Nanoparticle-Enhanced Radiotherapy to Trigger Robust Cancer Immunotherapy

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External radiotherapy is extensively used in clinic to destroy tumors by locally applied ionizing-radiation beams. However, the efficacy of radiotherapy is usually limited by tumor hypoxia-associated radiation resistance. Moreover, as a local treatment technique, radiotherapy can hardly control tumor metastases, the major cause of cancer death. Herein, core–shell nanoparticles based poly(lactic-co-glycolic) acid (PLGA) are fabricated, by encapsulating water-soluble catalase (Cat), an enzyme that can decompose H$_2$O$_2$ to generate O$_2$, inside the inner core, and loading hydrophobic imiquimod (R837), a Toll-like-receptor-7 agonist, within the PLGA shell. The formed PLGA-R837@Cat nanoparticles can greatly enhance radiotherapy efficacy by relieving the tumor hypoxia and modulating the immune-suppressive tumor microenvironment. The tumor-associated antigens generated postradiotherapy-induced immunogenic cell death in the presence of such R837-loaded adjuvant nanoparticles will induce strong antitumor immune responses, which together with cytotoxic T-lymphocyte associated protein 4 (CTLA-4) checkpoint blockade will be able to effectively inhibit tumor metastases by a strong abscopal effect. Moreover, a long term immunological memory effect to protect mice from tumor rechallenging is observed post such treatment. This work thus presents a unique nanomedicine approach as a next-generation radiotherapy strategy to enable synergistic whole-body therapeutic responses after local treatment, greatly promising for clinical translation.

External beam-based cancer radiotherapy is a mainstream cancer treatment strategy that has been extensively used in clinic to treat $65\text{–}75\%$ of local solid tumors at different stages. During radiotherapy, ionizing radiation beams such as high-energy X-ray, γ-ray, or electron beams are applied locally onto tumors to kill cancer cells. It is well known that the degree of cancer cell damage during radiotherapy is highly dependent on the concentration of available oxygen, which can stabilize radiation-induced DNA damages to prevent DNA self-repairing by cells, so as to improve the efficacy of radiotherapy. However, the rapid growth of cancer cells and the distorted blood vessels would lead to hypoxia inside solid tumors, resulting in therapeutic resistance and failure of radiotherapy. Thus, it is important to develop effective strategies to relieve tumor hypoxia so as to enhance the efficacy of radiotherapy. Another limitation of radiotherapy is that as a type of local treatment, conventional radiotherapy is not able to inhibit the growth of distantly spreading tumors and thus ineffective to control tumor metastases, which are often the ultimate cause of cancer deaths. Therefore, there would be an urgent clinical demand to develop next generation cancer radiotherapy strategies that are able to effectively destruct local solid tumors with hypoxic regions, as well as inhibit tumor metastases to realize a whole-body systemic therapeutic outcome.

Cancer immunotherapy by utilizing and training the patients’ own immunological systems to attack cancer cells, has emerged as a powerful new generation cancer therapeutic strategy showing tremendous promises in recent years.

Among various types of cancer immunotherapy strategies, checkpoint blockade strategies by blocking cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), or programmed-cell-death protein 1 (PD1) and its ligand PD-L1, have received massive attentions in recent years. CTLA-4 and PD-1/PD-L1 blockades by their respective antibodies have recently been approved by the U.S. Food and Drug Administration for treatment of several different types of cancers. Despite their remarkable progresses, checkpoint blockade treatment by triggering nonspecific immune responses still suffer from their side effects, and appear to be effective for only $\approx20\%$ of cancer patients as uncovered in many clinical studies. Therefore, it is hoped that combination of checkpoint blockade with other types of therapeutic strategy may bring new fortune for cancer therapy.

Immune responses after radiotherapy have also attracted substantial attentions in recent years. It has been found after radiotherapy-induced cancer cell destruction, tumor-associated antigens may be released to trigger certain levels of antitumor immune responses. In a latest study, Min et al. reported that poly(lactic-co-glycolic acid) (PLGA) nanoparticles with certain functional groups may show antigen-capturing functions...
to enhance immune responses after radiotherapy, which in combination with anti-PD1 blockade shows an abscopal therapeutic effect. However, the cure rate achieved by this strategy remains to be relatively low (20%), likely owing to the limited immune-stimulating effect of bare PLGA nanoparticles, as well as the inability of their strategy to overcome hypoxia-associated radiation resistance.

In this work, to realize radiotherapy triggered cancer immunotherapy, we fabricate multifunctional core–shell PLGA nanoparticles through a water/oil/water (w/o/w) double emulsion method. Within those nanoparticles, catalase (Cat), a water-soluble enzyme that triggers rapid decomposition of H$_2$O$_2$, is encapsulated inside the aqueous cavity, whereas hydrophobic imiquimod (R837), a Toll like-receptor-7 agonist as an immune adjuvant, is loaded into the PLGA shell (Figure 1a).

The formed PLGA-R837@Cat nanoparticles under X-ray radiation could cause destruction of primary tumors with enhanced tumor killing efficacy compared to X-ray radiation alone, owing to the Cat-triggered tumor hypoxia relief and modulation of immune-suppressive tumor microenvironment. After radiotherapy with PLGA-R837@Cat to trigger the immunogenic cell death (ICD) for tumor cells, the generated tumor debris could act as tumor-associated antigens, which with the help of R837-containing PLGA-R837@Cat nanoparticles as immune adjuvants could induce robust antitumor immune responses. With further combination use of anti-CTLA4 (αCTLA4) checkpoint blockade, such treatment could further effectively inhibit the growth of distant metastatic tumors with a very strong abscopal effect. Moreover, it is further uncovered that radiotherapy with PLGA-R837@Cat offers a robust long term immune memory effect to protect mice from rechallenged cancer cells. Therefore, by employing multifunctional nanoparticles with full biocompatibility, we are able to combine radiotherapy with immunotherapy to realize whole body systemic antitumor therapeutic outcomes triggered by enhanced radiotherapy of local tumors, promising in inhibiting tumor metastases and preventing tumor relapse.

A classical double emulsion (water/oil/water) method was utilized to prepare core–shell PLGA-based nanoparticles. Catalase, a water-soluble enzyme, was encapsulated into the hydrophilic core of PLGA core–shell nanoparticles during the first emulsion (w/o), while hydrophobic R837 was loaded into
the PLGA shell during the second emulsion process (w/o/w). Transmission electron microscope (TEM) image indicated that the synthesized PLGA-R837@Cat exhibited well-defined spherical shape and homogenous sizes at ≈100 nm (Figure 1b). The average hydrodynamic diameter of PLGA-R837@Cat was measured by dynamic light scattering (DLS) to be ≈100 nm (Figure 1c). After optimization, the final loading percentages of catalase and R837 (with respect to PLGA by weight) on PLGA-R837@Cat core–shell nanoparticles were measured to be ≈2% and ≈2.4%, as determined by bicinchoninic acid Protein Assay Kit and high performance liquid chromatography (HPLC), respectively. The release behavior of R837 was also carefully investigated using HPLC (Figure S1, Supporting Information), with ≈50% R837 released from PLGA-R837@Cat in 5 days. For control samples, PLGA, PLGA-R837, and PLGA@Cat core–shell nanoparticles were prepared following the same w/o/w double emulsion method but without addition of catalase or R837.

Considering that catalase has the ability to rapidly decompose H$_2$O$_2$ into H$_2$O and O$_2$, we first studied the generation of O$_2$ in the solution of H$_2$O$_2$ (100 × 10$^{-6}$ m) after addition of PLGA-R837 or PLGA-R837@Cat. As measured by an oxygen probe (JPBJ-608 portable Dissolved Oxygen Meters, Shanghai REX Instrument Factory), rapid generation of O$_2$ could be observed in the H$_2$O$_2$ solution in the presence of PLGA-R837@Cat. By contrast, PLGA-R837 without catalase exhibited no catalytic ability to H$_2$O$_2$ (Figure 1d). Furthermore, we evaluated the enzyme stability of PLGA-R837@Cat against proteases, which would widely exist in the complex physiological environment, by the G6th method.[13] Compared to free catalase, which was quickly digested and lost its activity after incubation with protease K for 4 h, catalase encapsulated in PLGA-R837@Cat was well protected and maintained ≈60% of its original enzyme activity after incubation with protease K for as long as 12 h (Figure 1e). Therefore, the enzyme activity of catalase could be effectively protected after it is encapsulated into PLGA-shelled nanoparticles, beneficial for its further in vivo applications.

Dendritic cells (DCs), the most important type of antigen-presenting cells, are responsible for activating and regulating the innate and adaptive immune responses.[14] Immature DCs would engulf foreign substances such as antigens and then process them into peptides, which are presented to major histocompatibility complex (MHC) on the cell surface during DC maturation.[14,15] The MHC-antigen complexes on DC surface could then be recognized by T cell receptors to activate T cells, triggering the subsequent immune response. We thus first studied the in vitro DC stimulation effects of different types of nanoparticles. Bone marrow derived DCs harvested from Balb/c mice were incubated with bare PLGA, free R837, PLGA@Cat, PLGA-R837, or PLGA-R837@Cat nanoparticles for 12 h. Then, flow cytometry was used to determine the DC maturation by analyzing the upregulation of costimulatory molecules, CD80 and CD86, which are the typical markers for DC maturation. The percentages of matured DCs (CD80$^+$CD86$^+$) incubated with PLGA-R837 or PLGA-R837@Cat appeared to be obviously higher than that incubated with free R837 at the same dose, whereas PLGA or PLGA@Cat without R837 showed no obvious stimulation effect to DCs (Figure S2, Supporting Information). Notably, all of those nanoparticles exerted no in vitro cytotoxic effects to a number of different cell lines including murine breast cancer cells (4T1), colon adenocarcinoma cell line cells (CT26) and mouse embryonic fibroblast cells (NIH-3T3), even at high nanoparticle concentrations up to 1 mg mL$^{-1}$ (Figure S3, Supporting Information).

Since catalase has the ability to decompose H$_2$O$_2$ into H$_2$O and O$_2$, we then exploited the ability of PLGA-R837@Cat to reduce the tumor hypoxia in vivo by immunofluorescent staining assay (Figure 2a,b; Figure S4, Supporting Information). Balb/c mice bearing CT26 tumors were sacrificed and their tumors were collected 4 h postinjection of different nanoparticles for immunofluorescence staining. It was found that the signals of both hypoxia-probe (pimonidazole) and hypoxia-inducible factor (HIF)-1α (green) signals showed dramatic decrease in tumors injected with PLGA-R837@Cat, owing to the decomposition of H$_2$O$_2$ to generate O$_2$ in the tumor microenvironment by catalase loaded inside those nanoparticles. By contrast, similar to the untreated tumors, tumors from mice injected with PLGA-R837 exhibited large hypoxia areas.

Based on previous studies, hypoxia in the tumor would lead to the recruitment of large numbers of macrophages and their subsequent polarization from M1 to M2 phenotype to promote growth of tumor cells.[16] Thus, the relieved hypoxia in the tumor microenvironment after PLGA-R837@Cat treatment may regulate the phenotype of tumor associated macrophages (TAMs). 3 days after injection of different nanoparticles, the phenotype of TAMs was carefully studied. An obvious reduction of M2-polarized TAMs (CD206hiCD11b$^+$F4/80$^+$) was observed after PLGA@Cat or PLGA-R837@Cat injection (Figure 2c,d). The polarization of TAMs from M2 to M1 was also confirmed by the level of cytokine in the tumor and the serum (Figure 2e–i). Therefore, we hypothesized that the greatly relieved tumor hypoxia by our nanoparticles would be desired not only for overcoming hypoxia-associated radiation resistance, but also for reversing the immunosuppressive tumor microenvironment to synergistically enhance the cancer treatment outcome.

It has been reported that many ablative treatment methods such as photothermal therapy, photodynamic therapy, as well as radiotherapy of tumors, are able to induce tumor-specific immune responses by producing tumor-associated antigens.[12,17] Thus, we wonder whether radiotherapy with PLGA-R837@Cat, as an immune adjuvant, would be able to trigger effective immunological responses in vivo. Considering the fact that local administration is usually used for immunotherapy in order to avoid the risk of cytokine storms, in our experiments, mice bearing subcutaneous CT26 tumors were intratumorally (i.t.) injected with PLGA-R837, Cat@PLGA or PLGA-R837@Cat (0.6 mg kg$^{-1}$ R837, 0.5 mg kg$^{-1}$ Cat). After 4 h, the tumors of these mice were exposed to X-ray at the dose of 8 Gy. Three days after the radiotherapy, we carefully studied the triggered immunological responses including in vivo DC activation in inguinal lymph nodes, and several key cytokine levels in mouse sera. Nanoparticle-injected mice without radiotherapy treatment were used as the controls. Compared to the untreated group, both PLGA-R837 and PLGA-R837@Cat could significantly promote the in vivo DC maturation, while PLGA@Cat showed negligible DC maturation stimulation effect. Moreover, radiotherapy applied after injection of PLGA-R837@Cat induced further increased DC.
maturation (~63%), to a level much higher than that induced by radiotherapy with PLGA-R837 or PLGA@Cat, as well as that in mice injected with PLGA-R837@Cat alone (Figure 3a,b). Therefore, R837-containing nanoparticles could act as an effective immune adjuvant, which is able to enhance the immune responses postradiotherapy treatment of tumors. To further study the long-term toxicology of PLGA-R837@Cat, serum biochemistry assay and complete blood panel test were carried out at day 1 and day 7 after injection of PLGA-R837@Cat. All measured parameters appeared to be normal, indicating that PLGA-R837@Cat would not induce significant systemic side effects to mice (Table S1, Supporting Information). Notably, we observed stronger immune stimulation effect of PLGA-R837@Cat in comparison to PLGA-R837 for mice postradiotherapy, and comparable effects of the two for those without radiotherapy. We speculate that such a phenomenon would likely be attributed to the existence of Cat in the former formulation to relieve tumor hypoxia and enhance radiation-caused tumor destruction,[3a] producing more tumor-associated antigens for stronger immune stimulation.

Figure 2. Modulation of tumor microenvironment after i.t. injection of PLGA-R837@Cat. a) Representative immunofluorescence images of tumor slices after hypoxia staining. The hypoxia areas and blood vessels were stained by antipimonidazole antibody (green) and anti-CD31 antibody (red), respectively. b) The relative hypoxia positive areas and blood vessel densities as recorded from more than ten images for each sample using the ImageJ software. c,d) Representative flow cytometric plots (c) and the corresponding quantification (d) of M2-macrophages (CD206+) in F4/80+ CD11b+ CD45+ cells. e–f) Cytokine levels in sera or tumors from mice after various treatments. Those results suggested the polarization of TAM from immune-suppressive M2 to immune-supportive M1 phenotype after hypoxia relief induced by Cat-loaded nanoparticles. Error bars indicate s.e.m. Statistical significance was calculated by one-way ANOVA using the Tukey post-test. P-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3. Immune responses after radiotherapy with PLGA-R837@Cat. a, b) FACS plots (a) and the statistic data (b) for in vivo DC maturation. Cells in the tumor-draining lymph nodes were collected from CT26 tumor-bearing mice after various treatments for assessment by flow cytometry after staining with CD11c, CD80, and CD86. Cytokine levels of c) TNF-α, d) IFN-γ, and e) IL-12p40, in sera from mice isolated on day 3 post various treatments. f) Confocal images showing the induction of the ICD marker CRT (stained as green by anti-CRT) on CT26 cells after radiotherapy. g) Flow cytometry plots and h) the corresponding quantification showing percentages (gated on CD4+ cells) of CD4+FoxP3+ Treg cells in tumors after various treatments indicated. i) The schematic illustration showing the various immune responses after radiotherapy with PLGA-R837@Cat, including TAM polarization posthypoxia relief, radiotherapy-induced ICD, the subsequent DC activation, as well as upregulated Tregs in the tumors. Among those mechanisms, the first three are immune-supportive and the last one is immune-suppressive. Error bars indicate s.e.m. Statistical significance was calculated by one-way ANOVA using the Tukey post-test. P-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Moreover, to better understand how PLGA-R837@Cat based radiotherapy interact with immunological system, we harvested the sera of mice 72 h after different treatments and analyzed interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), and interleukin 12 (IL-12p40) by enzyme-linked immuno-sorbent assay (ELISA). While IL-12 plays important roles in activating natural killer cells, which are important in the activation of innate immunity,[18] IFN-γ and TNF-α are critical markers of cellular immunity and play critical roles in tumor immunotherapy.[19] It was found that although PLGA-R837@Cat injection alone as well as radiotherapy with PLGA-R837 or Cat@PLGA was able to increase the secretion of proinflammatory cytokines, the cytokines levels in sera from mice after radiotherapy with PLGA-R837@Cat appeared to be the highest among all tested group (Figure 3c–e). These results taken together indicated that radiotherapy enhanced by PLGA-R837@Cat was able to generate strong in vivo immunological stimulation effects, owning to the effective adjuvant function of those nanoparticles as well as tumor-associate antigens released after such enhanced radiotherapy.

It is known that ablative treatment methods such as radiotherapy may induce ICD characterized by high expression of calreticulin (CRT) on the surface of dying cancer cells, thereby inducing effective immune responses.[20] We studied the expression of CRT on the surface of cancer cells after different treatments. At 4 h after X-ray irradiation at the dose of 6 Gy, CT26 cancer cells were stained with Alexa Fluor 647-conjugated anti-CRT antibody for confocal imaging. Obvious expression of CRT on the surface of cells was observed after X-ray irradiation, indicating that radiotherapy would induce ICD of cancer cells (Figure 3f). While the elicitation of ICD is favorable for antitumor immune responses, there exist immune-suppressive mechanisms within the tumor to compensate antitumor immunities.[21] Immune-suppressive regulatory T cells (Treg) are often enriched in the tumor to protect tumor cells from being attacked by the immune system.[22] As assessed by flow cytometry, obvious increased Treg (Foxp3+CD4+T) levels were observed in the tumors after radiotherapy, especially in the presence of PLGA-R837@Cat nanoparticles (Figure 3g,h). Therefore, we speculate that the antitumor immune responses generated by radiotherapy-induced ICD alone may be compromised by Tregs recruited into the tumor postradiotherapy (Figure 3i).

The blockade of CTLA4, which is an important negative T-cell stimulatory receptor, could inhibit the activity of Treg.[23] Moreover, the CTLA-4 blockade in combination with other ablative treatments could effectively induce tumor-specific immune responses, consequently inhibiting cancer metastases.[10,17c] Thus, we then explored the in vivo combination of radiotherapy enhanced by PLGA-R837@Cat together with CTLA-4 blockade therapy. In our experiments, colorectal cancer CT26 cells were inoculated on the left flank of each mouse as the primary tumor. A week later, the second tumor, as an artificial mimic of metastasis, was inoculated on the right flank of the same mouse. Four days later, when the first tumors reached ∼250 mm³, mice were randomly divided into nine groups (n = 6 per group): (1) untreated; (2) X-ray radiation alone; (3) X-ray + αCTLA4 treatment; (4) X-ray + intratumoral (i.t.) injection of PLGA@Cat (PC); (5) X-ray + PC + αCTLA4; (6) X-ray + PLGA-R837 (PR); (7) X-ray + PR + αCTLA4; (8) X-ray + PLGA-R837@Cat (PRC); and (9) X-ray + PRC + αCTLA4. PLGA-based nanoparticles were locally injected into each tumor at the doses of 0.5 mg kg⁻¹ for catalase and/or 0.6 mg kg⁻¹ for R837, while the X-ray dose was 8 Gy. Afterward, mice were intravenously (i.v.) injected with αCTLA4 (clone 9H10) at the dose of 10 µg per mouse on day 1, 3, 5, and 7 post X-ray radiation (Figure 4a).

The tumor sizes on both sides were closely monitored afterward. For the primary tumors, while the X-ray radiation alone or i.v. injected with αCTLA4 showed no appreciable inhibition effect on such large tumors (∼250 mm³), radiotherapy with PLGA@Cat was found to be more effective in inhibiting the primary tumor growth (Figure 4b; Figure S5, Supporting Information), suggesting that the introduction of catalase could enhance the radiotherapy efficacy by improving the tumor oxygenation (Figure 2a). However, those tumors after PLGA@Cat-based radiotherapy recovered later. When in combination with αCTLA4, the growth of the primary tumors was significantly inhibited. Moreover, as the immune-adjuvant R837 loaded in PLGA could trigger strong immune responses as mentioned above, PLGA-R837 based radiotherapy could delay the tumor growth later, and its antitumor effect could be further increased after combination with αCTLA4. Interestingly, whereas radiotherapy with PLGA-R837@Cat could significantly suppress the tumor growth at the early days after treatment, the combination of PLGA-R837@Cat-based radiotherapy with αCTLA4 resulted in complete elimination of the primary tumors.

As far as the secondary tumors are concerned (Figure 4c), mice with their primary tumors treated by X-ray, even with injection PLGA@Cat, showed rather rapid growth. Notably, the for mice with radiotherapy of their primary tumors injected with PLGA-R837 or PLGA-R837@Cat, their secondary tumors showed obviously reduced growth speed, indicating the important role of immune-adjuvant R837 to induce strong antitumor immune responses. However, such treatments could only suppress the tumor growth within the first 12 days, after when the tumors still showed rapid growth. As expected, i.v. injection with αCTLA4 could offer remarkably improved the abscopal effects for the respective groups, especially the “PLGA-R837 + αCTLA4” group and “PLGA-R837@Cat + αCTLA4” group. The most excitingly, the second tumors on mice with their primary tumors treated by PLGA-R837@Cat-based radiotherapy completely disappeared after injection with αCTLA4. Those results together strongly demonstrated that radiotherapy with PLGA-R837@Cat in combination with CTLA4 blockade would induce strong systemic antitumor immune responses to suppress the growth of distant tumors, promising for metastasis inhibition.

The mechanisms of antitumor immune responses after combined radiotherapy-immunotherapy with our nanoparticles were then carefully studied. Cytotoxic T lymphocytes (CTLs, CD3+CD4+CD8+) could directly kill cancer cells by releasing cytotoxins such as IFN-γ, granzymes, granulysin, and perforin.[24] Therefore, the secondary tumors were collected with the immune cells analyzed by flow cytometry on day 8. It was found that the percentages of CD8+ CTLs in the secondary tumors of mice after radiotherapy with either of the three types of nanoparticles showed obviously increase compared to those with radiotherapy alone, and αCTLA4 treatment could further enhance CTL infiltration into secondary tumors. Notably, the
Figure 4. Antitumor immune effects of radiotherapy in combination with checkpoint blockade. a) Schematic illustration of our experiment design. Mice with CT26 tumors on both sides were used. Tumors on the left side were designated as “primary tumors” for radiotherapy, and those on the right side were designated as “distant tumors” without direct exposure to X-ray. Growth curves for b) primary tumors and c) distant tumors on mice after various treatments indicated. PC, PR, and PRC refer to PLGA@Cat, PLGA-R837, PLGA-R837@Cat, respectively. The dose of X-ray was 8 Gy. d) Representative flow cytometry plots showing different types of T cells (in all cancer cells) in secondary tumors from different groups of mice. e) Proportions of tumor-infiltrating CD8+ killer T cells and CD4+ T cells among all cancer cells. f) Proportions of FoxP3+ Tregs among CD4 + T cells. Error bars indicate s.e.m. Statistical significance was calculated by one-way ANOVA using the Tukey post-test. P-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
CTL infiltration level in the secondary tumors for mice with their primary tumors treated by PLGA-R837@Cat-based radiotherapy in combination with αCTLA4 was found to be the highest among all tested group (Figure 4d,e).

On the other hand, helper T cells (CD4+CD4+CD8-) play important roles in regulating the adaptive immune responses. It is known that CD4+ helper T cells could be classified into effector T cells (Teff) (CD3+CD4+Foxp3-) that are in favor of immune responses, together with regulatory T cells (Tregs) (CD3+CD4+Foxp3+) that instead are able to hamper effective antitumor immune responses.[19] It was found that although radiotherapy with PLGA@Cat, PLGA-R837, or PLGA-R837@Cat could promote the infiltration of CD4+ helper T cells into the secondary tumors, most of these CD4+ helper T cells were immunosuppressive Tregs. Excitingly, after introduction of αCTLA4 antibody, the percentage of Tregs in the secondary tumor showed significant decrease, indicating that CTLA-4 blockade was able to effectively abrogate the activity of Tregs, which were recruited into distant tumors after the radiotherapy treatment of the primary tumors (Figure 4f).

Based on the above data, the mechanism is proposed as follows. After relief of tumor hypoxia by Cat encapsulated inside PLGA-R837@Cat nanoparticles, the radiotherapy induced destruction of primary tumors under direct X-ray radiation would be enhanced, generating a large amount of tumor debris. Afterward, the released tumor associated antigens in the presence of R837-loaded adjuvant nanoparticles are able to induce strong immune responses, such as the effective recruitment of both CTLs and helper T cells into distant tumors. Meanwhile, CTLA4 blockade is able to effectively suppress the activity of immunosuppressive Tregs, further helpful to promote antitumor immunity to inhibit the growth of distant metastatic tumors.

Encouraged by the outstanding therapeutic efficacy achieved on the subcutaneous tumor model, we further assessed the efficacy of this combination therapy to treat a more aggressive metastatic tumor model. In this experiment, 4T1 murine breast cancer cells expressing firefly luciferase (fluc-4T1) were inoculated into the breast pad of each mouse to form the orthotopic murine breast tumor. A week later, when the tumors reached ≈50 mm³, fluc-4T1 tumor cells were i.v. injected into the mice to simulate the whole-body spreading of tumor cells. In the following day, the orthotopic tumor was removed by either surgery or radiotherapy. Then, in vivo bioluminescence imaging was used to track the growth and metastasis of fluc-4T1 cancer cells in different groups of mice (Figure 5a). For mice with their primary breast tumors removed by surgery, obvious bioluminescence signals, indication of tumor metastases, showed up 14 days after i.v. injection of fluc-4T1 cells, even if those mice were treated with αCTLA4, or the combination treatment of PLGA-R837@Cat and αCTLA4 (Figure 5b). By contrast, while mice after radiotherapy with PLGA-R837@Cat in the absence of αCTLA4 showed slightly delayed metastases, further combination with αCTLA4 treatment for mice after PLGA-R837@Cat-based radiotherapy could strongly inhibit tumor metastasis (Figure 5b).

Then, the survival of mice after different treatments was closely monitored (n = 10 for each group). It was found that 60% of mice could survive for 60 days after the combination therapy of PLGA-R837@Cat-based radiotherapy plus αCTLA4. In marked contrast, no single mouse from the other five control groups could survive in 40 days (Figure 5c). Our experiments further evidence that radiotherapy with PLGA-R837@Cat to treat the primary tumor plus the CTLA4 blockade could induce strong whole-body antitumor immunological responses, which would be able to effectively inhibit cancer metastases and greatly prolong the survival of mice with whole-body spreading tumors.

Immunological memory could enable the immune system to protect organisms from previously encountered pathogens, including cancer cells.[26] To study the immunological memory induced by radiotherapy with PLGA-R837@Cat nanoparticles (abbreviated as “RT”), we rechallenged mice which had no detectable tumors after surgery or RT 40 days later with the second batch of cancer cells. Mice were divided into four groups (n = 5 per group): (1) surgery; (2) surgery + αCTLA4 (pre & post); (3) RT + αCTLA4 (pre); and (4) RT + αCTLA4 (pre & post). Herein, αCTLA4 (10 μg per mouse for each injection) was treated for two rounds, with the first round (pre) given on day 1, 3, and 5, and 7 after eliminating their first tumors, and the second round (post) given right after reinoculation of the secondary tumors on day 41, 43, 45, and 47 (Figure 6a). For mice with surgical removal of their primary tumors, the growth of their rechallenged tumors was not significantly inhibited even plus two rounds of αCTLA4 treatment (pre & post). In marked contrast, for mice with their first tumors treated by RT plus one round of αCTLA4 treatment (pre & post), the tumor regrowth occurred for only 1 out 6 mice. For those mice with two rounds of αCTLA4 treatment (pre & post), no tumor was detectable for all mice (Figure 6b). Our results demonstrated the strong long-term immune protective effect induced by radiotherapy with PLGA-R837@Cat plus αCTLA4 treatment.

To investigate the mechanism of antitumor immune memory induced by the treatment, central memory T cells (T(CM)), and effector memory T cells (T(EM)) in the spleen were analyzed on day 40 in different groups of mice. Different from T(CM) cells, which would expand and differentiate at the very beginning of antigen-stimulation, T(EM) cells could induce strong immune memory protection effect by producing important cytokines such as TNF-α and IFN-γ.[27] Notably, the percentage of T(EM) cells (CD3+CD8+CD62L-CD44+) after PLGA-R837@Cat-based radiotherapy plus αCTLA4 was much higher than that in mice treated by surgery with or without αCTLA4 (Figure 6c,d). On day 40 and day 47 (a week after inoculating the secondary tumor), ELISA was used to measure the cytokines in sera of mice after different treatments. The levels of IL-12p70, an important marker of innate immunity, TNF-α and IFN-γ, the typical markers of cellular immunity, were all obviously higher in mice treated by PLGA-R837@Cat-based radiotherapy plus αCTLA4, in comparison to surgery or surgery plus two rounds of αCTLA4 (Figure 6e–j). Thus, radiotherapy with PLGA-R837@Cat plus αCTLA4 treatment indeed could induce strong immune memory to effectively inhibit the recurrence of cancer.

In this work, multifunctional PLGA-R837@Cat nanoparticles are fabricated by using fully biocompatible components for combined radio-immunotherapy of cancer. In our current PLGA-R837@Cat system, Cat is able to relive tumor hypoxia,
modulate the immune-suppressive tumor microenvironment, and enhance the destruction efficacy of primary tumors under direct X-ray radiation, while R837 loaded inside those nanoparticles could further stimulate the robust immune responses of tumor debris after radiotherapy-induced ICD. By further combining PLGA-R837@Cat-based radiotherapy with the clinically approved CTLA-4 checkpoint blockade therapy, we not only achieve a remarkable synergistic whole-body therapeutic effect to inhibit tumor metastases, but also realize effective long-term immune memory protection to prevent cancer recurrence.

Although the abscopal effect of radiotherapy has been observed in previous studies,\[12\] those effects often are not robust enough to dramatically impress tumor metastases. One possible reason is that although radiotherapy is able to elicit ICD for tumor cells, the increased Treg levels in the tumor after radiotherapy could compensate the antitumor abscopal effect in conventional radiotherapy. The immunotherapeutic outcome demonstrated by radiotherapy with PLGA-R837@Cat nanoparticles with both the tumor hypoxia relief function and the immune-adjuvant activity, and in further combination with CTLA4 checkpoint blockade to suppress Tregs, appears to be superior to that achieved in most of previous preclinical animal studies with conventional radiotherapy.\[2a\]

On the other hand, compared to immunotherapy triggered by nanoparticle-based photothermal or photodynamic therapy reported in our and others’ recent studies,\[10a,28\] triggering immunotherapy with radiotherapy would be more practical. Unlike light with limited tissue penetration depth, radiation beams are able to effectively destruct deeply buried intern tumors with large sizes. Considering
the extensive clinical use of radiotherapy, and the fully biocompatible components of our nanoparticles, our strategy by bridging radiotherapy with immunotherapy using nanotechnology may indeed have tremendous potential for clinical translation. The idea of triggering whole-body systemic immunotherapeutic effects with local treatment of radiotherapy could thus pave the way for the next generation cancer radiotherapy, especially for treatment of late-stage cancer patients that are not curable by conventional surgery or radiotherapy strategies.

Figure 6. Long-term immune-memory effects induced by PLGA-R837@Cat-based radiotherapy. a) Schematic illustration of PLGA-R837@Cat-based radiotherapy (abbreviated at RT) and αCTLA4 combination therapy to inhibit cancer relapse. b) Tumor growth curves of rechallenged tumors inoculated 40 days postelimination of their first tumors (eight mice per group) by surgery or RT plus αCTLA4. c–d) Proportions of effector memory T cells (TEM) in the spleen analyzed by flow cytometry (gated on CD3+CD8+ T cells) at day 40 right before rechallenging mice with secondary tumors. e–f) Cytokine levels in sera from mice isolated at day 40 right before rechallenging mice with secondary tumors. h–j) Cytokine levels in sera from mice isolated 7 days after mice were rechallenged with secondary tumors (at day 47). Error bars indicate s.e.m. Statistical significance was calculated by one-way ANOVA using the Tukey post-test. P-value: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Q.C. and J.C. contributed equally to this work. This article was supported in part by the National Basic Research Programs of China (973 Program) (2016YFA0021020), the National Natural Science Foundation of China (51525203 and 51761145041), Collaborative Innovation Center of Suzhou Nano Science and Technology, and a Project Funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

checkpoint blockade, immunotherapy, nanoparticles, radiotherapy, tumor hypoxia relief

Received: April 8, 2018
Revised: October 15, 2018
Published online:


