Synergistic STING activation by PC7A nanovaccine and ionizing radiation improves cancer immunotherapy

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ABSTRACT

Solid cancers are able to escape immune surveillance and are resistant to current treatment in immunotherapy. Recent evidence indicates the critical role of the stimulator of interferon genes (STING) pathway in antitumor immunity. STING-targeted activation is extensively investigated as a new strategy for cancer therapy. Previously, we reported a safe and efficacious STING-activating nanovaccine to boost systemic tumor-specific T cell responses in multiple tumor models. Local radiotherapy has been reported to not only reduce tumor burden but also enhance local antitumor immunity in a STING-dependent manner. In this study, we demonstrate that combination of these two modalities leads to a synergistic response with long-term regression of large established tumors in two mouse tumor models. The percentage of CD8+ T cells increased significantly in primary tumors after combination therapy. Mechanistically, the augmented T cell responses of radiotherapy and nanovaccine is STING pathway dependent. Furthermore, nanovaccine synergizes with radiotherapy to achieve a better therapeutic effect in distal tumors. These findings suggest that combination of local radiotherapy with systemic PC7A nanovaccine offers a useful strategy to improve the therapeutic outcome of late stage solid cancers.

1. Introduction

Stimulation of innate immune pathways plays an important role in T cell production and tumor infiltration [1]. Among various innate pathways, stimulator of interferon genes (STING) is emerging as a unique mediator protein for host defense. STING exists in many cell types, including all antigen-presenting cells, epithelial cells, endothelial cells, and fibroblasts [2], which provide a broad platform for effective innate stimulation. Activation of the STING pathway upregulates the transcription of genes that encode type I interferons (IFNs) and proinflammatory cytokines and chemokines. STING activation in tumor tissues can lead to accumulation and infiltration of CD8+ T cells. Recent studies show after tumor cell inoculation in STING-deficient mice, tumors grew more rapidly than in wild-type or TRIF-deficient mice. CD8+ T cells against tumors were also defective in mice lacking STING, but not in those lacking Toll like receptor, myeloid differentiation primary response 88 (MyD88) or mitochondrial antiviral signaling protein (MAVS), suggesting the essential role of STING in antitumor immunity [3].

We previously reported a synthetic polymeric nanoparticle, PC7A NP, which generated a robust cancer-specific T cell response with low systemic cytokine expression [4]. Antigen-loaded PC7A NP was systemically administered by subcutaneous injection to target the lymphoid organs. The nanoparticle formulation (20–30 nm in diameter) allowed efficient cytosolic delivery of tumor antigens to the dendritic cells inside draining lymph nodes, while stimulating the innate pathway for T cell activation. T cell activation is exclusively dependent on STING, but not on TLR or MAVS pathways. The PC7A nanovaccine

Abbreviations: STING, stimulator of interferon genes; IFN, interferon; RT, radiotherapy; LN, lymph node; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; PBS, phosphate-buffered saline

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illustrated efficacious anti-tumor response in multiple mouse tumor models, including B16 melanoma, MC38 colon carcinoma, and TC-1 cervical tumors. Despite the therapeutic promise, tumor resistance to PC7A nanovaccine is observed in established tumors (e.g., > 100 mm³). Combination of other therapeutic modalities with PC7A nanovaccine may prove necessary in the eradication of large, immunosuppressive tumors.

Radiotherapy (RT) is widely used in the treatment of various solid tumors. Increasing evidence has shown that local irradiation not only reduces tumor burden [5,6], but also augments adaptive T cell response against tumors [7,8]. Deng et al. reported that ionizing radiation produces a type I IFN-dependent antitumor response via the STING pathway. The DNA of dying irradiated cancer cells are found in the cytoplasm of dendritic cells, which is responsible for the activation of the cGAS-STING-type I IFN pathway [9]. Direct activation of STING by intra-tumoral injection of STING agonists also led to potent immune responses and systemic tumor regression [10]. Therefore, targeting the STING pathway in tumors offers an effective strategy to enhance adaptive antitumor immunity.

In this study, we investigated the synergy between the systemic cancer-specific T cell response initiated by STING-activating PC7A nanovaccine, and local STING activation by ionizing radiation for increased T cell infiltration in tumors. The combined STING activation strategy produced a synergistic therapeutic outcome against large, established tumors compared to either treatment alone. STING-deficient mice treated with the same strategy showed significantly less CD8⁺ T cells both systemically and locally in the tumor. In addition to the response seen in the primary irradiated tumors, we also observed an abscopal effect from this combination, which is rarely found in patients treated with radiotherapy alone. These data suggest combining PC7A nanovaccine with local radiation offers a useful strategy in treating both primary and metastatic cancers.

2. Materials and methods

2.1. Chemicals

Antigenic peptides E7 45-62 (GQAEPDRAHYNVFTCCKD), OVA257-262 (SIINFEKLTEWTSSNVMEERKKIV), were synthesized by Biomatik. Fetal bovine serum, penicillin streptomycin, and cell culture media were obtained from Invitrogen Inc. (OR, USA). Amicon ultra-15 centrifugal filter tubes (MWCO = 100 K) were from Millipore. Other solvents and reagents were purchased from Sigma-Aldrich or Fisher Scientific Inc.

2.2. Syntheses and preparation of PC7A micelle nanoparticles

2-(Hexamethyleneimino) ethyl methacrylate (C7A-MA) monomer was synthesized following previous procedures [11,12]. PEG-b-PC7A copolymer was synthesized by atom transfer radical polymerization (ATRP) method [13]. Micelles were prepared following a solvent evaporation method [12]. Briefly, 10 mg of PEG-b-PC7A copolymer was first dissolved in 1 mL methanol and then added into 4 mL distilled water dropwise under sonication. The mixture was filtered 4 times to remove methanol using the micro-ultrafiltration system (MW = 100 KD). Then distilled water was added to adjust the polymer concentration to 10 mg/mL as a stock solution. After micelle formation, the nanoparticles were characterized by dynamic light scattering (DLS, Malvern ZetaSizer model) for hydrodynamic diameter (Dh) measurement.

2.3. Animals and cells

All animal procedures were performed with ethical compliance and approval by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Female C57BL/6 mice (6-8 weeks) were obtained from the University of Texas Southwestern breeding core. STINGmut/mut mice were purchased from the Jackson laboratory. B16-OVA cells were kindly provided by Dr. Patrick Hwu at MD Anderson Cancer Center and TC-1 cells by Dr. T. C. Wu at John Hopkins University. Both cell lines were routinely tested using mycoplasma contamination kit (R&D). Cells were cultured in DMEM medium (10% fetal bovine serum, 100 U/mL penicillin G sodiuim and 100 μg/mL streptomycin (Pen/Strep), non-essential amino acids, and 20 μM β-mercaptoethanol (β-ME)) at 37 °C in 5% CO₂ and the normal level of O₂.
3. Results

3.1. Established tumors develop resistance to PC7A nanovaccine

We previously demonstrated that STING-activating PC7A nanovaccine generated potent tumor-specific T cell responses in several mouse tumor models [4]. However, the dependence of antitumor efficacy of the vaccine therapy on the different size and stage of tumor development is not clear. To address this question, we immunized mice at several time points after TC-1 tumor cell inoculation. When mice were immunized with E743-62–PC7A vaccine 5 days after tumor cell inoculation, 70% of mice showed complete regression 2 weeks after vaccination, and 60% mice showed tumor free survival 60 days after tumor inoculation (Fig. 1, Supplementary Fig. 1). In contrast, when mice were immunized on day 10 after tumor cell inoculation, the tumors were well-established (about 100–200 mm³). The tumors showed some shrinkage a week after the first vaccination, but the effect was not adequate to reject the tumors, which finally recurred a month later. When mice were immunized 13 days after tumor cell inoculation, this group showed continuous tumor growth and shorter life span compared to the other two groups. At the same time, CD8⁺ T cells in tumors at different time points after tumor inoculation were examined. Results showed that larger tumors contained lower percentage of T cells (Supplementary Fig. 2A), and PD-L1 was expressed on tumor cells and showed that larger tumors contained lower percentage of T cells (Supplementary Fig. 2A). Tumor-free mice were rechallenged with 1 × 10⁶ TC-1 tumor cells (Supplementary Fig. 3E), and animals showed long-term memory to reject the transplanted tumors, whereas tumors grew robustly in naive mice.

This synergistic effect was also evaluated in B16-OVA melanoma tumor model. After the tumor size reached 70 mm³ (about 10 days after inoculation), the mice received a single dose of 20 Gy local radiation, followed by subcutaneous injection of PC7A nanovaccine, with a boost 7 days post initial vaccination (Fig. 2A). Nanovaccine alone and radiation alone were used as controls to assess the effect of monotherapy. The result showed that radiation alone and nanovaccine alone deterred the tumor growth marginally compared with the E7 peptide only control, but were unable to suppress eventual tumor growth. In contrast, combination of nanovaccine and radiation therapy showed significant therapeutic synergy over either monotherapy alone, as 50% of mice were tumor-free 60 days after tumor inoculation (Fig. 2B–C, Supplementary Fig. 3A–D). Tumor-free mice were rechallenged with 1 × 10⁶ TC-1 tumor cells (Supplementary Fig. 3E), and animals showed long-term memory to reject the transplanted tumors, whereas tumors grew robustly in naive mice.

To investigate whether local radiation can help overcome tumor resistance to PC7A vaccine, we employed established TC-1 tumors in C57BL/6 mice with tumor size reaching ~200 mm³ (in about 13 days after inoculation). Based on previous reports, T lymphocytes are highly sensitive to ionizing radiation and can be cleared rapidly from the radiation site [14,15], so in the combination group, mice first received a single dose of 20 Gy local radiation, followed by subcutaneous injection of PC7A nanovaccine, with a boost 7 days post initial vaccination (Fig. 2A). Nanovaccine alone and radiation alone were used as controls to assess the effect of monotherapy. The result showed that radiation alone and nanovaccine alone deterred the tumor growth marginally compared with the E7 peptide only control, but were unable to suppress eventual tumor growth. In contrast, combination of nanovaccine and radiation therapy showed significant therapeutic synergy over either monotherapy alone, as 50% of mice were tumor-free 60 days after tumor inoculation (Fig. 2B–C, Supplementary Fig. 3A–D). Tumor-free mice were rechallenged with 1 × 10⁶ TC-1 tumor cells (Supplementary Fig. 3E), and animals showed long-term memory to reject the transplanted tumors, whereas tumors grew robustly in naive mice.

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3.2. Radiation synergizes with nanovaccine to effectively control established tumors

To investigate whether local radiation can help overcome tumor resistance to PC7A vaccine, we employed established TC-1 tumors in C57BL/6 mice with tumor size reaching ~200 mm³ (in about 13 days after inoculation). Based on previous reports, T lymphocytes are highly sensitive to ionizing radiation and can be cleared rapidly from the radiation site [14,15], so in the combination group, mice first received a single dose of 20 Gy local radiation, followed by subcutaneous injection of PC7A nanovaccine, with a boost 7 days post initial vaccination (Fig. 2A). Nanovaccine alone and radiation alone were used as controls to assess the effect of monotherapy. The result showed that radiation alone and nanovaccine alone deterred the tumor growth marginally compared with the E7 peptide only control, but were unable to suppress eventual tumor growth. In contrast, combination of nanovaccine and radiation therapy showed significant therapeutic synergy over either monotherapy alone, as 50% of mice were tumor-free 60 days after tumor inoculation (Fig. 2B–C, Supplementary Fig. 3A–D). Tumor-free mice were rechallenged with 1 × 10⁶ TC-1 tumor cells (Supplementary Fig. 3E), and animals showed long-term memory to reject the transplanted tumors, whereas tumors grew robustly in naive mice.

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3.3. STING pathway is required for the therapeutic synergy of combination therapy

Since either PC7A nanovaccine or local radiation were shown to depend on STING pathway for antitumor immunity, we sought to determine the role of STING in the combination strategy. We first determined the ratio of tumor-infiltrating T cells over cancer cells from various treatment groups. Tumor tissues were removed for analysis
5 days after the second vaccination in mice inoculated with the TC-1 tumors. Tumor tissues were dissociated into single cells by collagenase treatment. Flow cytometry was then utilized to detect the percentage of infiltrating T cells (Supplementary Fig. 4). The results show that compared to non-treated WT mice, both radiation alone and vaccine alone treatment increased tumor infiltrating T cells (CD45+CD3+cells) and antigen-specific CD8+ T cells (E7 tetramer+CD8+CD3+cells). Moreover, combination of radiation and vaccine treatment resulted in significant increase of both T cell populations over single arm controls, demonstrating clear therapeutic synergy (Fig. 3A–B). T cell infiltration in tumors correlated with anti-tumor efficacy (Fig. 2B–C).

In STING mutant mice, results show that majority of the T cell accumulation and infiltration especially tumor specific CD8+ T cells were abolished compared to radiation alone and vaccine alone groups from...
the wild type animals (Fig. 3A–B), which is consistent with our previous reports [4,9]. For the combination therapy group, compared to wild type mice, STING mutant mice showed significantly decreased lymphocytes in tumor after treatments (P < .01). Furthermore, we used immunohistochemistry assay to detect the percentage of CD8+ T cells infiltration into the tumors (Fig. 4C). In wild type mice, a higher percentage of tumor-infiltrating CD8+ T cells were detected compared to STING mutant mice after combination therapy. To further corroborate these findings, we evaluated the systemic T cell activation 9 days after radiation and the first dose of vaccine in both wild type and STING mutant mice (Fig. 4). Spleens were removed and dissociated into single cells for flow cytometry analysis (Supplementary Fig. 5). The treated group showed distinctive increase of E7-specific T cells in CD8+ T cell population compared to the untreated group and STING mutant group (Fig. 4B–C). These experiments demonstrate that STING pathway is required for both elevated systemic T cell response and local tumor infiltration in the combination therapy.

3.4. Local radiation synergizes with PC7A nanovaccine to control distal tumors

Ionizing radiation has been reported to reduce tumor growth outside the treatment field in rare clinical cases, referred to as the abscopal effect [16,17]. Enhancement of the abscopal effect has great promise in treatment of patients with metastatic tumors. Historically, preclinical evidence has supported the notion that distal tumor (metastatic tumor) regression as a result of radiation is immune-mediated [18–20]. In this study, we used the dual TC-1 tumor model to test the abscopal effect. Primary tumors were first initiated by subcutaneous injection of cells (1.5 × 10⁵ cells) on the left flanks of mice, and 4 days later, distal tumors (2 × 10⁵ cells) were introduced on the right flanks (Fig. 5A).

In the untreated control group, distal tumors grew both later and smaller than the primary tumors (Supplementary Fig. 6A), resembling metastatic tumor nodules in patients. When primary tumor size reached ~200 mm³ (in about 13 days), mice received a single dose of 20 Gy local radiation on primary side and PC7A vaccine subcutaneously. We measured tumor growth on both the primary and distal tumors. Results show radiation alone was effective at inhibiting the growth of the irradiated primary tumor (Fig. 5B), but it had no effect on distal tumors compared to non-treated group. PC7A nanovaccine alone showed increased inhibitory effect on distal tumors over the non-treated control, and combination of both nanovaccine and radiation achieved additional growth inhibition in distal tumors (Fig. 5C–D, Supplementary Fig. 6B). These results illustrate radiation alone failed to prime a durable immune response to attack distal TC-1 tumors, but combination with STING vaccine led to a significant improvement in systemic T cell response against distal tumors.

4. Discussion

Therapeutic vaccines are designed to harness the immune system to induce potent tumor-specific cytotoxic T cells for cancer immunotherapy. New trends in therapeutic cancer vaccines focus on the mode of antigen delivery and the type of immune-stimulating adjuvants [21–23]. Our PC7A nanovaccine allowed stable antigen loading within a small size confinement (< 50 nm) that facilitates antigen delivery to the lymph nodes. So this nanovaccine platform can be rapidly adopted to incorporate many existing tumor-associated antigens as well as tumor neoantigens. Equally important, early endosomal release of tumor antigens into the cytosol avoids lysosomal degradation, leading to increased antigen cross-presentation on the cell surface. Uniquely, this synthetic nanoparticle itself stimulated innate cellular immunity through the STING-type I IFN pathway, which induced a long term anti-tumor response. Compared to several established adjuvants (CpG, polyI:C and Alum), PC7A NP was able to induce better T cell response and anti-tumor efficacy with lower systemic cytokine expressions[4]. In large, established tumors, this vaccine showed decreased antitumor efficacy. Historically, clinical trial outcomes of many cancer vaccines have also been disappointing [24–27] with similar limitations in treating late stage tumors. Therefore, developing strategies to reduce tumor burden and overcome resistance of established tumors to cancer vaccines has the potential to improve the clinical result of therapeutic vaccines.

Tumor radiation can rapidly reduce tumor burden without directly suppressing vaccine-induced systemic T cell responses. Increasing evidence shows that radiation can augment adaptive T cell responses to tumors [7,8]. First, radiation can damage the tumor tissue and reorganize tumor-associated blood vessels, which presumably allows for better T cell or cytokine penetration [28,29]. Second, it has been shown that the therapeutic effect of ablative radiation therapy depends largely on CD8+ T cells, as radiation increases T cell priming [30]. Furthermore, local radiation induces antigen release and cross-presentation...
mainly through STING-Type I IFNs pathway [20]. Previous reports showed that only antigens released by dead tumor cells were not sufficient to stimulate T cell activation. Under non-inflammatory conditions, these dead cells failed to induce antitumor immune responses [31]. Radiation not only induces antigens' release from tumor cells, but also triggers innate sensing, stimulates and attracts antigen-presenting cells (APCs) to uptake and present tumor antigens, as well as increases expression of co-stimulators, finally activates tumor specific T cell response. Among different innate sensing pathways that modulate tumor inflammation, it has been shown that STING-type I IFNs were required for radiation-induced inflammation and subsequent adaptive immune responses [3,9]. Due to the increasing recognition of the importance of STING pathway in T cell priming and anti-tumor effects, we combined our nanovaccine with local ionizing radiation to synergize STING function in both the lymphatic system and tumor site. This combination showed an obvious synergy effect in established tumor models. In STING mutant mice, the T cell response produced by this combination was markedly decreased, indicating that the STING pathway and subsequent pro-inflammatory cytokines expressed in the tumor are crucial for allowing T cells to home to tumors and attack. Our data show encouraging antitumor efficacy in relatively large tumors by combining radiation and vaccine therapy. Kelly et al. reported using a combination of four immunotherapeutic agents to eliminate large tumor burdens [32]. Inclusion of additional agents that target independent immune suppressive mechanisms (e.g., anti-PD-1 or PD-L1) may be beneficial to augment the antitumor response in the current regimen.

In this study, we further investigated the combination therapy on distal tumors, and observed that distal tumors displayed a similar synergistic response to the combined treatments as the primary tumors, but were more resistant. In several mice, while the primary large tumors showed obvious regression after treatment, the distal tumor still persisted and eventually grew (Fig. 5 and Supplementary Fig. 6), as reported in similar studies [33]. One possible explanation for this diminished response might be the intact immunosuppressive environment of the distal tumors (i.e., without radiation-induced inflammation). Another possibility is the attraction of more circulating T cells to the primary tumor site, which resulted in fewer T cells accumulating in the distal tumor. Further work is necessary to elucidate the mechanism of vaccine resistance in distal tumors to achieve optimal abscopal effect.

5. Conclusions

We previously reported a STING-activating nanovaccine to elicit a robust T cell response against multiple tumor types. In this study, we combined the nanovaccine with radiation therapy to treat large, established solid tumors. The combination regimen showed a synergistic

Fig. 5. Synergistic effect of PC7A nanovaccine and radiation therapy on distal TC-1 tumor growth. (A) Schematic design of the experiments. (B) Tumor growth curve of primary tumors after different treatments. (C) Distal tumor growth in C57BL/6 mice was analyzed for different treatment groups. (D) Individual tumor growth curves for control group, radiation alone, E7p-PC7A NP alone and E7p-PC7A NP combined with radiation. Synergistic outcome was observed in growth inhibition of distal tumors by combination therapy. ***P < .001, **P < .01, *P < .05.
effect in both established and distal tumors. Mechanistically, local radiation effectively reduced the tumor burden and reverted immunosuppressive environment through the activation of STING pathway. The spatial orchestration of STING activation, systemically through the PC7A nanovaccine and locally by tumor radiation, resulted in significantly improved tumor growth inhibition and long-term survival in TC-1 and B16-OVA tumor-bearing mice. The antitumor efficacy was greatly diminished in STING mutant mice. Results from this study indicate the importance of synergizing STING activation of the lymphoid organs and solid tumors for cancer immunotherapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2019.02.036.

References


