Peptide nanotube loaded with a STING agonist, c-di-GMP, enhance cancer immunotherapy against melanoma

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ABSTRACT

The activation of the stimulating factor of the interferon gene (STING) pathway can enhance the immune response within the tumor. Cyclic diguanylate monophosphate (c-di-GMP) is a negatively charged, hydrophilic STING agonist, however, its effectiveness is limited due to the poor membrane permeability and low bioavailability. Herein, we introduced KL-7 peptide derived from Aβ amyloid fibrils that can self-assemble to form nanotubes to load and deliver c-di-GMP, which significantly enhanced c-di-GMP’s effectiveness and then exhibited a robust “in situ immunity” to kill melanoma cells. KL-7 peptide nanotube, also called PNT, was loaded with negatively charged c-di-GMP via electrostatic interaction, which prepared a nanocomposite named c-di-GMP-PNT. Treatment of RAW 264.7 cells (leukemia cells in mouse macrophage) with c-di-GMP-PNT markedly stimulated the secretion of IL-6 and INF-β along with phospho-STING (Ser365) protein expression, indicating the activation of the STING pathway. In the unilateral flank B16-F10 (murine melanoma cells) tumor-bearing mouse model, compared to PNT and c-di-GMP, c-di-GMP-PNT can promote the expression of INF-β, TNF-α, IL-6, and IL-1β. At the same time, up-regulated CD4 and CD8 active T cells kill tumors and enhance the immune response in tumor tissues, resulting in significant inhibition of tumor growth in tumor-bearing mice. More importantly, in a bilateral flank B16-F10 tumor model, both primary and distant tumor growth can also be significantly inhibited by c-di-GMP-PNT. Moreover, c-di-GMP-PNT demonstrated no obvious biological toxicity on the main organs (heart, liver, spleen, lung, and kidney) and biochemical indexes of mice. In summary, our study provides a strategy to overcome the barriers of free c-di-GMP in the tumor microenvironment and c-di-GMP-PNT may be an attractive nanomaterial for anti-tumor immunity.

KEYWORDS
cyclic diguanylate monophosphate, stimulating factor of the interferon gene (STING), peptide nanotubes, in situ immunity, tumor immunotherapy

1 Introduction

Cancer remains one of the leading causes of death in the world. Surgical resection, radiotherapy, and chemotherapy are still the traditional methods of treating tumors, but most of these methods lead to adverse reactions [1]. As an alternative to traditional treatment, immunotherapy is to inhibit and kill tumor cells by mobilizing the body’s immune system and enhancing the anti-tumor immunity of the tumor microenvironment [2]. Cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS) is a DNA sensor. It triggers innate immune responses through producing the second messenger cyclic GMP-AMP (cGAMP), which binds and activates the adaptor protein stimulator of the interferon gene (STING). Recent studies have shown that the innate immune cyclic GMP-AMP synthase-STING (cGAS-STING) pathway may play an important role in anti-tumor immunity [3–7]. It was reported that the STING-deficient mice have a higher susceptibility to tumor formation, diminished anti-tumor T cell immunity, and decreased immunotherapeutic effects [8]. Previous studies have demonstrated that activation of the STING signaling pathway promotes the production of type I interferons (IFNs) and other proinflammatory cytokines, thereby activating DCs, NK cells, and CD8+ T cells in tumor-bearing mice to enhance anti-tumor effects [9, 10]. In addition, the delivery of exogenous cyclic dinucleotide agonists of the STING pathway is a promising type of immunotherapy, which can activate innate immunity to augment tumor immunogenicity [11].

Cyclic dinucleotides (CDNs), such as c-di-GMP [12], c-di-AMP [13], and cGAMP [14, 15] are natural agonists of STING. They have been proved to be important pathogen-associated molecular
patterns (PAMP) molecules [16] to stimulate anti-tumor response to inhibit tumor progression. Specifically, when the STING binds to CDNs, STING transfers from the endoplasmic reticulum to the Golgi apparatus and is followed by the recruitment of protein kinase (TBK1). The nuclear factor κB (NF-κB) and the transcription factor interferon regulatory factor 3 (IRF3) [17, 18] are further activated and lead to the transcription of type I interferons and other proinflammatory cytokines. The STING pathway mediates type I IFN, can stimulate antigen-presenting cells (APC) and promote the infiltration of tumor-infiltrating lymphocytes (TIL) in the tumor microenvironment (TME) to kill the tumor cells [19–21]. Therefore, CDNs could serve as a promising potent adjuvant to trigger the STING pathway bridging the innate and adaptive immunity, resulting in the success of tumor immunotherapy. However, the deficiencies of CDNs such as electronegativity, hydrophilicity, and instability hinder further application in anti-tumor immunity. The phosphate group on the CDNs limits its ability to enter the cytoplasm and bind to STING [22, 23]. Phosphodiesterase degrades CDNs, resulting in a low biological half-life and thus low bioavailability [24]. In addition, since STING agonists inaccurately target tumor tissue [23], this may cause systemic inflammation or strong auto-immunity [25].

To overcome CDNs’ weakness, nano-drug delivery systems (NDDSs) provide a new chance to improve their immune activity and bioavailability. Wang’s team [25] used iRGD (a tumor-penetrating peptide that can bind to neuropilin-1 (NRP-1) and trigger tumor tissue penetration) to covalently bind camptothecin (CPT) to form amphiphile peptide nanotube hydrogels to deliver c-di-AMP to promote immune-stimulated TME, induce tumor regression of breast cancer (4T1), and prevent tumor recurrence and metastasis. Hanson et al. [26] designed polyethylene glycol (PEG)-encapsulated lipid nanoparticles for c-di-GMP in combination with HIVgp1 polyethylene vaccine, which promoted c-di-GMP accumulation in lymph nodes, enhanced CD8+ T cell response, and enhanced anti-tumor immunity. Chen [27] synthesized mesoporous silica nanoparticles with Rhodamine B isothiocyanate (RITC) for in vivo fluorescent labeling. It bounds c-di-GMP, which has a significant therapeutic effect on mice with 4T1 transplanted tumors. Wu et al. [28] design a supramolecular cytosolic delivery system based on controllable recognition of calixarene, namely CALixarene-STING (CASTING), to improve CDN druggability. CASTING efficiently enhances the immunostimulatory potency of modified cyclic di-GMP to generate an immunogenic microenvironment for melanoma regression, anti-PD-1 response rate increase, and durable memory formation against tumor recurrence.

With the development of molecular self-assembly, an increasing number of inorganic materials (e.g., carbon nanotubes) and low-toxic organic nanotubes (e.g., peptide nanotubes) have been synthesized. Compared with nanostructures in other morphologies (e.g., nanospheres, nanocubes, and liposomes), nanotubes as drug nanocarriers have been reported to have a higher aspect ratio that can prolong blood circulation and retention time in tumors, nanotubes particles have a larger surface area in contact with target cells and therefore adhere better to cells and are internalized more efficiently, demonstrating improved transport and delivery throughout the body [29–33]. This enhanced surface area of nanotubes improves drug loading efficiency meaning more drugs can reach target sites at a lower therapeutically administered dose. This should improve drug efficacy at lower concentrations and reduces the likelihood of drug-induced side effects at equivalent doses [34]. All these features promote nanotubes as ideal nanocarriers for tumor intracellular imaging, drug delivery, and cancer therapy. Lai reported that peptide nanotube-templateed biominalization of Cu2S nanoparticles for the combination treatment of metastatic tumors [35]. Hilbich et al. [36–38] found that theAc-KLVFFAL-NH2 (KL-7) peptide derived from Aβ amyloid fibrils can self-assemble to form nanotubes at pH 7.0 through antiparallel β-folding through electrostatic, π–π stacking, and hydrophobic interaction [39–41]. Therefore, we hypothesize that using KL-7 peptide nanotube, also called PNT, to load and deliver c-di-GMP can significantly overcome the weakness of CDNs and enhanced its effectiveness resulting in a robust “in situ immunity” to kill melanoma cells. The nanocomposite of c-di-GMP loaded in PNT, called c-di-GMP-PNT, was assembled and characterized. The encapsulation efficiency and release profile of c-di-GMP were determined by ultraviolet absorption spectrophotometer (UV-2450) and high-performance liquid chromatography (HPLC). The immune response triggered by c-di-GMP-PNT was investigated. After its injection in murine melanoma cells (B16-F10) tumor xenografts mice, anti-tumor activity was explored and confirmed. More importantly, in a bilateral flank B16-F10 tumor model, both primary and distal tumor growth can also be significantly inhibited by c-di-GMP-PNT. We use c-di-GMP-PNT as an efficient adjuvant system to trigger immune responses, followed by the inhibition of tumor growth.

2 Experimental

2.1 Materials

Hexafluoroisopropanol (HFIP) was purchased from Shanghai Myrell Chemical Technology Co., Ltd. (purity > 98%). Thiazolyl blue tetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) staining solution were purchased from Solarbio (Beijing, China). Mouse interleukin 6 (IL-6) ELISA kit (E-EL-M0033C) was purchased from Wuhan Elabscience Biotechnology Co., Ltd. α-Tubulin and STING (Catalog number: 19851-1-AP) were purchased from Wuhan Sanying Biotechnology Co., Ltd. p-STING (Catalog number: #72971s) was purchased from Cell Signaling Technology (USA). ECL (Catalog number: P0018AS) was purchased from Shanghai Beyotime Biotechnology Co., Ltd. Alanine aminotransferase (ALT) assay kit, aspartate aminotransferase (AST) assay kit, blood urea nitrogen (BUN) assay kit, and creatinine (CRE) assay kit were purchased from Jiancheng Biological Co., Ltd.

2.2 Cell cultures and animals

B16-F10 cells and leukemia cells in mouse macrophase (RAW 264.7) were purchased from ATCC. Cells were cultured in RPMI 1640 medium (Gibco) or Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing penicillin/streptomycin, 10% fetal bovine serum (FBS, Gibco), and glutamine at 37 °C with 5% CO2. Healthy male BALB/c mice (18–22 g, 4–6 weeks) were purchased from Slac & Jingda Corporation of laboratory animals, Changsha, China. All animals were acclimatized for 3 days before being used. Adequate water and food (standard laboratory meals) were provided throughout the study unless otherwise stated. All animal procedures were approved by the Institution Animal Care and Use Committee of Hunan Normal University (Ethics Approval No.: HUNNU-IACUC-2018034).

2.3 Synthesis of KL-7 peptide

KL-7 was synthesized by 9-fluorenyl-methoxycarbonyl (FOMC) solid-phase peptide method [42] and purified by RP-HPLC. After the completion of the peptide chain, we removed the FOMC group at the N-terminal, added V_acetic anhydride-V_DMSO-V_DIAH = 5:50:1, and sealed the reaction for 20 min. The mixed solution of trifluoroacetic acid/phenylenedimide/dimercaptoethane/
phenylene sulfide (90%/5%/3%/2% (v/v)) was used for polypeptide pyrolysis. After reacting at room temperature for 2 h, the pyrolysis solution was precipitated with 40 mL glacial ethyl ether (~20 °C), centrifuged at 4 °C, and centrifuged at 4,000 rpm to obtain KL-7 crude product. KL-7 was purified by RP-HPLC (Milford, MA, USA) (column, C18, 300 Å, 10 mm × 250 mm, Welch Materials, Inc.) via a 0.1% TFA/acetonitrile gradient (TFA: trifluoroacetic acid) (0%–60% for 35 min) at a flow rate of 3 mL/min. A 0.5 μL sample was placed on the 384-well mass spectrometry point sample, and the same volume of matrix solution (containing α-cyano-4-hydroxycinnamic acid (CCA), 50% ACN, and 0.1% TFA) was added. Then, at 25 kV acceleration voltage, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (AB, SCIEX) was used for analysis and detection.

2.4 Cell toxicity assay
In short, RAW 264.7 and B16-F10 cells were incubated with DMEM and 1640 medium (PS+, FBS−) containing different concentrations of c-di-GMP, PNT, and c-di-GMP-PNT, at 37 °C and in 5% CO2 incubator for 24 h. Then we added 10 μL MTI (0.5 mg/mL) solution to each well, then added 100 μL dimethyl sulfoxide (DMSO) solution, and incubated at 37 °C for 4 h. The absorbance was measured at 490 nm by SpectraMax i3x Multi-Mode Microplate Reader.

2.5 Preparation of PNT
KL-7 (15.6 mg, 0.018 mmol) was added with 2 mL hexafluorosopropanol (HFIP), then oscillated in a vortex oscillator for 2 min, ultrasonicated for 30 s, and cooled in the ice bath for 30 min. Then, HFIP was removed in the atmosphere of nitrogen to obtain a polypeptide film attached to the wall of the centrifugation tube. The polypeptide membrane was dissolved in 0.1% acetonitrile aqueous solution (VHFIP/V2O3 = 2:3), and the pH of the polypeptide membrane solution was adjusted to 7.0 with 200 mM NaOH. After incubation for 24 h in a constant temperature water bath at 37 °C, the PNT were prepared with a concentration of 3.5 mM.

2.6 Preparation of c-di-GMP-PNT complexe
c-di-GMP-PNT was combined from c-di-GMP and PNT in the molar ratio of 1:1.4, incubated for 24 h in a constant temperature water bath at 37 °C, and then dispersed by ultrasonication for 2 h to obtain the aqueous solution of c-di-GMP-PNT complex.

2.7 Fluid dynamics dimensions and zeta potential
The solution of PNT and c-di-GMP-PNT were diluted to 500 μM with MQ, and the final volume was 1 mL. The average size, polydispersity index (PDI) distribution, and zeta potential of PNT and c-di-GMP-PNT were measured by a dynamic light scattering system (Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK) at 25 °C with two measurements per set and one measurement set to three.

2.8 Transmission electron microscopy (TEM)
The morphology and structure of PNT and c-di-GMP-PNT were observed by TEM (Tecnai G20 F20, American). 0.5 μL of each sample was dropped onto a copper wire coated with a FormaVar/carbon supporting film, and the excess solution was absorbed with a filter paper. The copper grid was then covered with a drop of 2% uranyl acetate aqueous solution. The sample was air-dried and photographed.

2.9 Determination of encapsulation rate
The prepared c-di-GMP-PNT solution was placed in a high-speed centrifuge, centrifuged at a speed of 4 °C, 10,000 rpm for 20 min, and the supernatant was collected and detected by HPLC (Milford, MA, USA) (column, C18, 300 Å, 10 mm × 250 mm, Welch Materials, Inc.), the peak area was recorded and repeated three times, and the average value was taken. We substituted it into the standard curve to calculate the content of c-di-GMP, and calculated the encapsulation rate according to the following formula

\[
\text{Encapsulation rate} = \frac{\text{Quality of free c-di-GMP}}{\text{Total quality of c-di-GMP}} \times 100\%
\]

where C0 is the concentration of c-di-GMP in the dialysate at the n-th sampling, μg/mL; V is the volume of dialysate, mL; C is the concentration of c-di-GMP in the dialysate at the n-th sampling, μg/mL; V is the volume of dialysate taken out each time, mL; C0 is the concentration of c-di-GMP in the drug-loaded micelles, μg/mL; and V is the volume of loaded c-di-GMP micelles, mL.

2.10 Drug release
The c-di-GMP-PNT were placed in a 3,500 Da dialysis bag and sealed with a dialysis clip. Then put them into a phosphate buffered solution (PBS) (80 mL) with pH 7.4 and stirred slowly with a magnetic stirrer (37 °C, 120 rpm). In a certain time interval (0, 0.5, 1, 1.5, 2, ..., 24 h), we took 1 mL of PBS dialysate solution, and added 1 mL of fresh PBS solution. Then the absorbance at 255 nm was measured on ultraviolet-visible (UV–vis) spectrophotometry (Unocal 4802), and calculated the cumulative release rate according to the formula

\[
\text{Cumulative release rate} = \frac{C_0 V + \sum_{i=1}^{n} C_i V_i}{C_0 V} \times 100\%
\]

where C0 is the concentration of c-di-GMP in the dialysate at the n-th sampling, μg/mL; V is the total volume of dialysate, mL; C0 is the concentration of c-di-GMP in the dialysate at the n-th sampling, μg/mL; V is the volume of dialysate taken out each time, mL; C0 is the concentration of c-di-GMP in the drug-loaded micelles, μg/mL; and V is the volume of loaded c-di-GMP micelles, mL.

2.11 Cell uptake
The RAW 264.7 cells were incubated with c-di-GMP-DY547 (3.44 μM), c-di-GMP-DY547-PNT (3.44 μM c-di-GMP-DY547) at 37 °C, and in 5% CO2 incubator for 1, 2, and 12 h, and then washed three times with PBS. 400 μL of 4% paraformaldehyde solution was added to each confocal dish. After fixing the cells for 20 min, the cells were washed three times with PBS. The nuclei were stained with DAPI (diluted 1:100 with PBS) for 15 min and then washed lightly with PBS three times. Finally, observations and imaging were performed using a laser confocal fluorescence microscope (Leica SP8, Germany). DAPI emitted light blue fluorescence (excitation: 340 nm, emission: 488 nm) and c-di-GMP-DY547 emitted red fluorescence (excitation: 557 nm, emission: 574 nm).

The RAW 264.7 cells were plated into a 6 well plate and were incubated with c-di-GMP-DY547 (3.44 μM), c-di-GMP-DY547-PNT (3.44 μM c-di-GMP-DY547) at 37 °C and in 5% CO2 incubator for 1 h. Then, cells were washed three times with PBS solution to remove free c-di-GMP-DY547 or c-di-GMP-DY547-PNT. All cells of each well were harvested and resuspended with the 500 mL PBS solution for flow cytometry analyses.

2.12 Inflammatory cytokines were detected by ELISA kit
RAW 264.7 cells were inoculated into 6-well plates and each well was added with 1,200 μL DMEM medium (PS+, FBS+) containing c-di-GMP, PNT, and c-di-GMP-PNT, respectively. The control group was added with DMEM medium containing fresh serum without drugs. And then placed it in a constant temperature incubator at 37 °C with 5% CO2 for 6, 12, and 24 h. At the end of time, the cell supernatant was collected. Finally, the contents of IL-6 and INF-β in the cell supernatant were determined according to
the procedures of the Elabscience ELISA kit (E-EL-M0033C).

2.13 Western blot
RAW 264.7 cells (2 × 10^6) were seeded on 6-well plates and incubated for 6, 12, and 24 h at 37 °C. Cells were then washed three times with PBS, scraped into 2×sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and boiled at 100 °C for 5 min. Whole-cell lysates were resolved by 10% SDS-PAGE (80 V for 30 min and then 120 V for 1 h) followed by electron transfer to a polyvinylidene difluoride membrane (PVDF, 200 mA for 1.5 h). Membranes were blocked using 5% (w/v) skim milk in TBST (PBS/0.1% Tween 20) and then incubated with STING (1:2,000), p-STING (1:1,000), or α-tubulin (1:5,000) primary antibodies overnight at 4 °C. On the following day, after washing three times with TBST, the membrane was incubated with secondary antibodies (1:10,000) in a fresh 5% milk blocking solution for 1.5 h. Then cleaned and transferred to ECL solution, and scanned with a chemiluminescence imaging system (Tanon 5200) to obtain a developed image.

2.14 Tumor model and treatment
A total of 2 × 10^4 B16-F10 cells (200 μL) were injected subcutaneously into the right-back of BALB/c mice. When the tumor volume reached 100 mm^3 (day 0), the mice were randomly divided into the normal saline group, c-di-GMP group, PNT group, and c-di-GMP-PNT group. On the 4th, 6th, and 8th day, mice were treated with 60 μL of saline, 60 μL of free c-di-GMP (1, 5, and 10 μg per mouse), and 60 μL of c-di-GMP-PNT (equivalent to 1, 5, and 10 μg c-di-GMP per mouse) via intratumoral injection, respectively. The tumor sizes were tracked and the mice were sacrificed on day 13 post-tumor inoculation. Blood was collected from the eyeballs of each group, centrifugation (4 °C, 16,000 rpm) was conducted for 30 min, and serum was collected. The tumor tissue and main organs (heart, liver, spleen, lung, and kidney) were dissected and fixed in 4% paraformaldehyde.

B16-F10 cells (2 × 10^6 cells, 200 μL) were subcutaneously inoculated into the right flank of BALB/c mice. When the tumor volume grew to 100 mm^3 (day 0), the mice were randomly divided into the saline group, c-di-GMP group, PNT group, and c-di-GMP-PNT group. On the 4th, 6th, and 8th day, each group was injected with 60 μL of saline, 60 μL of PNT, 60 μL of free c-di-GMP (10 μg per mouse), and 60 μL of c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse). Survival rate and tumor growth were monitored every day in all groups. Tumor volume: 0.5 × length × width. Mice were euthanized when the tumor volume reached 1,500 mm^3.

2.15 In situ and distal tumor-bearing mouse models and treatment
The bilateral B16-F10 tumors were cultured on the left and right sides of the mice, and the tumors that were administered were named primary tumors, and the tumors that were not administered were named distal tumors. 2 × 10^6 B16-F10 cells (200 μL) were injected subcutaneously into the right-back of BALB/c mice, and 2 × 10^6 B16-F10 cells (100 μL) were injected subcutaneously into the left-back of BALB/c mice for tumor. When the volume grew to 60–80 mm^3 (day 0), the mice were randomly divided into the saline group, c-di-GMP group, PNT group, and c-di-GMP-PNT group. On the 4th, 6th, 8th, and 10th day, each group was injected with 60 μL of saline, 60 μL of PNT, 60 μL of free c-di-GMP (10 μg per mouse), and 60 μL of c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse). Then, the tumor volume and the body weight of the mice were monitored daily. On the 18th day, the mice were sacrificed, and the tumor tissue and main organs (heart, liver, spleen, lung, and kidney) were dissected and fixed in 4% paraformaldehyde.

2.16 Immunofluorescence staining assay
Tumor tissue sections were blocked with 3% bovine serum albumin solution for 1 h at room temperature, then deparaffinized and antigen retrieval was performed. Antibodies, Alexa-Floor 467 anti-IL-6 (Beijing BIOSS Company), Alexa-Floor 488 anti-IL-1β (Biologent Company, USA), Anti-TNF-α (Servicebio Company, China), Anti-CD4 (Servicebio Company, China), and Anti-CD8 (Servicebio Company, China) were incubated at 4 °C, and images were acquired using CaseViewer.

2.17 Biosafety assessment
On the 13th day, when the tumor volume of mice in the saline group was close to 2,000 mm^3, blood was collected from the eyes of each group, centrifugation was conducted for 10 min, and serum was collected. According to the kit operation instructions, ALT, AST, BUN, and CRE were detected. The main organs (heart, liver, spleen, lung, and kidney) were dissected and fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining to assess toxicity.

2.18 Statistical analysis
Data were expressed as mean ± standard deviation by Student’s test or one-way analysis of variance (ANOVA). p-values less than 0.05 were considered statistically significant (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

3 Results and discussion
3.1 Preparation and characterization of c-di-GMP-PNT
The amphiphilic KL-7 peptide was synthesized using the standard FMOC solid-phase peptide synthesis method [39]. KL-7 was purified by C18 RP-HPLC and determined by MALDI-TOF MS. The results indicated that KL-7 has high purity (Fig. S1(a) in the Electronic Supplementary Material (ESM)) and the average molecular weight of KL-7 is 878.1 Da which is consistent with the calculation (Fig S1(b) in the ESM). According to the reported procedures [39], as shown in Fig. 1(a), KL-7 was self-assembled into PNT in an acetonitrile aqueous solution (pH = 7.0). The c-di-GMP-PNT was assembled by a drug-loading strategy based on the electrostatic interaction between the negatively charged STING agonist c-di-GMP and the positively charged PNT. As shown in Fig. 1(b), the TEM image demonstrated KL-7 peptide exhibited an obvious peptide nanotube structure. The diameter of PNT is about 58.96 ± 3.32 nm, and the length is about 323.71 ± 58.13 nm (Fig. 1(b)). c-di-GMP-PNT also showed a peptide nanotube structure with a diameter of about 57.75 ± 2.99 nm, and a length of about 342.04 ± 89.30 nm (Fig. 1(c)). Due to the exposed −NH₂ of the side chain of lysine, PNT exhibits electropositivity. The surface zeta potential of PNT is about 42.13 ± 1.85 mV, which is suitable for electronegative drug loading. When c-di-GMP was added, the zeta potential shifted from 42.13 ± 1.85 mV of c-di-GMP-PNT, indeed indicating a successful complexation between c-di-GMP and PNT (Fig. 1(d)). In addition, the average hydrodynamic diameters of PNT and c-di-GMP-PNT are 196.01 ± 1.6 and 211.45 ± 2.8 nm, respectively (Fig. 1(e)). The PDI values are 0.215 and 0.212, respectively, which are all less than 0.3, showing that PNT and c-di-GMP-PNT have good dispersibility in water. The encapsulation rate of c-di-GMP was 51.64% determined by HPLC measurement. Then, the cumulative release rate curve was determined by UV–vis absorption (Table S1 in the ESM and Fig. 1(f)). At 37 °C, pH = 7.4,
the cumulative release rates of c-di-GMP were 47.1%, 80.3%, and 98.8% at 7.5 h, 11 h and 23 h, respectively. Taken together, these results exhibited that we successfully prepared c-di-GMP-PNT and can continue to release c-di-GMP.

3.2 PNT increases the immune cytokine secretion of c-di-GMP by STING-dependent signaling pathway

Macrophages are immune effector cells that have a variety of immune functions, including immune defense [43], immune surveillance, and immune regulation, playing an important role in the body’s innate immune system. Activated macrophages can secrete INF-β, IL-6, and other cellular inflammatory factors [44–46], which are helpful to improve the body’s immunity. Therefore, the mouse macrophage cell line RAW 264.7 was selected in this experiment. After RAW 264.7 cells were incubated with different concentrations of PNT and c-di-GMP-PNT for 24 h, no significant cytotoxicity was observed in the range of 0–50 μM (Figs. S2(a)–S2(c) in the ESM). In order to verify the ability of PNT to effectively deliver c-di-GMP into cells, fluorescently labeled c-di-GMP (c-di-GMP-DY547) was loaded into PNT to give c-di-GMP-DY547-PNT and the RAW 264.7 cells were incubated with c-di-GMP-DY547-PNT several hours, and the fluorescence intensity of c-di-GMP-DY547 in the cells was detected at different time points (Fig. 2(a)). The results showed that the fluorescence intensity was time-dependent. The fluorescence intensity of c-di-GMP-PNT treated cells was stronger than that of free c-di-GMP, which indicated PNT can enhance intracellular delivery of c-di-GMP (Fig. 2(b)). Compared with the blank and free c-di-GMP group, the fluorescence intensity of the c-di-GMP-PNT in cells increased at 1 h (Fig. 2(c)). The experiments on cellular uptake and drug release proved that PNT could promote the uptake of c-di-GMP by RAW 264.7 cells, and then, as demonstrated in vitro (Fig. 1(f)), c-di-GMP-PNT could release c-di-GMP continuously and slowly into the cells.

In addition, when the c-di-GMP was transferred into cells, it can activate the STING signaling pathway. To clarify the enhanced ability of c-di-GMP-PNT to activate the STING signaling pathway, we treated RAW 264.7 cells with 15 μM c-di-GMP and c-di-GMP-PNT for 6, 12, and 24 h, and then detected the expression levels of STING protein and the phosphorylation of STING protein. As shown in Fig. 2(d), like the c-di-GMP, c-di-GMP-PNT also can up-regulate the STING protein expression and increase phosphorylation of protein STING. More importantly, Western blot quantitative analysis showed that the phosphorylation expression of c-di-GMP-PNT was significantly higher than that of control, c-di-GMP, and PNT groups at 24 h (Fig. 2(e)). To confirm that the immune response was triggered by c-di-GMP-PNT through the STING-dependent signaling pathway, the immune cytokines were detected in RAW 264.7 cells (Figs. 2(f) and 2(g)). The IL-6 and INF-β factors related to STING significantly increased after treatment by c-di-GMP-PNT, but didn’t show obvious change after treatment by PNT or free c-di-GMP alone. The activation of immune cytokine production of free c-di-GMP is limited due to the inherent poor membrane permeability and instability [24]. Therefore, increased IL-6 and INF-β and up-regulation of p-STING protein were observed in c-di-GMP-PNT-treated RAW 264.7 cells, suggesting that PNT-mediated c-di-GMP delivery is essential for efficient activation of the STING pathway. The above results indicate that PNT can significantly enhance the ability of c-di-GMP to activate the STING pathway, leading to the production of immune cytokines.

3.3 PNT enhances the anti-tumor effect of c-di-GMP in vivo

Due to the enhanced immune activation performance of c-di-GMP-PNT in vitro through the STING signaling pathway, we were wondering if c-di-GMP-PNT could also enhance anti-tumor properties in vivo. Thus, BALB/c mice were injected subcutaneously with about 2 × 10^7 B16-F10 cells to construct the unilateral flank tumor-bearing mouse model. As shown in Fig. 3(a), on the 4th, 6th, and 8th day, normal saline, free c-di-GMP, and c-di-GMP-PNT were injected into the tumor, respectively. The
tumor volume and body weight were monitored and recorded. At the end of the therapeutic experiment, mice were euthanized and tumors were excised for histological examination. Proinflammatory factors IL-6 and IL-1β, T cell indicators CD4 and CD8, interferon factors (INF-β), and tumor necrosis factors (TNF-α) were detected by immunofluorescence, as they are important indicators of immune activation.

Whether c-di-GMP (1 μg per mouse) or c-di-GMP-PNT (equivalent to 1 μg c-di-GMP per mouse) has no significant difference in inhibiting tumor growth (Fig. 3(a) in the ESM). However, compared to the gradually increased tumor volume in the PNT and saline groups, the 5 μg c-di-GMP and c-di-GMP-PNT treatment groups demonstrated significant tumor growth inhibition (Figs. 3(c) and 3(d) in the ESM), and these effects were also observed in the 10 μg c-di-GMP and c-di-GMP-PNT treatment (Figs. 3(b) and 3(c)). More importantly, on day 7, both c-di-GMP and c-di-GMP-PNT (equivalent to 5 and 10 μg c-di-GMP per mouse) started to develop tumor ulcers, where the c-di-GMP-PNT group was most pronounced. The anti-tumor effect was positively correlated with the dose of c-di-GMP or c-di-GMP-PNT. Expectedly, the anti-tumor effect of c-di-GMP-PNT was significantly better than that of c-di-GMP at a dose of both 5 and 10 μg (Fig. 3(c)). The result of tumor weight further confirmed that c-di-GMP and c-di-GMP-PNT performed a significant anti-tumor profile, and c-di-GMP-PNT’s performance is better than c-di-GMP’s (Fig. S3(d) in the ESM and Fig. 3(d)). After 5 and 10 μg drug treatment, there was no significant effect on the weight of the mice indicating that there was no systemic toxicity after the treatment (Fig. S3(e) in the ESM and Fig. 3(e)). TUNEL results showed that after the 10 μg c-di-GMP-PNT treatment, more apoptotic or necrotic cells than those in the saline and c-di-GMP treatment group were observed (Fig. 3(f)). H&E staining analysis showed that after treatment with c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse), nuclear shrinkage and cell-free areas of tumor cells can be clearly observed, indicating that the rate of apoptosis was high (Fig. 3(g)).

As previously reported [10, 46], it has been demonstrated that c-di-GMP in the tumor microenvironment can stimulate innate and adaptive immunity through the STING signaling pathway, thereby inhibiting tumor growth in vivo. Furthermore, it is widely recognized that the internalization of STING agonists in the immune cells can stimulate STING/type I IFN signaling, which is responsible for the cross-presentation and subsequent killing-tumor CD8+ T cell activation [47, 48]. Therefore, the reason for the enhanced anti-tumor effect of c-di-GMP-PNT maybe that PNT increased the ability of c-di-GMP to activate the immune response.
PNT enhances the anti-tumor effect of c-di-GMP in vivo. (a) Schematic diagram of the therapeutic unilateral tumor-bearing mouse model. BALB/c mice were subcutaneously injected with 2 × 10^6 B16-F10 cells (n = 5 per group) in the flank at day 0. Intratumoral injections of c-di-GMP (10 μg per mouse) and c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse), were started on days 4, 6, and 8. All mice were sacrificed on day 13, photograph of the tumors in each group (b) and the average tumor weight (d) were examined. The tumor volume (c) and body weight (e) were monitored during treatments. (f) TUNEL and (g) H&E staining of tumor sections. (h)–(m) Immunofluorescence staining of CD4, CD8, TNF-α, INF-γ, IL-6, and IL-1β in tumor sections was exhibited and (n)–(s) quantitative analysis by Image J. Data are shown as mean ± SD (*p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001). (t) Survival curves of B16-F10-bearing mice subcutaneously injected with saline, PNT, free c-di-GMP, and c-di-GMP-PNT.

To confirm this hypothesis, CD4, CD8, INF-γ, TNF-α, IL-6, and IL-1β were detected in tumors, as they are important indicators of immune activation. Most importantly, the expression of CD4, CD8, INF-γ, TNF-α, IL-6, and IL-1β factors in tumor tissues was detected by immunofluorescence. As shown in Figs. 3(h)–3(m), compared with the saline and c-di-GMP groups, enhanced fluorescence intensity of INF-γ, TNF-α, IL-6, IL-1β, CD4, and CD8 was observed in the c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse) group. This result was further confirmed quantitatively analyzed by Image J. As shown in Figs. 3(n)–3(s), the free c-di-GMP had small amounts of INF-γ, TNF-α, IL-6, and IL-1β expressed in tumor tissues. c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse) significantly up-regulated INF-γ, TNF-α, IL-6, and IL-1β, suggesting that PNT can increase the level of c-di-GMP production of these factors and enhance the anti-tumor immune response. More interestingly, compared to saline and free c-di-GMP, the expression of c-di-GMP-PNT (equivalent to 10 μg c-di-GMP per mouse) was significantly increased in CD4 and CD8.

We calculate the survival rate of mice. As illustrated in Fig. 3(t), the saline group and PNT groups died within 30 days. In contrast, the c-di-GMP and c-di-GMP-PNT treatment groups demonstrated significant tumor growth inhibition, and 100% of them survived longer than 50 days. More importantly, on day 7, both c-di-GMP and c-di-GMP-PNT started to develop tumor ulcers, where the c-di-GMP-PNT group was most pronounced. Notably, the c-di-GMP-PNT group displayed the smallest tumor volume and survived without evidence of residual tumor burden.

Taken together, these results demonstrated that both the c-di-GMP and the c-di-GMP-PNT can activate the mice’s immunity to inhibit tumor growth, which was confirmed by the increased proinflammatory cytokines production, interferon factors, tumor necrosis factors, and up-regulate T cells. Furthermore, the enhanced anti-tumor performance of c-di-GMP-PNT may be...
attributed to the fact that PNT can effectively deliver c-di-GMP, which may activate STING more effectively, produce more cell inflammatory factors, and then improve the anti-tumor activity of c-di-GMP.

3.4 In vivo, the systemic antitumor immune response to c-di-GMP is enhanced by PNT

To further investigate the c-di-GMP-PNT induced systemic immune response to inhibit the distal tumor growth, the bilateral B16-F10 tumor-bearing mouse model was constructed. As shown in Fig. 4(a), the bilateral B16-F10 tumors were cultured at the left and right flanks of mice and renamed as primary and distal tumors, respectively. On the 4th, 6th, and 8th day, saline, PNT, free c-di-GMP (10 μg per mouse), and c-di-GMP-PNT (10 μg c-di-GMP per mouse) were injected into the primary tumors, respectively. The mice’s primary and distal tumor volume and body weight was monitored and recorded. All mice were euthanized and tumors were excised for tissue sections on day 18. H&E and TUNEL staining were used to evaluate the apoptosis and necrosis of tumor cells, and immunofluorescence staining was used to analyze the antitumor activity of immune activation.

As expected, the primary tumor of mice treated with saline and PNT rapidly grows. Although there was no significant difference in the volume of the tumor in situ between c-di-GMP and c-di-GMP-PNT, tumor elimination rate was faster after c-di-GMP-PNT treatment (Fig. 4(c)). More importantly, the distal tumors of mice treated with c-di-GMP-PNT were significantly smaller than those treated with c-di-GMP as described below. The weight of the primary tumors on the last day in the c-di-GMP-PNT group was the lightest (Fig. 4(e)). More interestingly, the distal tumor in the c-di-GMP and c-di-GMP-PNT was also obviously inhibited (Fig. 4(d)). In addition, the anti-distal tumor effect of c-di-GMP-PNT was better than that of c-di-GMP (Figs. 4(b) and 4(f)). No obvious weight losses were observed in all groups after 18 days of treatment, indicating that there was no systemic toxicity to bilateral tumor-bearing mice via c-di-GMP-PNT (Fig. 4(g)). As shown in Figs. 4(h) and 4(i), H&E and TUNEL analysis of the primary and distal tumor also showed apoptotic and necrotic tumor cells in the c-di-GMP-PNT treatment group. In order to confirm the immune-activated anti-tumor effects, immunofluorescence staining analysis of primary and distal tumors treated with c-di-GMP-PNT was performed to examine the expression levels of IFN-β, TNF-α, IL-6, IL-1β, CD4, and CD8.

![Figure 4](image_url)
In primary and distal tumors, the fluorescence intensity of IFN-β, TNF-α, IL-6, IL-1β, CD4, and CD8 was enhanced by c-di-GMP-PNT’s treatment (Figs. S4(a)–S4(d) in the ESM and Figs. 4(j)–4(k)). To further confirm these results, we used Image J to conduct a quantitative analysis of the images. It showed that compared to free c-di-GMP, the expression of IFN-β and TNF-α were observed in c-di-GMP-PNT significantly increased (Figs. S4(e) and S4(f) in the ESM). More importantly, compared with the saline and c-di-GMP groups, the expression of CD4 and CD8 significant upregulation was observed in primary and distal tumors treated with c-di-GMP-PNT (Figs. 4(l) and 4(m)). In conclusion, the significantly enhanced distal tumor growth inhibition of c-di-GMP-PNT probably due to PNT can effectively improve the systemic immune response ability of c-di-GMP to inhibit the distal tumor growth as described in the unilateral flank tumor-bearing mouse model (Fig. 3).

To further evaluate the biosafety of c-di-GMP-PNT in vivo, H&E staining analysis was performed to analyze the heart, liver, spleen, kidney, and lung of tumor-bearing mice after 18 days of treatment. After treatment with c-di-GMP and c-di-GMP-PNT, there was no significant difference in the morphology of the heart, liver, spleen, lung, kidney, and other organs, and there was no histopathological abnormality (Fig S5(a) in the ESM). The ALT and AST values were all within the reference range by blood test analysis, and the results showed that the treatment had no obvious liver toxicity in the mice (Fig S5(b) in the ESM). At the same time, the renal function indicators urea nitrogen and CRE were also within the normal range. In conclusion, c-di-GMP-PNT has good biosafety in vivo and has potential application in tumor therapy.

4 Conclusions

In summary, the c-di-GMP-PNT complex was designed for immune anti-tumor therapy. Thanks to the slender shape and high aspect ratio structure, PNT has the advantages of prolonging blood circulation and good biocompatibility. PNT delivers c-di-GMP, which solves the problem of poor c-di-GMP membrane permeability and promotes more effective STING activation. Importantly, the c-di-GMP-PNT complex can effectively regulate the expression of STING and p-STING proteins, and promote the secretion of IL-6 proinflammatory cytokines. In the tumor-bearing mouse model, c-di-GMP-PNT can significantly inhibit the growth of tumors in a dose-dependent manner and activate the immune response. More importantly, in situ injections of the c-di-GMP-PNT complex can activate the anti-tumor immune response and significantly inhibit the growth of tumors in situ and distal tumors. This proves that PNT can enhance the therapeutic efficacy of c-di-GMP. Our strategy highlights the advantage that facilely being constructed by PNT with c-di-GMP via electrostatic interactions and anti-tumor effect in situ. Therefore, c-di-GMP-PNT is very promising in enhancing cancer immunotherapy.

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Electronic Supplementary Material: Supplementary material (synthesis and characterization of KL-7 peptide; the encapsulation rate and cumulative release rate of c-di-GMP-PNT; cytotoxicity of PNT, c-di-GMP, and c-di-GMP-PNT; anti-tumor effect of c-di-GMP-PNT (equivalent to 1 and 5 μg c-di-GMP per mouse); representative immunofluorescence images; and biosafety analysis) is available in the online version of this article at https://doi.org/10.1007/s12274-022-5102-z.

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