Polymersome-mediated cytosolic delivery of cyclic dinucleotide STING agonist enhances tumor immunotherapy

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Cyclic dinucleotides (CDNs) as stimulator of interferon genes (STING) agonists capable of inducing strong antitumor innate immune response are highly promising for tumor immunotherapy. The efficacy of these CDNs is, however, reduced greatly by their fast clearance, poor cell uptake and inefficient cytosolic transportation. Here, we report that reduction-responsive biodegradable chimaeric polymersomes (CPs) markedly enhance tumor retention and cytosolic delivery of a synthetic CDN, ADU-S100, and bolster STING pathway activation in the tumor microenvironment and tumor draining lymph nodes, giving significantly better tumor repression and survival of B16F10 melanoma-bearing mice compared with free CDN control. The superiority of CPs-mediated CDN delivery is further verified in combination therapy with low-dose fractionated radiation, which brings about clearly stronger and longer-term immunotherapeutic effects and protection against tumor re-challenge. The development of nano-STING agonists that are able to overcome the delivery barriers of CDNs represents an effective strategy to potentiate cancer immunotherapy.

1. Introduction

Immunotherapy has turned out to be an attractive way to cure cancer or defer tumor evolution, characterizing long-term effects [1–3]. In recent years, immune checkpoint blocking (ICB) therapy using CTLA-4 antibodies [4,5], PD-1 antibodies [6–8] or PD-L1 antibodies [9,10] has been approved for the treatment of melanoma, lung cancer, and liver cancer. However, the response rate is only about 20% [11–15]. Many researchers have focused on further improving ICB therapy by combining chemotherapy drugs, stereotactic radiosurgery or other immune checkpoint inhibitors [16–19]. Recently, much attention has focused on stimulator of interferon genes (STING), which is the major innate immune pathway involved in the generation of spontaneous antitumor T cell response. The activation of STING pathway by STING agonists, such as cyclic dinucleotides (CDN) including c-diGMP, c-diAMP, and cGAMP, drives the production of type I IFN and other cytokines, and stimulates the maturation of dendritic cells (DCs) and cross-presentation of tumor antigens for subsequent T cell priming [20,21], leading to effective anti-cancer therapy via intratumoral (i.t.) injection [22–24]. A synthetic CDN, ADU-S100, combining with PD-1 antibody has moved to clinical trials in patients with triple-negative breast cancer or relapsed/refractory melanoma. CDNs activate STING pathway by binding to STING protein on endoplasmic reticulum (ER). However, CDNs are anionic and hydrophilic, which renders fast clearance following intratumoral injection and poor internalization by antigen presenting cells (APCs), leading to low bioavailability and reduced efficacy [25].

Local delivery and nanodelivery systems have been investigated to improve the performance of STING agonist CDNs [26]. Hartgerink et al. reported that STINGel implanted subcutaneously could sustain CDN release and achieve enhanced cancer immunotherapy in murine oral tumor model [27], though cell entry problem was still not addressed.

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CDNs via electrostatic interaction have shown to enhance cytosolic delivery. For instance, cationic liposomes loading therapy can release tumor-associated antigens and enhance cytoplasmic delivery of CDNs [31, 32]. Wilson et al. reported that pH-sensitive polymersomes could chaperone cGAMP into tumor cells and greatly enhance STING activation and therapeutic activity via i.t. or i.v. administration [33, 34]. Given STING agonists taking effect in the cytosols, robust and strong local inflammation and tumor cell death [30]. In vivo release was studied using Cy3-diAMP as a model CDN. CPs-Cy3-diAMP prepared as above was transfected into dialysis tubes (MWCO 140 kDa) and dialyzed against 20 mL PB (10 mM, pH 7.4) with or without 10 mM GSH at 37 °C with mild stirring. At preset intervals, 5 mL medium was taken out to quantify the released dinucleotide by fluorometry (n = 3) and 5 mL fresh medium was supplemented.

2.2. The endocytosis and endosomal escape behavior of CPs-CDN in BMDCs

The cellular uptake of CPs-CDN was studied in bone marrow derived dendritic cells (BMDC), bone marrow derived macrophages (BMDM), B16F10 and L929 cells using Cy5 labeled CPs-CDN. In brief, into BMDC (1 × 10⁵ cells/well), BMDM (1 × 10⁵ cells/well), B16F10 (5 × 10⁵ cells/well), or L929 cells (5 × 10⁵ cells/well) in 6-well plates, were added 200 μL Cy5 labeled CPs-CDN (Cy5: 3.2 nM). After 4 h incubation at 37 °C, the cells were trypsinized, centrifuged (1000 rpm, 5 min), washed with PBS and analyzed by flow cytometry.

To study the endosomal escape behavior, BMDCs were cultured in 24-well plate (2 × 10⁵ cells/well) for 24 h. 100 μL CPs-Cy3-diAMP or free Cy3-diAMP (300 nM Cy3 in PB) was added and incubated for 4 h at 37 °C. The culture medium was removed and Lysotracker-deep-red (100 nM) was added to stain endosomes/lysosomes for 1 h. The cells were then fixed with 4% paraformaldehyde for 15 min and washed with PBS, and stained with DAPI for 5 min. The fluorescence images were obtained by using confocal laser scanning microscope (CLSM).

2.3. The maturation, cytokine secretion and protein expression of BMDCs

BMDCs cultured in a 6-well plate (1 × 10⁵ cells/well) were added with CPs-CDN, free CDN (1 μg CDN/mL), empty CPs or PBS. After incubation for 24 h, the cells were treated with mouse antibodies PerCP/Cy5.5- or Cy5-dCD45, FITC-αCD11c, APC-αCD80, and PE-αCD86 for analysis of DC maturation using flow cytometry. The culture medium was collected and centrifuged (3000 rpm, 10 min), and the concentrations of IFN-β and TNF-α were measured with mouse IFN-β and TNF-α ELISA kits.

For Western blot assays, BMDCs cultured in 6-well plate (2 × 10⁵ cells/well) were treated with CPs-CDN, free CDN (200 μL, 1 μg CDN/mL) or PBS for 6 h, and then washed, digested, and lysed using standard procedure before adding antirabbit pIRF3 (1:1000), pTBK1 (1:1000) or GAPDH (1:1000) antibodies followed by secondary antibody goat anti-rabbit IgG (1:2000) (supporting information).
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(a) Long PEG as outer shell
Biodegradable polycarbonate
Disulfide-crosslinks
Short PEI as inner shell
for CDN loading
CDN encapsulated
CPs-CDN

(b) 45-50 nm

(c) Poor cytosolic
delivery
dsDNA

(d) GSH
cGAMP

(e) Type I IFNs

(f) Antigen presenting cell

(g) DC Maturation (%)

(h) IFN-β conc (ng/mL)

(i) TNF-α conc (ng/mL)

(j) pIRF3
pTBK1
GAPDH

(caption on next page)
blood was withdrawn from ophthalmic vein of mice into heparinized tubes, and immediately centrifuged at 5000 rpm for 5 min. 20 μL plasma was taken and incubated at 37 °C overnight with 180 μL acetonitrile, 150 μL H2O (1:1, v/v) containing diisobutyltetrathiol (DTT), 20 mM α-cysteine to extract Cy7-AMP. After centrifugation (15000 rpm, 20 min), Cy7 concentration in the plasma was quantified using a microplate reader (Thermo, USA).

2.6. Antitumor therapy in mice bearing B16F10 tumor

To investigate the anti-tumor effect of CPs-CDN, the mice bearing B16F10 tumors (tumor volume of ~50 mm³) were i.t. injected with 50 μL CPs-CDN or free CDN (1.0 mg CDN/kg) on day 0, 3, and 7 using PBS as a control (n = 7). Tumor volume, body weight and survival rate of the mice were recorded. Body weight and tumor size were measured every two days, and relative to their initial values on day 0. Tumor volume was calculated from the formula: V = L*W²/2 (L and W are tumor length and width, respectively). Mice were designated dead when the mice died, body weight loss was over 15%, or tumor volume was over 2000 mm³.

To investigate the anti-tumor effect of local fractionated X-ray combined with CPs-CDN, the mice bearing B16F10 tumors (~50 mm³) were locally radiated with 3 Gy or 5 Gy X-ray on day 0, 3, and 6, followed by i.t. injection of 50 μL CPs-CDN (0.5, 1.0 or 1.5 mg CDN/kg), CDN (1.0 mg/kg), empty CPs or PBS on day 1, 4 and 8 (n = 7).

The cured mice were re-challenged with secondary tumors by subcutaneous injection of 1.5 × 10⁶ B16F10 cells into the left flank on day 176 post initial tumor inoculation using healthy mice as control. Tumor volume was monitored every two days without any additional treatment.

2.7. Analysis of the immune cells and cytokines

The mice bearing B16F10 tumors (~100 mm³) were locally radiated with 3 Gy or 5 Gy X-ray on day 0, 3, and 6, and i.t. injected with 50 μL CPs-CDN, CDN (1.0 mg CDN/kg) or PBS on day 1, 4 and 8 (n = 4). At 6 h after last administration, the mouse blood was collected and plasma concentrations of IFN-β and TNF-α were measured with mouse IFN-β and TNF-α ELISA kits.

At 48 h after last administration, the mice were sacrificed and the tumors and tumor draining lymph nodes (TDLN) were harvested to obtain single cell suspensions in PBS containing 1% FBS. The cells were treated with ACK buffer for 10 min at 4 °C to lyse red blood cells, then stained with corresponding antibodies at 4 °C for 20 min in the dark (10⁶ cells/tube). To prevent cell sinking to the bottom, cell suspensions were gently tapped every 5 min. Mouse antibodies PerCP-Cy5.5-αCD45, FITC-αCD11c, APC-αCD80, and PE-αCD86 were used for DC maturation analysis. Mouse antibodies PerCP-Cy5.5, FITC-αCD11b and APC-αCD206 were used for macrophage phenotype analysis. Mouse antibody APC-αCD3, FITC-αCD8a and PE-αCD4 were used for CD8 T and CD4 T cell labeling. The cells were washed twice with PBS containing 1% FBS before evaluation using flow cytometer.

To study hematolymph and eosin (H&E) staining and immunohistochemistry, at 48 h after last administration one mouse from each group was randomly selected. Tumors and major organs (heart, liver, spleen, lung, kidney) were excised, washed, fixed, embedded, sectioned (5 μm) and stained with H&E for pathological analysis. For immunohistochemical staining of tumor slices, paraffin sections (5 μm) were immune-stained with rabbit αCD8 (1:500; Abcam) or rabbit αCTB antibody (1:200; Abcam) for 1 h at 37 °C, followed with Alexa Fluor 633-conjugated goat anti-rabbit secondary antibody (1:500; Invitrogen). The nuclei were finally stained with DAPI, and fluorescence images were taken by using a fluorescence microscope (Olympus BX41).

2.8. Statistical analysis

Data were presented as the mean ± standard deviation and analyzed using GraphPad Prism. Significant differences among groups were determined by one-way ANOVA with Tukey multiple comparison tests, and survival rate was analyzed by Kaplan-Meier technique with a log-rank test for comparison. *p < 0.05 was considered significant, **p < 0.01, ***p < 0.001 were considered highly significant.

3. Results and discussion

3.1. Preparation of CPs-CDN

CDNs are water-soluble, small and rigid molecules which renders few nanosystems capable of efficiently loading and delivering them to DCs [42,43]. Here, we investigated chimaeric polymersomes (CPs) based on PEG-P(TMC-DTC)-PEI copolymer, which has shown to mediate efficient RNAi therapy of solid tumors [44], as a tool for cytoplasmic delivery of ADU-S100, a CDN STING agonist under clinical trial. PEG-P (TMC-DTC)-PEI was synthesized with Mθ of 5.0 (15.5±2.1) kDa/mole (¹H NMR shown in Fig. S1), from which CPs self-assembled exhibited a unimodal size distribution profile with a hydrodynamic size of 47 nm (Fig. 1b), neutral zeta potential (Table S1), and a spherical and vesicular structure (Fig. 1b). CPs could efficiently load ADU-S100 to afford a CDN loading content of 17.2 wt% (Table S1). The loading efficiency for CDN was 86.0%, which was similar to CPs for siRNA and CpG CDN [37,44,45] and much higher than other nanoparticles and liposomes for CDNs (~45%) [31,34,46]. Notably, CDN-loaded CPs (CPs-CDN) revealed excellent colloidal stability in buffer containing 10% FBS or upon storage for 3 weeks (Fig. 1c). In vitro release studies using Cy3-diAMP as a model CDN showed that ca. 7.4% and 58.9% CDN was released from CPs in 12 h under physiological condition and 10 mM glutathione environment, respectively (Fig. 1d), verifying that CPs can stably load CDN while trigger CDN release under cytosolic reductive condition, similar to previous reports for siRNA and protein therapeutics [35–37].

3.2. Cytoplasmic CDN delivery and STING pathway activation

The STING pathway is closely related to the innate immune system, especially the function of antigen-presenting cells (APCs) [32,47,48]. To activate immune response, STING agonist CDNs have to be delivered to APCs and bind with STING proteins located on endoplasmic reticulum. We studied the cellular uptake of CPs-CDN in BMDC, BMDM, B16F10 and L929 cells by flow cytometry using Cy5 labeled CPs-CDN. Fig. 1e shows that at 4 h incubation, BMDCs endocytosed one to three orders of magnitude more CPs-CDN than B16F10, BMDM and L929 fibroblasts, supporting efficient and preferential internalization of CPs-CDN by DCs. To study the intracellular trafficking behavior of CPs-CDN using CLSM,
we stained the endo/lysosomes with lysotracker. Fig. 1f shows that BMDCs after 4 h incubation with CPs-Cy3-diAMP had much stronger Cy3 fluorescence and higher level of separation from endo/lysosomes than free Cy3-diAMP. Pearson’s coefficient was calculated to be significantly decreased for CPs-Cy3-diAMP compared to free Cy3-diAMP (**p), confirming an efficient internalization and cytosolic release of CDNs.

By activating STING pathway, CDNs could induce production of type I IFN and promote DC maturation [49], which plays an important role in antigen cross-presentation and T cell priming [50,51]. To investigate STING activation ability of CPs-CDN, we incubated BMDCs with CPs-CDN for 24 h and analyzed DC maturation and cytokine release. Fig. 1g shows that empty CPs did not stimulate DC maturation, although PEI was reported as adjuvant for interferon induction and DC maturation [52,53]. This low DC maturation supports that PEI is located inside the polymersomes. Notably, CPs-CDN increased the proportion of mature BMDCs (CD80+CD86+) to 80.5%, which was significantly higher than free CDN (59.3%). IFN-β secretion, as a hallmark of STING activation [54], in free CDN group was 410-fold higher than that in CPs and PBS groups, while CPs-CDN further doubled the IFN-β secretion compared with free CDN (**p) (Fig. 1h). CPs-CDN induced also significantly higher TNF-α production than free CDN (Fig. 1i). We further analyzed pTBK1 and pIRF-3 expression, as STING after binding with CDNs will translocate from ER to Golgi and subsequently recruit TANK-binding kinase 1 (TBK1) and activate IFN regulatory factor 3 (IRF3) via a phosphorylation dependent mechanism [55,56]. Fig. 1j reveals that CPs-CDN drastically promoted both pTBK1 and pIRF-3 expression as compared to PBS group and free CDN, further verifying the activation of STING pathway. These results confirm that CPs-CDN can efficiently deliver CDNs to cytosol of BMDCs, leading to enhanced

Fig. 2. In vivo pharmacokinetics, tumor distribution and antitumor performance of CPs-CDN in B16F10 melanoma-bearing mice. a Plasma concentration of Cy7 after a single i.t. injection of free Cy7-AMP or CPs-Cy7-AMP (10 µg Cy7/mouse, n = 3). b CLSM images of tumor slices at 24 h after single i.t. injection of free Cy3-diAMP or Cy5-CPs-Cy3-diAMP. Scale bars: 20 µm. c Treatment scheme of CPs-CDN against B16F10 melanoma-bearing mice (1 mg CDN/kg). At tumor volume of ca. 50 mm³, the mice were i.t. administered CPs-CDN, CDN, or PBS on day 0, 3 and 7. d Tumor growth curves (n = 7) and individual spider plots. e Survival rates (n = 7). Kaplan-Meier survival analyses with two-tailed Mantel-Cox tests. f Relative body weights (n = 7). Statistical analysis: one-way ANOVA with Tukey’s multiple comparisons. Data are represented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
activation of STING pathway and production of type I IFNs thus promoting DC maturation.

3.3. Antitumor effect of CPs-CDN

The tumor retention and distribution of CPs-CDN after intratumoral (i.t.) administration was investigated in B16F10 melanoma-bearing mice using Cy7-labeled penta-nucleotide (Cy7-AMP) and Cy3-diAMP as CDN models. Fig. 2a shows that free Cy7-AMP quickly diffused into blood circulation from tumor sites yielding a $C_{\text{max}}$ in 5 min. In contrast, Cy7-AMP loaded polymersomes, CPs-Cy7-AMP, revealed ca. 10 times lower Cy7-AMP in blood at 5 min and gradually reached $C_{\text{max}}$ at 30 min post i.t. administration. CPs-Cy7-AMP had over 2 times lower AUC than free Cy7-AMP, supporting that CPs-CDN hinders CDN leakage from the injection site. Free CDNs, even clinically investigating CDNs, suffer from suboptimal cell uptake and bioavailability [33]. To study tumor retention, CLSM images of tumor slices acquired at 24 h post i.t. injection of Cy3-diAMP loaded Cy5-labeled CPs exposed strong and widespread Cy3 and Cy5 fluorescence (Fig. 2b). The merged images showed partial separation of Cy3-diAMP (green) from Cy5-labeled CPs (red). In contrast, weak Cy3 fluorescence was seen in tumor slices from free Cy3-diAMP group. The rapid tumor diffusion and extended tumor retention of CPs-CDN is likely due to its optimal particle size of ca. 50 nm. It was reported that small particles of 20–50 nm could penetrate deep into tumor while nanoparticles of less than 20 nm would be quickly cleared from the body [57,58]. These results concluded that CPs-CDN not only prolongs tumor retention and restrains diffusion into blood, but also possibly enhances CDN delivery to tumor infiltrating immune cells as well as tumor cells in vivo. Barber et al. reported that dying tumor cells that contain STING agonists could also induce effective STING signaling activation in engulfing immune cells like macrophages to trigger immune response, antigens cross-presentation and CTLs generation [59].

The immunotherapeutic effect of CPs-CDN was investigated using ADU-S100 as a therapeutic CDN model in B16F10 melanoma-bearing mice. When tumor volume reached 50 mm$^3$, mice were i.t. administered with CPs-CDN, free CDN, or PBS on day 0, 3 and 7 (Fig. 2c). The results displayed clearly that CPs-CDN induced significantly better tumor inhibition than free CDN though free CDN also effectively retarded tumor growth (Fig. 2d). Notably, the median survival time (MST) of CPs-CDN group was extended to 37 d, which was significantly longer than free CDN and PBS groups (26 and 15 d, respectively) (Fig. 2e). Unlike PBS group which showed rapid increase of body weight in 15 d due to aggressive tumor growth, CPs-CDN group had little increase in body weight within 25 d (Fig. 2f). H&E analyses further displayed no tissue damage and infiltration of inflammatory cells to main organs (Fig. 52). The typical toxicity associated with STING pathway is cytokine storm and will cause systemic inflammatory response in the host, multiple tissue and organ damage, or even death, which were all not seen during CPs-CDN treatment. These results indicate that CPs-CDN elicits potent immunotherapy of melanoma without inducing adverse effects.

3.4. Combination of CPs-CDN with X-ray for melanoma radioimmunotherapy

X-ray radiotherapy (RT) as a critical and auxiliary antitumor therapy is widely used in treating patients [60]. The anti-tumor responses elicited by RT mainly include DNA damage, damage-associated molecular patterns (DAMPs) release, and cGAS/STING activation, leading to innate and adaptive immune response [38–40]. The activation of STING pathway is, however, transient, resulting in temporary anti-tumor effect [41]. Herein, we investigated the combination of CPs-CDN and fractionated low-dose X-ray irradiation for strong and durable radioimmunotherapy of B16F10 melanoma (Fig. 3a). When tumor volume reached 50 mm$^3$, tumors were irradiated with 3 Gy X-ray on day 0, 3 and 6, followed by i.t. administration of CPs-CDN, free CDN, empty CPs or PBS on day 1, 4 and 8 (CDN: 20 μg/mouse). The results showed that 3 Gy

![Fig. 3](image-url)

The combination of CPs-CDN (1 mg CDN/kg) and 3 Gy X-ray for radioimmunotherapy of B16F10 melanoma-bearing mice (n = 7). a Tumor inoculation and treatment scheme. When tumor volume reached 50 mm$^3$, mice were treated by irradiating the tumors with X-ray (3 Gy) on day 0, 3 and 6 following i.t. administration of CPs-CDN, CDN, CPs or PBS on day 1, 4, and 8. b Tumor growth curves and individual spider plots. c Survival rates. Kaplan-Meier survival analysis (two-tailed Mantel-Cox test). d Relative body weights. Statistical analysis: one-way ANOVA with Tukey’s multiple comparisons. Data are represented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
X-ray alone or combined with empty CPs could induce inhibition of B16F10 tumor growth to a low extent, and tumors grew exponentially after stopping X-ray irradiation (Fig. 3b). The combination of 3 Gy X-ray with CPs-CDN or free CDN greatly improved the inhibition of melanoma growth, in which CPs-CDN was significantly more potent than free CDN (**p). Accordingly, 3 Gy X-ray + CPs-CDN group brought about the best survival benefit with an MST of 42 d, which was significantly longer than 3 Gy X-ray + free CDN and CPs-CDN groups (Fig. 3c). It is noticed that treatment with 3 Gy X-ray + CPs-CDN at CDN dose of 20 μg/mouse was much better than combo groups at CDN dose of 10 or 30 μg/mouse (Fig. S3). Of note, 3 Gy X-ray alone had only limited improvement of MST over PBS (23 vs 15 d). The group of 3 Gy X-ray + CPs-CDN showed less body weight increase than other groups in 25 d (Fig. 3d), supporting its high potency and low adverse effects.

We further studied the efficacy of radioimmunotherapy of melanoma at a higher X-ray dose of 5 Gy (Fig. 4a). The results showed that 5 Gy X-ray irradiation had better tumor inhibitory effect than 3 Gy (Fig. 4b). However, the mice survival rate was not significantly improved compared with 3 Gy X-ray (MST = 26 vs 23 d) (Fig. 4c), in line with short-term STING activation and transient anti-tumor immune response [41]. The combination of 5 Gy X-ray with CPs-CDN or free CDN markedly further halted melanoma growth. Remarkably, CPs-CDN gave significant survival benefits compared to free CDN (MST: 54 vs 39 d). Three out of seven mice (3/7) became tumor free for both combo groups over an observed period of 176 d post tumor inoculation (Fig. 4c), supporting that combination of RT and STING agonists can elicit durable and strong immunotherapeutic effects [47,61,62]. Importantly, all treatments were well tolerated (Fig. 4d). The activation of STING pathway plays a crucial role in both immune cells and tumor cells, and can regulate multiple stages in the cancer-immunity cycle and induce spontaneous initiation of anti-tumor CD8+ T cell response [62]. The STING agonists need to be delivered to endoplasmic reticulum where STING locates. The efficient cytoplasmic delivery of CPs-CDN makes it a great system for boosting anti-cancer microenvironment. In comparison, toll-like receptors (TLRs)

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Fig. 4. The combination of CPs-CDN (1 mg CDN/kg) and 5 Gy X-ray for radioimmunotherapy of the mice bearing B16F10 melanoma (n = 7). a Tumor inoculation, treatment, and rechallenge scheme. When the tumor volume reached 50 mm3, the mice were treated by locally irradiating tumors at 5 Gy on day 0, 3 and 6, and i.t. administration of CPs-CDN, CDN or PBS on day 1, 4, and 8. b Tumor volume and spider plots of individual tumor growth curves (n = 7). c Survival rates. Kaplan-Meier analysis (two-tailed Mantel-Cox test). d Relative body weights. Statistical analysis: one-way ANOVA with Tukey’s multiple comparisons. e The cured mice were rechallenged with B16F10 cells on the opposite side on 175 d after the first inoculation (n = 3). f Analysis of tumor sections of 5 Gy X-ray + CPs-CDN group. From left to right: immunofluorescence staining of CRT, CD8+ T cells, CD80+ DCs and H&E staining images. Scale bar: 50 μm. For b-e, blue symbols and lines: PBS, red: 5 Gy X-ray, green: 5 Gy X-ray + CDN, purple: 5 Gy X-ray + CPs-CDN. *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
are pattern recognition receptors (PRRs) of the innate immune system, and the delivery of agonists of intracellular TLRs such as TLR3/7/8/9 need to be released in endosomes/lysosomes.

The cured mice were further re-challenged with subcutaneous inoculation of $1.5 \times 10^5$ B16F10 cells at the opposite flank on day 176. The results demonstrated that one mouse from 5 Gy X-ray + CPs-CDN group rejected tumor, while the other two mice exhibited greatly retarded tumor growth compared with PBS group (Fig 4e). The mice from 5 Gy X-ray + CDN group also revealed delayed tumor growth compared with PBS group; nevertheless, all three mice re-grew tumors and died in 23 d after tumor rechallenge. The immunofluorescence staining of tumor slices of 5 Gy X-ray + CPs-CDN group displayed extensive tumor cell necrosis, tremendous calreticulin (CRT) exposure, CD80+ DCs and CD8+ T cell infiltration (Fig 4f, Fig S4), in agreement with RT triggered DNA damage and release of DAMPs (CRT) as tumor associated antigens (TAA) that serve as "eat-me" signal for maturation of APCs and enhancing tumor-specific T cell response [38–40]. These results confirm that radioimmunotherapy with 5 Gy X-ray + CPs-CDN induces strong and durable adaptive immune response.

3.5. Radioimmunotherapy with CPs-CDN and X-ray promotes proinflammatory TME

The successful immunotherapy depends not only on the activation of APCs but also on the effective elimination of the immunosuppressive TME, which largely counteracts the anti-tumor immunity [34]. Here, we evaluated the effect of CPs-CDN and its combination with X-ray on immune microenvironment of B16F10 melanoma. Giving that DCs are the major immune cells in activation of STING pathway, DC maturation in the tumor draining lymph node (TDLN) at 48 h after the last injection was analyzed by flow cytometry. TDLN plays a major role in accommodating mature DCs that present antigen information to specific T cells. The results showed that i.t. injection of free CDN stimulated a great increase of mature DCs (CD11c+CD80+CD86+) in TDLN than PBS (Fig 5a), whereas DC maturation in CPs-CDN group was further more significant than free CDN group (67.7% vs. 56.1%, **p), which is consistent with in vitro BMDC activation results. The efficient TDLN transportation of CPs-CDN is likely due to its small size (47 nm) and easy access to lymphatic lumen from the interstitial space [63]. While 3 Gy X-ray alone had limited ability to promote DC maturation, its combination with free CDN and in particular CPs-CDN significantly augmented DC maturation. Interestingly, 5 Gy X-ray + CPs-CDN could further improve the proportion of mature DCs in TDLN to 89.9%, which was significantly higher than 3 Gy X-ray + CPs-CDN group (*p) and CPs-CDN group (**p). This could be ascribed to an increased release of ICD and DAMPs (CRT, HMGB1 and ATP) inducing specific activation of effector T cells to inhibit tumor growth [38,39], and to enhanced STING pathway activation caused by 5 Gy X-ray than 3 Gy X-ray [64].

Cytotoxic T lymphocytes (CTLs) are the key effector cells in cancer immunotherapy. The proportions of tumor-infiltrating CD8+ and CD4+ T cells after immunotherapy and combination therapy were studied and analyzed (Fig 5b, Fig S5). The gating strategies were shown in Fig S6. Fig S5 shows that there were no significant differences in CD4+ T cells among groups. Although stimulating DC maturation, free ADU-S100 did not increase the CTL proportion in TME, nor did 3 Gy X-ray. In sharp contrast, CPs-CDN greatly promoted tumor infiltration of CTLs (*p),

![Fig. 5.](image-url) Anti-tumor immune response induced by the combination of CPs-CDN and X-ray in B16F10-bearing mice (n = 4). a Typical histograms and quantitation of DC maturation in TDLN analyzed by FACS. b The proportion of CD8+ T cells in tumors (n = 4). c The positive expression of CD206 (mean fluorescence intensity of CD206-Alexa Fluor 647) on macrophages in TME (n = 4). a-c: TDLN or tumors were harvested at 48 h after the last injection. d Plasma concentrations of IFN-β and TNF-α of the mice at 6 h after last injection (n = 3). Statistical analysis: one-way ANOVA with Tukey’s multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.
showing the benefits of polymersomal formulation in recruiting CTL to TME. The combination of CPs-CDN with X-ray further significantly more increased CTLs than free CDN + X-ray (**p), in accordance with the results of DC maturation. Notably, compared with PBS, a significant reduction of immunosuppressive M2 phenotype macrophages (M2M, CD11b + CD206 hiatus) was observed for free CDN (**p) and CPs-CDN (**p) groups (Fig. 5c, Fig. 5S). The combination with X-ray with CPs-CDN led to a further decrease of M2M contents. These results indicate that the combination of CPs-CDN with X-ray not only enhances immunostimulatory effects but also alleviates immunosuppressive environment in B16F10 tumors. In this study, total dose of 15 Gy (3 × 5 Gy) was applied in combination with CPs-CDN, inducing strong and durable antitumor effects but also alleviates immunosuppressive environment in combination of CPs-CDN with X-ray not only enhances immunostimulatory effect and migration of DCs, enhance cytotoxicity mediated by CD8+ T cells, and bridge innate and adaptive immunity, besides direct inhibitory effect on tumor cells [65,66]. It should be noted that under stimulation, multiple cells in tumor including DCs, tumor cells, macrophages, and T cells are capable of producing IFN-β in vivo. APCs were reported to induce the highest IFN-β production while much lower IFN-β production by B16F10 cells [34].

4. Conclusion

We have shown that polymersome-mediated cytosolic delivery of cyclic dinucleotide STING agonist (CPs-CDN) greatly enhances tumor immunotherapy and radioimmunotherapy via augmenting tumor retention, tumor draining lymph node uptake, dendritic cell uptake and maturation, and intracellular trafficking and release. Interestingly, in contrast to free ADU-S100 and 3 Gy X-ray that induce little tumor infiltration of CTLs, CPs-CDN leads to greatly increased CTLs in tumor microenvironment, illustrating a unique role of polymersome-mediated cytosolic delivery. The combination of CPs-CDN with 5 Gy X-ray further re-enforces STING activation, promotes the secretion of IFN-β and TNF-α, increases the proportions of mature DCs and effector T cells, and reduces immunosuppressive M2M in TME, yielding further enhanced and durable anti-tumor immune responses with significant survival benefits and cure of 3/7 B16F10-bearing mice. The reduction of systemic exposure of STING agonist enabled by polymersomes, on the other hand, might also alleviate their adverse effects. This polymersome-facilitated cytosolic delivery of STING agonists appears to be an attractive strategy to boost cancer immunotherapy. Besides local I.t. injection, polymersomal CDN might also be delivered systemically as reported for CpG and siRNA [37,44,45]. The dense PEGylation, neutral surface charge and favorable size of CPs-CDN facilitate effective bypass of phagocytosis [67,68]. This study has proven the concept that CPs-CDN can elegantly solve the issues of free CDN such as fast clearance, poor cell uptake and inefficient cytotoxic transportation and boost the immunotherapy of B16F10 melanoma mouse model. B16F10 melanoma is a typical model used for immunotherapeutic studies because it is highly malignant and immunogenic. Advanced metastatic melanoma remains highly challenging to treat in the clinics. In the future, we will further investigate the efficacy of CPs-CDN in more and clinically relevant models including denser solid tumors in which tumor penetration is another limiting factor.

Declaration of competing interest

The authors declare no known competing financial interest.

CRediT authorship contribution statement

Huan Zheng: Methodology, Investigation, Validation, Writing – original draft. Beibei Guo: Investigation, Data curation. Xinyun Qiu: Methodology, Investigation. Yifeng Xia: Investigation, Data curation. Yan Qu: Data curation. Liang Cheng: Resources, Visualization. Fenghua Meng: Conceptualization, Supervision, Writing – review & editing. Zhiyuan Zhong: Conceptualization, Supervision, Writing – review & editing.

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Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.02.029.

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