incubated for 12 h. Then, the cells were incubated with TDN (50, 100, 200, and 400 nM) and TDN modified with/without aptamer PL1 or siRNA (400 nM) for 24 h. Subsequently, Cell Counting Kit 8 (CCK8) reagent (B34304, Bimake, Houston, Texas) was used to replace the medium and the cells were cultured for an additional 2 h at a determined time. The measurement of the absorbance unit at 450 nm was taken with a microplate reader (Thermo Fisher Multiscan FC). The cells that were not treated were regarded as the control group. Three independent experiments were performed for each assay. The viability of the cell was determined using the following formula: cell cytotoxicity (%) = (A\textsubscript{sample} − A\textsubscript{blank}) / (A\textsubscript{control} − A\textsubscript{blank}).

2.5. Analysis of TDN-FA with/without PL1 or Pcsk9 siRNA on T Cell Function. We prepared single-cell suspension cells of the spleen using a mechanical method. Then, according to the standard procedure of the CD4+ T Cell Isolation Kit (480005, MojoSort), we incubated a 100 μL aliquot containing 10\textsuperscript{6} cells on ice with the preprepared cocktail consisting of biotin-labeled antibody for 15 min and preprepared 10 μL of streptavidin nanobeads for 15 min. Then, 500 μL of MojoSort Buffer was added for column separation, and the CD4+ T cells were separated using magnetic separation with medium capacity columns (# 130-122-729).

Both anti-mouse CD3 antibody (1 μg/mL, Sino Biological) and anti-mouse CD28 antibody (1 μg/mL, Sino Biological) in a 100 μL volume were added to each test well in the 96-well plate, followed by incubation at 4 °C incubation overnight. After washing three times with PBS, 10 mg/mL recombinant PD-L1 (CJ88, Novoprotein) in a 100 μL volume was added, followed by incubation at 37 °C for 4 h, and then washed twice with PBS. Afterward, 100 μL of TDN-FA, TDN-FA/PL1, or TDN-FA/PL1/Pcsk9 siRNA nanomedicine (400 nM) was added, followed by incubation at 37 °C for 2 h. Afterward, the T cells (5 × 10\textsuperscript{6} cells per well) obtained from magnetic separation were added, and the plate was incubated at 37 °C for 72 h. Then, we collected the culture medium and used the mouse IFN-γ ELISA Kit (EK280/3, MULTISCIENTES) to quantify the IFN-γ level. The CCK8 Cell Proliferation assay was adopted to assess the proliferation of cultured T cells.

2.6. Evaluation of Antitumor Efficacy in a Murine Syngeneic Tumor Model. Animal experiments were done under the approval of the Animal Use and Care Committee of the First Affiliated Hospital, Sun Yat-sen University. BABL/C mice were purchased from GemPharmatech Co., Ltd. (Foshan, Guangdong, China). Age-matched 6-week-old female BABL/C mice were treated with syngeneic CT26 cells (2 × 10\textsuperscript{5} per mouse) subcutaneously. When the tumor’s long axis reached ~5 mm, the mice were randomly divided into four groups. Typically, 400 nM TDN-FA, TDN-FA/PL1, or TDN-FA/PL1/Pcsk9 siRNA in a 100 μL volume was, respectively, injected into the corresponding group every 2 days. Each treated group comprised at least five BABL/C mice. Tumor growth and body weight were monitored, tumor size was observed using a caliper, and its corresponding volume was calculated based on the formula by which the volume equals half of (length) (width)\textsuperscript{3}. The point after 10 times treatment was defined as the end of the animal experiments; mice blood was obtained for biochemical analysis, and tumors were collected for detecting DNA and protein expression. The major organs of mice, including livers, hearts, lungs, spleens, and kidneys were also gained to further perform hematoxylin and eosin (H&E) staining.

2.7. Blood Biochemistry Analysis. Blood was obtained from the eyeballs of mice, and then the serum was obtained via centrifugation at 2000 rpm for 10 min for biochemistry analysis. Alanine aminotransferase (ALT, C009), aspartate aminotransferase (AST, C010), alkaline phosphatase (AKP, A059), creatinine (CRE, C011),
urea nitrogen (BUN, C013), and uric acid (UA, C012) (Nanjing Jiancheng Bioengineering Institute, China) Kits were purchased to analyze the serum samples.

2.8. Stability Evaluation of PL1, Pcsk9 siRNA, and TDN-FA/PL1/Pcsk9 siRNA. TDN-FA/PL1/Pcsk9 siRNA was incubated in a culture medium containing 10% FBS at 37 °C for 0, 2, or 4 h, followed by vortexing at 100 rpm for 10 min. The concentrations of DNA or RNA in the supernatant were used to evaluate the degradation rate. The stability was estimated using 8% native polyacrylamide gel electrophoresis (PAGE).

2.9. Tumor Targeting Analysis of TDN Nanomedicine In Vivo. The Cy7-labeled TDN with/without FA, PL1, or Pcsk9 siRNA was administered to mice bearing CT26 colon carcinoma via intravenous tail injection. The noninvasive NIR fluorescence imaging device coupled with an excitation optical source and a cooling charge facility (IVIS Lumina LT Series III, PerkinElmer), which consists of a was administered to mice bearing CT26 colon carcinoma via intravenous tail injection. The noninvasive NIR fluorescence imaging device coupled with an excitation optical source and a cooling charge facility (IVIS Lumina LT Series III, PerkinElmer), which consists of a

Figure 1. Illustration of the synthesis of the multimodal DNA tetrahedron nanoassembly. (A) Strategy of folic acid conjugated to DNA. Chemical structure formula of folic acid (left); aminocaproic acid (middle); monodeoxyribonucleotide (right). (B) DNA hybridization induced accurate self-assembly of TDN-FA/PL1/Pcsk9-siRNA.

2.12. Western Blotting Analysis. After washing three times, the cells were then subjected to the radioimmunoprecipitation assay (RIPA) lysis buffer supplied with a protease inhibitor (9803S, CST). Proteins with different kDa values in the lysates were separated via SDS-PAGE electrophoresis, followed by transfer to the poly-(vinylidifluoride) (PVDF, Sigma-Aldrich) membrane. Proteins could be detected by corresponding antibodies and horseradish peroxidase (HRP)-coupled secondary antibodies. Electrochemiluminescence (ECL) was used to develop the HRP signal. Protein quantification was analyzed via ImageJ (National Institutes of Health, NIH). The antibodies included the rabbit anti-PCSK9 antibody (catalog number b185194, Abcam), anti-HLA class 1 ABC antibody (catalog number 15240-1-AP, Proteintech), anti-GAPDH antibody (catalog number 60004-1-Ig, Proteintech), and anti-β-actin antibody (catalog number 20536-1-AP, Proteintech).

2.13. Immunohistochemistry Staining. Immunohistochemistry (IHC) staining was carried out based on the previously described studies. The primary antibody included anti IFN-γ (catalog number A12450, 1:100 dilution, ABclonal), Granzyme B (catalog number bs-1351R, 1:500 dilution, Bioss), and Ki-67 (catalog number bs-1351R, 1:500 dilution, Bioss), and Ki-67 (catalog number BS130-3, 1:1000 dilution, Servicebio). After diaminobenzidine (ZLI9107, ZSGB-BIO, Beijing, China) staining and hematoxylin counterstaining, the tissue photos were captured with an Olympus microscope (Olympus, Tokyo, Japan).

2.14. Statistical Analysis. All statistical data were analyzed by GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). A one-way analysis of variance (ANOVA) test and two-tailed Student’s t-test were used as appropriate. To ensure statistical power, the number of experimental replicates was at least three replicates in vitro, and the mice experimental groups were typically composed of five mice each. The results were represented as mean ± standard deviation (SD). P < 0.05 was regarded as statistical significance.
3. RESULTS AND DISCUSSION

3.1. Characterization of the Multifunctional DNA Tetrahedron Nanoassembly. The conjugation of folic acid to S1 and S2 was mediated by a scaffold consisting of hydroxyprolinol, where the −COOH of FA was conjugated to the prolinol with a stretched aminocaproic acid tether (Figure 1A). Then, the multifunctional TDN-FA/PL1/Pcsk9 siRNA was programmed based on self-assembly by six short DNA strands with overhangs, which complemented with others through a careful annealing process at the 3′ ends at the same proportion. The number of base pairs of every six structural edges was 30, and thus the calculated TDN height was ~8 nm. Additionally, the location where the 3′ and 5′ ends of an oligonucleotide meet would form a gap in the neutral position of each edge. The gap accompanied by an overhang on the edge was capable of complementing with aptamers PL1 or double Pcsk9 siRNA strands (Table S1). Thus, two aptamers and two siRNAs are bound per folic acid-conjugated nanoparticle (one per edge) (Figure 1B).

As shown in Figure 2A, the microstructure of TDN-FA and TDN-FA/PL1/Pcsk9-siRNA was obtained in an aqueous buffer with atomic force microscopy imaging equipment, and the images with a high resolution not only showed the appearance of an individual particle with three upper edges but also a height of ~2.4 or 2.8 nm. The lower height detected compared with the theoretical value might be related to the few oligonucleotide strains without sufficient force, resulting in the failure to form a rigid construction, and the higher height of packed TDN-FA/PL1/Pcsk9-siRNA than TDN-FA alone indicated the loading of PL1 and siRNA-modified sequence may stretch the backbone outside. Typically, 8% native PAGE was used to show the stepwise assembly process of the TDN-FA/PL1/Pcsk9-siRNA nanoparticles, and the results demonstrate a distinct and slower-moving band shift obtained with an increasing number of assembly DNA strands, which validates its successful package without side products (Figure 2B). The hydrodynamic diameter of assembled TDN-FA/PL1/Pcsk9-siRNA nanoparticles was measured to be 28.6 nm with a narrow size distribution (Figure 2C). The ζ-potential was close to −40.89 ± 6.098 mV when they were prepared at 1 μM (Figure 2D). Next, to determine the stability of assembled TDN-FA/PL1/Pcsk9-siRNA against enzymatic degradation, we incubated the DNA nanostructure in a medium containing 10% FBS. PAGE analysis showed that either naked aptamers or siRNAs were almost completely degraded after 2 h of incubation, while the TDN-FA/PL1/Pcsk9-siRNA still exhibited a partial (almost 80%) integrated band (Figure 2E). The result suggests that TDN could protect PL1 and Pcsk9-siRNA from enzymatic degradation, which indicates that the TDN backbone could enhance the stability of aptamer and siRNA therapeutic.

3.2. Biocompatibility and Cellular Internalization of TDN-FA/PL1/Pcsk9-siRNA. To assess the cytocompatibility of TDN nanoassembly, we treated colon carcinoma mouse cell lines (CT26 and MC38) with TDN-FA alone, TDN-FA/PL1, or TDN-FA/PL1/Pcsk9-siRNA. CT26 or MC38 cells treated with TDN nanoassembly were captured, and the images did not show alteration in the morphology compared with the control group (Figure S1). Cytotoxicity resulting from the TDN nanoparticles was measured to be 28.6 nm with a narrow size distribution (Figure 2C). The ζ-potential was close to −40.89 ± 6.098 mV when they were prepared at 1 μM (Figure 2D). Next, to determine the stability of assembled TDN-FA/PL1/Pcsk9-siRNA against enzymatic degradation, we incubated the DNA nanostructure in a medium containing 10% FBS. PAGE analysis showed that either naked aptamers or siRNAs were almost completely degraded after 2 h of incubation, while the TDN-FA/PL1/Pcsk9-siRNA still exhibited a partial (almost 80%) integrated band (Figure 2E). The result suggests that TDN could protect PL1 and Pcsk9-siRNA from enzymatic degradation, which indicates that the TDN backbone could enhance the stability of aptamer and siRNA therapeutic.

It is well known that chemotherapy for various cancers has side effects. The characteristics of nanoparticles and physiological features of tumors contribute to the enhanced permeability and retention (EPR) effect, which results in the presence of passive targeting. What is more, it has been proven that nanoparticles could easily transfer into the tumor tissue via the EPR effect. Therefore, as the optimum approach, the active projects could increase targeted specificity based on passive...
methods. For targeted cancer therapy, the key question was whether the modified folate could increase the TDN nanoassembly amount in folic acid receptor (FAR) positive cells. To address this issue, flow cytometry (FCS) analysis was performed to observe the intracellular fluorescence intensity after treating the CHO, MC38, and CT26 cell lines with FAM-labeled TDN alone, FAM-labeled TDN-FA, FAM-labeled TDN-FA/PL1, or FAM-labeled TDN-FA/PL1/Pcsk9-siRNA. For FAR-positive cells, the fluorescent signals generated by FAM-labeled TDN-FA, FAM-labeled TDN-FA/PL1, or FAM-

Figure 3. Biocompatibility, targeted ability, and cell distribution of TDN-FA/PL1/Pcsk9 siRNA in colon carcinoma cell lines. (A) Biocompatibility of various concentrations of TDN-FA/PL1/Pcsk9 siRNA toward CT26 (left) and MC38 (right) cells at 24 h. (B) Relative cell viability of CT26 and MC38 cells after various treatments at 24 h. (C) Flow cytometry showed the uptake of FAM-labeled TDN, FAM-labeled TDN-FA, FAM-labeled TDN-FA/PL1, or FAM-labeled TDN-FA/PL1/Pcsk9 siRNA in CHO (left), MC38 (middle), and CT26 (right) cells at 37 °C for 2 h. (D) Confocal laser scanning microscopic images demonstrating the cellular distribution of TDN-FA/PL1/Pcsk9 siRNA. Images were captured in CT26 (top) and MC38 cells (bottom) after 2 h of incubation (scale bar: 50 μm). Cell nuclei were stained with DAPI (blue), FAM dye-labeled TDN-FA/PL1/Pcsk9 siRNA (green), and lysotracker was stained with DND99 (red).
labeled-TDN-FA/PL1/Pcsk9-siRNA were similarly higher than $10^4$ absorbance unit (AU), whereas signals generated by the FAM-labeled TDN alone was remarkably lower than $10^4$ AU as shown on the scale interval of abscissa (Figure 3C). The result indicates that FA/FAR mediates the targeted cellular uptake. TDN-FA/PL1 and TDN-FA/PL1/Pcsk9-siRNA were efficiently taken up by FAR-positive CT26 and MC38 cells for subsequent intracellular delivery.

Most nanocarriers enter the cells through endocytosis, followed by the endosome and lysosome pathway; thus, we analyzed TDN-FA/PL1/Pcsk9-siRNA in subsequent intracellular delivery by confocal laser scanning microscopy (CLSM). The obtained results demonstrated that the FAM-labeled TDN/PL1/Pcsk9-siRNA and lysosome of both CT26 and MC38 cells were co-located after 2 h (Figure 3D), which was consistent with the lysosome pathway reported previously. Although TDN-FA/PL1/Pcsk9-siRNA showed an effective internalization, it would be destroyed if localized in the lysosome for a long time, and thus escaping from the lysosome is necessary for improving its bioavailability. The proton sponge effect is the most studied escaping method of such endocytosis from the lysosome. However, the mechanism of TDN-FA/PL1/Pcsk9-siRNA escaping needs further study.

3.3. Bioactivity of TDN-FA/PL1/Pcsk9-siRNA In Vitro
To detect the bioactivity of TDN-FA/PL1/Pcsk9-siRNA in vitro, we performed T cell activation assays to confirm whether TDN-FA/PL1/Pcsk9-siRNA has the effect of blocking PD-L1 and activating CD4+ T cells. We also measured the production of IFN-γ and proliferation of cells. As shown in Figure 4, CD4+ T cells purified from mice spleen were activated in the presence of IL2 and CD3/CD28 antibody, followed by exhibiting a significantly higher T cell multiplication level as well as IFN-γ yield compared to the nonactivated cells. These effects were reversed by the 10 μg/mL recombinant mouse PD-L1 protein. The PD-L1-mediated suppression of CD4+ T cells functions was rescued when TDN-FA/PL1 or TDN-FA/PL1/Pcsk9-siRNA was added. Both multiplication of CD4+ T cells and IFN-γ yield increased significantly, as much as 1.69- or 1.9-fold compared with the control group, respectively, but no obvious variation was observed between the two groups. These results manifested that the aptamer PL1 packed on TDN succeeded in rescuing the activity of T cells caused by the suppression of the PD-1/PD-L1 pathway.

To confirm the silencing efficiency of Pcsk9 siRNA specific for the targeted gene, we examined Pcsk9 mRNA and protein levels in CT26 and MC38 cells treated with Lipo-ctrl, lipo-siRNA, TDN-FA, TDN-FA/PL1, and TDN-FA/PL1/Pcsk9-siRNA. The results showed that Pcsk9 mRNA expression in Lipo-Pcsk9 siRNA and TDN-FA/PL1/Pcsk9-siRNA group was sufficiently silenced, achieving approximately 68.13 and 41.55% in CT26 cells (Figure 4C), 61.51 and 52.45% in MC38 cells (Figure 4D) compared with the control group, respectively. Western blotting manifested that the PCSK9 protein was suppressed in CT26 and MC38 cell lines treated with Lipo-Pcsk9 siRNA and TDN-FA/PL1/Pcsk9-siRNA compared with the control group, respectively (Figure 4E,F).
Since MHC I was proven to be an important downstream molecule of PCSK9 in previous studies, we also detected MHC I mRNA changes in the above samples. As shown in Figure 4C, MHC I mRNA expression in Lipo-Pcsk9-siRNA and TDN-FA/PL1/Pcsk9-siRNA groups was upregulated by 2.16-fold and 2.09-fold in CT26 cells but by 2.03-fold and 1.78-fold in MC38 cells, respectively (Figure 4D).

3.4. Antitumor Efficiency and Security of TDN-FA/PL1/Pcsk9-siRNA Therapy in Mice Bearing CT26 Colon Carcinoma. To assess the antitumor efficiency of TDN-FA, TDN-FA/PL1, and TDN-FA/PL1/Pcsk9-siRNA nanomedicine or PBS via tail vein injection every 2 days (10 cycles), the model of CT26 colon carcinoma in BABL/C mice was used. When the tumor’s long axis reached $\sim$5 mm, the mice were then administered an intravenous injection of PBS, TDN-FA, TDN-FA/PL1, and TDN-FA/PL1/Pcsk9-siRNA every 2 days (Figure 5A). The mice were then euthanized for antitumor evaluation, and the samples were collected for pathological assessments. The result demonstrated that the tumor volumes in TDN-FA/PL1 and TDN-FA/PL1/Pcsk9-siRNA groups were much smaller than those in either the control or the TDN-FA group. The tumor growth inhibition rates of TDN-FA/PL1 and TDN-FA/PL1/Pcsk9-siRNA groups were 93.5 and 83.48%, respectively (Figures 5B,C and S2). Furthermore, we detected proliferation marker Ki-67 levels and apoptosis indicators in tumor tissues by immunohistochemistry staining and TUNEL staining, respectively. We found that the Ki-67 positive index decreased in the TDN-FA/PL1/Pcsk9-siRNA group and the TDN-FA/PL1 group compared with the control and TDN-FA groups; moreover, we found that the Ki-67 positive index in the TDN-FA/PL1/Pcsk9-siRNA group was even lower than Ki-67 in the TDN-FA/PL1 group (Figure S3). In addition, TUNEL staining revealed that the positive signals were much higher in the TDN-FA/PL1/Pcsk9-siRNA group than in other groups (Figure S4). These results suggest that aptamer PL1 and Pcsk9-siRNA cofunctional TDN-FA have a remarkable antitumor effect on CRC in vivo.

To further examine the security of TDN nanoassembly in mice bearing colon carcinoma, the average weight of mice in the TDN nanoassembly treatment group did not show a significant reduction compared with the control group (Figure 5D).
In addition, serum and major organs were harvested from all mice in the experiment. The tissues from organs were sliced and stained using H&E to evaluate the histologic morphology changes. The concentrations of serum BUN, CRE, and UA, which are biomarkers for evaluating kidney function in the treatment groups, were similar to those in the control group (Figure 5E). Meanwhile, no remarkable concentration variation of serum AKP, ALT, and AST, which are indicators of liver function, was observed between the treatment and control groups (Figure 5F). The histologic morphology of the liver, heart, lung, kidney, and spleen in the treatment groups was identical to the control group (Figure 5G). The result suggests that TDN nanoassembly treatment has no toxicity in vivo.

To further assess the tumor targeting of TDN nanoparticles mediated by FA in vivo, Cy7-labeled TDN, Cy7-labeled TDN-FA, Cy7-labeled TDN-FA/PL1, or TDN-FA/PL1/Pcsk9-siRNA was intravenously administered to the BABL/C mice bearing CT26 colon carcinoma. The tumor was monitored by noninvasive NIR fluorescence imaging. The result showed that Cy7-labeled TDN-FA nanoparticles were much more than that of non-FA modified TDN nanoparticles at 1 h after injection (Figure 5H). The data suggest that FA/FAR could effectively contribute to targeting tumor therapy.

Taken together, our results clearly demonstrated that TDN-FA/PL1/Pcsk9-siRNA therapy in mice model bearing CT26 colon carcinoma had remarkable efficiency and safety. In addition, DNA nanoparticles showed favorable accumulation in tumors, which improves the bioavailability of anticancer drugs.

3.5. TDN-FA/PL1/Pcsk9-siRNA Induces Intratumoral Infiltration of CD8⁺ Cells by Upregulating MHC1.

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Figure 6. Characteristics of gene silencing and IFN-γ and Granzyme expression in tumors. (A) Pcsk9, IFN-γ, and Granzyme mRNA expression in tumors detected by qRT-PCR. (B) PCSK9 and MHC1 protein expression in tumors detected by the Western blotting assay. (C) IFN-γ (top) and Granzyme B (bottom) expression in tumors of different treated groups by IHC (scale bar: 50 μm).
the mechanism of TDN/PL1/Pcsk9-siRNA in CRC therapy, our results manifested that MHC1 protein expression was much more in CT26 tumors treated with TDN-FA/PL1/Pcsk9-siRNA compared with the other groups by western blotting analysis (Figure 6A). To further verify if the activation of T cells took part in the suppressive effect of TDN-FA/PL1/Pcsk9-siRNA on tumors, we examined the Granzyme B and IFN-γ levels in tumor tissues from mice bearing CT26. As shown in Figure 6B,C, the qPCR and IHC identified that IFN-γ expression in the TDN-FA/PL1 and TDN-FA/PL1/Pcsk9-siRNA groups was much higher than that in the control group and the TDN-FA alone group, respectively. The data indicate that DNA aptamer PL1 packed on the TDN nanoparticle might be the main factor contributing to tumor growth inhibition by reactivating T cells, which mainly secrete IFN-γ. Similarly, the qPCR and IHC showed that Granzyme B expression in both TDN-FA/PL1 and TDN-FA/PL1/Pcsk9-siRNA groups was much higher than that in the control group and the TDN-FA alone group, respectively. Moreover, Granzyme B expression was higher in the TDN-FA/PL1/Pcsk9-siRNA treatment group than that in the TDN-FA/PL1 treatment group, which indicates that both aptamer PL1 and Pcsk9 siRNA packed on the TDN backbone could contribute to the increased Granzyme B expression. It is reasonable that the TDN-FA/PL1-treated tumors also acquired the cytotoxic activity marked by increased Granzyme B expression. The underlying mechanism needs further study.

Taken together, our results suggest that TDN-FA/PL1/Pcsk9 siRNA dissociated and released the Pcsk9 siRNA in CRC tumor cells by FA/FAR recognition, thereby reducing PCSK9-mediated MHC I degradation, inducing lymphocytic infiltration. Meanwhile, TDN-FA/PL1/Pcsk9 siRNA delivered aptamer PL1, restoring the T cell function from the inhibition of the PD-1/PD-L1 axis. The schematic representation of antitumor therapy of TDN-FA/PL1/Pcsk9 siRNA is shown in Figure 7.

4. CONCLUSIONS
In conclusion, we successfully fabricated the multifunctional DNA tetrahedron nanoparticle PL1 and tumor promoter gene Pcsk9 siRNA for colon carcinoma therapy. In vitro study showed that the constructed TDN-FA/PL1/Pcsk9-siRNA could not only reactivate T cells inhibited by PD-L1 but also effectively silence the Pcsk9 gene and protein expression. Importantly, in vivo experiment demonstrated that the multimodal DNA tetrahedron nanoassembly exhibited tumor accumulation and surprisingly excellent targeting therapeutic efficacy for mice bearing CT26 colon carcinoma without toxic side effects after 10 times intravenous injection. It is reasonable that the exciting therapeutic effects of DNA nanoassembly in CT26 colon carcinoma will open up novel avenues for the applications of DNA nanotechnology in various cancers.

ASSOCIATED CONTENT

Supporting Information

Sequences of main components in self-assembly multifunctional TDN-FA/PL1/Pcsk9 siRNA (Table S1); morphology of CT26 and MC38 cells with different treatments (Figure S1); tumor volume of the end time from the different treatment groups in vivo (Figure S2); and Ki-67 expression (Figure S3) by immunohistochemistry staining and TUNEL staining (Figure S4) in tumor tissues from different treatment groups (PDF)

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**Notes**

The authors declare no competing financial interest.

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