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Phototherapy using immunologically modified carbon nanotubes to potentiate checkpoint blockade for metastatic breast cancer

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Abstract

Metastasis is the major cause of cancer-death. Checkpoint inhibition shows great promise as an immunotherapeutic treatment for cancer patients. However, most currently available checkpoint inhibitors have low response rates. To augment the antitumor efficacy of checkpoint inhibitors, such as CTLA-4 antibodies, a single-walled carbon nanotube (SWNT) modified by a novel immunoadjuvant, glycosylated chitosan (GC), was used for the treatment of metastatic mammary tumors in mice. We treated the primary tumors by intratumoral administration of SWNT-GC, followed with irradiation with a 1064-nm laser to achieve local ablation through photothermal therapy (PTT). The treatment induced a systemic antitumor immunity which inhibited lung metastasis and prolonged the animal survival time of treated. Combining SWNT-GC-laser treatment with anti-CTLA-4 produced synergistic immunomodulatory effects and further extended the survival time of the treated mice. The results showed that the special combination, PTT +SWNT-GC+anti-CTLA-4, could effectively suppress primary tumors and inhibit metastases, providing a new treatment strategy for metastatic cancers.

Graphical Abstract

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Laser immunotherapy is an effective treatment modality for metastatic cancers. Single-walled carbon nanotubes (SWNTs), functionalized with an immunoadjuvant, glycated chitosan (GC), can increase the specificity and efficacy of the treatment, upon laser irradiation. Moreover, the treatment can potentiate the suppression of regulatory T cells checkpoint inhibitors such as anti-CTLA-4 antibodies. The combination treatment of laser-SWNT-GC enhances tumor antigen uptake and presentation, hence activating T cells to destroy residual tumor cells at the treatment sites as well as metastatic tumor cells at distant sites.

Keywords

Breast cancer; metastasis; photothermal therapy; checkpoint inhibition; immunotherapy; 1064-nm laser; glycated chitosan; single-walled carbon nanotube

Introduction

Although the treatment of cancer has been improving continuously, malignant tumors are still one of the major threats to human life. It is predicted that there will be over 1.7 million new cases of cancer and over 600,000 cancer-related deaths in the United States in 2018 [1]. Breast cancer has the highest frequency of incidence amongst women, and it is the second leading cause of cancer-related deaths worldwide. Many breast cancer patients already have metastases at the time of diagnosis, which is the main cause of breast cancer deaths [2, 3]. With continuous progress in immunological research, immunotherapy has shown great potential for clinical application to treat advanced cancers.

Current immunotherapy options include cytokine therapy [4], T-cell adoptive immunotherapy [5], checkpoint blockade therapy [6], and tumor vaccines [7]. Immune checkpoint blockade therapy targets the regulatory pathways in the immune system that are preventing antitumor immune responses, and has already been approved to treat chemotherapy-resistant, advanced solid tumors by the FDA. However, the clinical response rates of currently available checkpoint inhibitor were below 20%, due to their limitation of generating nonspecific immune response [8]. Cancer vaccines can induce specific anti-tumor immune responses against malignant tumors and trigger long-term immunological memory to suppress tumor recurrence [9,10]. Tumor vaccines using exogenous tumor-associated

antigens are inherently limited because of the heterogeneity and weak immunogenicity of many cancers [11].

Photothermal therapy (PTT) is a modality for local cancer treatment that uses heat generated from absorbed light energy to ablate tumors. PTT can trigger a host antitumor immune response by releasing tumor antigens into the local microenvironment. Commonly, PTT utilizes a light absorber directly injected into a tumor to increase the specificity and reduce the invasiveness. Because laser light in near-infrared (NIR) region has good tissue penetrability, ideal conditions for light absorbers include selective absorption in the NIR region [12]. In addition, light absorbers should have high concentration in tumors without direct cytotoxicity. In recent years, many nanomaterials with the above characteristics have been explored, and encouraging effects have been achieved in photothermal treatment of tumors using gold nanomaterials [13,14], carbon nanomaterials [15,16], and many others.

Laser immunotherapy has been developed to treat metastatic breast cancer, by inducing an *in situ* autologous vaccination, through the combination of local selective PTT and an immunoadjuvant, glycated chitosan (GC). Previous clinical results have indicated that local treatment with laser immunotherapy is capable of reducing and eliminating treated primary breast tumors as well as untreated metastases in the lungs, significantly prolonging patient survival [17].

Single-walled carbon nanotubes (SWNTs), a one-dimensional quantum wire, have been explored as both a light absorber and drug delivery vehicle for tumor theranostics [18]. In our designed anti-tumor therapy strategy, SWNTs enhance the selectivity of the treatment by locally absorbing NIR light in the tumor microenvironment and aiding the release of tumor antigens. By loading GC onto the SWNT surface, SWNT-GC further enhances the treatment by stimulating the host immune cells, inducing antitumor immune response. On this basis, the combined use with anti-CTLA checkpoint blockade to suppress the activity of immunosuppressive regulatory T cells (Tregs) should further enhance the immune response induced by laser+SWNT-GC [20]. In this study, we investigated the use of SWNT-GC with NIR laser irradiation to potentiate anti-CTLA checkpoint blockade therapy in a highly aggressive 4T1 murine breast cancer model.

2. Materials and methods

2.1. Preparation and characterization of SWNT-GC

The CoMoCAT® process produces SWNT using silica-supported bimetallic cobalt-molybdate catalysts [21]. The material consists of a narrow distribution of nanotube types with an average diameter of 0.81 nm and a length of 170 nm. The catalyst contained 6 wt% total metal with a Co:Mo molar ratio of 1:2 [22]. SWNT-GC solution was prepared as described in our previous work, the concentration of SWNT-GC solution was 5 mg SWNT per 25 mg GC per ml. GC combines with SWNT through strong non-covalent interactions to form a stable composite structure [23]. Briefly, 5 mg of SWNTs in powder form were mixed by sonication with 5 ml of 0.5% aqueous GC for 30 min using a ColeeParmer Ultrasonic Processor (CPX750) at 22% amplitude. The suspension was then centrifuged at 12,000 rpm for 30 min. The final concentration of SWNT-GC in the solution was determined by

comparing its optical absorbance with that of standard curve of SWNT solutions with known concentrations. The absorption spectra of SWNT-GC solution were measured by a UV-VIS-NIR spectrophotometer (VARIAN Cary 50 Bio, Agilent Technologies, Inc, USA). A transmission electron microscope (H-750, HITACHI, Tokyo, Japan) was used to examine the morphology and size characteristics of SWNT-GC. The photothermal conversion efficiency of SWNT-GC solution was determined by temperature increase using an infrared thermal imager (FLIR, Boston, MA, USA).

2.2. Cell culture

Murine tumor cell line 4T1 were cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA), and murine DC cell line DC 2.4 were cultured in DMEM (GIBCO), supplemented with 10% fetal bovine serum (FBS, ATCC, Manassas, VA, USA), penicillin (100 units/ml), and streptomycin (100 mg/ml) in 5% CO₂, 95% air at 37°C in a humidified incubator. All cell lines were tested and verified to be free of mycoplasma.

2.3. Animal model

Female BALB/c mice aged 6-8 weeks were purchased from Harlan Sprague Dawley Co. (Indianapolis, IN, USA). Mice were housed in the animal facility of the Department of Comparative Medicine at the University of Oklahoma Health Sciences Center (OUHSC). All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH) and approved by the OUHSC Institutional Animal Care and Use Committee (IACUC).

Tumor cells (4T1, 5×10^4 in 100 μ l PBS) were injected into the flank region of female BALB/c mice, aged 6-8 weeks. Animals were treated when the tumors reached a size of approximately 300 mm³.

2.4. Laser Treatment

For *in vitro* experiment, 4T1 cells cultured in 12-well plates (1×10^4 cells per well) were divided into control group, laser group and laser+ SWNT-GC group. PBS and SWNT-GC solution were added to different groups. After incubating for 12 hours, cells were washed with PBS, and irradiated with a 1064 nm laser at 1 W/cm² for 10 min.

For *in vivo* experiment, tumor-bearing mice were divided into different treatment groups. SWNT-GC solution (100 μ l, 5 mg-25 mg/ml) was directly injected into the center of each tumor 2 hours before laser irradiation. The laser light was delivered to the tumor using a fiber optic transmission system. The laser power was 1 W/cm² and the irradiation range included tumor and 0.5 cm surrounding skin with a treatment duration of 10 minutes. During laser irradiation, mice were anesthetized with a 4% isoflurane/96% oxygen mixture using a precision vaporizer during all treatments. Five mice in the control group, laser group, and laser+SWNT-GC group were sacrificed 15 days after treatment, and tumor size was measured.

2.5. The administration of laser treatment, SWNT-GC, IgG, and α -CTLA-4 antibody

For *in vitro* treatment, tumor cells were incubated with SWNT-GC at different concentrations for 2 h, rinsed with PBS, and then irradiated with laser light. The light source was a 1064 nm semiconductor laser (Laserglow, Toronto, ON, Canada).

For *in vivo* treatment, SWNT-GC solution (100 μ l) was directly injected into the center of tumor 2 h before laser irradiation. The laser was delivered to the tumor using a fiber optic delivery system with a power density of 1 W/cm² for 10 min. Tumor-bearing mice were anesthetized with 4% isoflurane/96% oxygen using a precision vaporizer during all the treatments. Anti-mouse IgG or anti-mouse α -CTLA-4 antibody (Leinco Technologies, MO, USA) were injected intraperitoneally 1, 4, or 7 days after laser treatment, at a dose of 200 μ g/kg. The mice were observed daily and euthanized when tumor diameter exceeded 20 mm.

2.6. Cell staining assays

To determine the effects of SWNT-GC on tumor cells, 4T1 cells cultured in 12-well plates (1 $\times$ 10⁴ cells per well), were divided into control and SWNT-GC groups, by incubating with PBS or SWNT-GC for 12h.

Trypan blue (Thermo Fisher Scientific, MA, USA) staining was used to detect the death cells. After mixing the Trypan blue solution and the cell suspension in a ratio of 1:1, the mixture (10 μ l) was added to the cell counting slide and counted using an automated cell counter (TC20, Bio-RAD, CA, USA). Calcein-AM (Thermo Fisher Scientific) and Propidium Iodide (PI) (Thermo Fisher Scientific) were also used to determine the survival and death of tumor cells. CM-H₂DCFDA (Thermo Fisher Scientific) was used to indicate the production of reactive oxygen species (ROS) in tumor cells. MitoTracker Orange CMTMRos (Thermo Fisher Scientific) was used to indicate mitochondrial membrane potential in tumor cells. Treated cells were washed with PBS, then stained with Calcein-AM (2 μ M) and PI (4 μ M), with CM-H₂DCFDA (10 mM) or CMTMRos (500 nM), for 30 mins at 37 °C. Then the cells were washed with PBS for 2-3 times and observed with a fluorescence microscope at 490 nm and 545 nm excitation wavelengths.

2.7. Wound healing assay

Cells were seeded in 6-well plate (1 $\times$ 10⁶ per well) for 24 h. Thereafter, a scratch (wound) was introduced in the cell layer using a pipette tip. Cells were washed three times with medium to remove detached cells. Then SWNT-GC was added, and the cells were incubated for 12 h. Images of the wounds were taken at 0 and 12h. The area of the wound in the images was measured using Image J.

2.8. ATP assay

Extra-cellular ATP was measured in the serum-free media via ATP bioluminescent assay kit (Sigma, St Louis, MO, USA) based on luciferin-luciferase conversion, following manufacturer's instructions. Bioluminescence was assessed by Cary Eclipse Spectrophotometer (Agilent Technologies, Inc, USA).

2.9. Detection of DCs activation

DC 2.4 cells (1×10^5) were incubated with treated tumor cells (5×10^5) in 24-well transwell plates (Sigma, MO, USA) for 24 hours in complete RPMI 1640. After incubation, DCs were collected and stained with CD40 or 80-FITC (eBioscience, CA, USA) for flow cytometry analysis, and the supernatants were collected for TNF α detection by an ELISA kit (R&D systems, Minneapolis, MN, USA).

2.10. ELISPOT assay of IFN- γ

Spleen cells were taken from the mice 7 days after treatments and incubated in the ELISPOT assay plate for 48 h. The cells were removed by washing, and the cytokine secretion was indicated by the blue spots. Pictures of the plates were taken, and the spots were counted.

2.11. Statistical Analysis

Values are expressed as mean + standard error of the mean. The data were analyzed with 1-way analysis of variance followed by Bonferroni posttest. Data between the two groups were compared using the unpaired T test. A *P* value of <0.05 was considered statistically significant. The statistical software used is SPSS 19.0.

3. Results

3.1. Characterization of SWNT-GC

The SWNT-GC solution showed a strong absorption capacity in the wavelength range of 950-1100 nm (Fig. 1A). Previous research by our team confirmed that SWNT-GC has similar light absorption characteristics to SWNT [19]. SWNT-GC has an elongated tubular structure when detected by transmission electron microscope (Fig. 1B). In order to detect the photothermal conversion by SWNT-GC, we examined the temperature change in SWNT-GC solution under laser irradiation and found that the temperature increase of SWNT-GC solution under laser irradiation depended on laser power densities (Fig. 1C) and SWNT-GC concentrations (Fig. 1D). To test the stability of the photothermal conversion efficiency of SWNT-GC, we performed intermittently repeated irradiation (5 min interval) on the SWNT-GC solution (100 $\mu\text{g/ml}$) using a 1064 nm laser (1 W/cm^2). The temperature curve showed that the temperature increase does not obviously change after repeated irradiation (Fig. 1E).

3.2. Cellular effects of SWNT-GC on 4T1 tumor cells

To determine the direct effect of SWNT-GC on 4T1 tumor cells, the changes in the reactive oxygen species (ROS) and mitochondrial membrane potential ($M\phi$) in the cells were observed by cell staining with indicators. The production of ROS in 4T1 cells was increased significantly in SWNT-GC group ($P < 0.01$) (Fig. 2A); however, there was no significant difference of $M\phi$ in SWNT-GC group (Fig. 2B). To further determine the effect of SWNT-GC on the biological behavior of tumor cells, wound healing experiments were performed. As shown in Fig. 2C, the migration speed of 4T1 tumor cells were significantly reduced by SWNT-GC.

To investigate the cytotoxicity of SWNT-GC on 4T1 cells, Calcein-AM/PI staining and trypan blue staining were performed. The cells were imaged using a fluorescent microscope

or counted using an automated cell counter. Both Calcein-AM/PI staining and trypan blue staining showed that the viability of 4T1 cells co-incubated with SWNT-GC were not significantly affected (Fig. 2D).

3.3. Cellular effects of laser+SWNT-GC

In order to test the cytotoxicity of SWNT-GC under laser irradiation, 4T1 tumor cells were co-incubated with SWNT-GC solution for 12 hours, followed by irradiation of the 1064 nm laser. Compared to laser only group, the group with SWNT-GC had a more pronounced temperature increase (Fig. 3A), which resulted in a high level of cell death (Fig. 3B). The ATP release assay also confirmed that the combination of laser and SWNT-GC released more ATP as compared to the control group (Fig. 3C). The Calcein-AM and propidium iodide (PI) staining results further confirmed that the combined use of SWNT-GC significantly enhanced the killing effect of laser irradiation on 4T1 cells (Fig. 3D).

To test the stimulatory effect of laser+SWNT-GC on dendritic cells (DCs), treated 4T1 cells were incubated with DC 2.4 cells for 24 h, and the activation of DCs was determined by flow cytometry and ELISA analysis. As shown in Fig. 4A–B, the expression of CD40 and CD80 on the DC surface were increased after the incubation with laser-treated 4T1 cells. However, the expression was much higher after the incubation with 4T1 cells treated by laser+SWNT-GC. The same results were confirmed by TNF α secretion from DC 2.4 cells under different treatments (Fig. 4C).

3.4. Therapeutic effects of Laser+SWNT-GC on 4T1 tumors

To investigate the effect of combination of SWNT-GC and 1064-nm laser, mice bearing 4T1 breast tumors were treated, when the tumor size reached 300 mm³. The temperature on the surface of the tumor was measured during treatment by an infrared thermal imager. The results showed SWNT-GC significantly increases the temperature on the tumors under the laser irradiation (Fig. 5A–B). Five days after the treatments, 5 mice in each group were sacrificed, and the tumor size and number of lung metastases were measured. The results showed that the tumors in the laser group was smaller than those of the control group; however, the diameter of the tumors in the laser+SWNT-GC group were significantly reduced compared to that of the laser group (Fig. 5C). The analysis of lung metastasis showed that there was no significant difference in the number of lung metastases in the laser group compared with the control group, while the number of lung metastases in the laser +SWNT-GC group was significantly less than that in the control group and the laser group (Fig. 5D).

The mice were closely observed, and the survival time was recorded for up to 50 days. Compared to the control group, the laser group did not show significant survival benefit (median survival time: control group vs. laser group: 27 days vs. 30 days). Compared with the control group and laser group, the survival time of laser+SWNT-GC group was significantly longer, and the median survival time was 42.5 days (Fig. 5E).

3.5 Efficacy enhancement of laser+SWNT-GC plus checkpoint inhibition

We next examined whether laser+SWNT-GC could be used to potentiate a checkpoint blockade therapy, such as anti-CTLA (α -CTLA4), to enhance the antitumor efficacy and antitumor immunity. The results showed that the survival time of mice in the treatment group using IgG and α -CTLA-4 antibody alone did not show a significant difference (median survival time: IgG group vs. α -CTLA-4 antibody group: 25 days vs. 25 days). The combination of laser+SWNT-GC with IgG or α -CTLA4 significantly increased the survival time of mice in both groups. In particular, the treatment of laser+SWNT-GC+ α -CTLA-4 achieved a significantly higher survival rate compared than that of laser+SWNT-GC+IgG treatment (median survival time: L+SWNT-GC+IgG group vs. L+SWNT-GC+ α -CTLA-4 antibody group: 46.5 days vs. 58 days) (Fig. 5F).

3.6. Antitumor immunity of combined Laser+SWNT-GC and checkpoint inhibition

To further determine the antitumor immune response induced by laser+SWNT-GC+ α -CTLA-4, an ELISPOT assay was performed to detect the presence of tumor antigen-specific T cells after treatment. Splenocytes were harvested from tumor bearing mice treated as described in Fig. 6A on day 7 and re-treated for another 48h. The production of IFN- γ by splenocytes from α -CTLA-4 treated mice was increased. However, the production of IFN- γ by splenocytes from laser+SWNT-GC treated mice was significantly increased. Especially, the combination of α -CTLA-4 with laser+SWNT-GC induced much higher IFN- γ production than that induced by laser+SWNT-GC or α -CTLA-4 alone (Fig. 6A).

The antitumor immune response elicited by laser+SWNT-GC was further confirmed by immunofluorescence assay. We found that laser+SWNT-GC instigated CD8⁺ T cell infiltration within treated tumor tissue. Particularly, tumor-infiltrating CD8⁺ T cells were significantly enhanced in the treatment group of laser+SWNT-GC+ α -CTLA-4. No tumor-infiltrating CD8⁺ T cells were observed in untreated mice (Fig. 6B).

4. Discussion

Metastasis is the main cause of death from breast cancer [24]. Therefore, it is of great clinical value to kill local tumors and control metastatic tumors at the same time. We developed a combination treatment strategy, using PTT combined with SWNT-GC, as well as anti-CTLA-4, to enhance therapeutic effects in metastatic mouse tumors. The combination of PTT+SWNT-GC can kill orthotopic breast tumors and release tumor antigens, effectively stimulating a specific anti-tumor immune response. Combining anti-CTLA checkpoint blockade treatment can significantly enhance the body's anti-tumor immune response, resulting in a stronger and more lasting inhibitory effect on metastatic tumors.

SWNT-GC consists of a photothermal agent and an immunoadjuvant, designed to generate antitumor immunological effects. It could maintain both the optical properties of the SWNT and the immunological functions of GC. Previous studies have confirmed that similar to SWNTs, SWNT-GC also have no direct cytotoxic effects on tumor cells [25]. Similar killing effects were obtained in tumor cells using SWNT or SWNT-GC combined with laser

treatment, whereas in tumor animal models, Laser+SWNT-GC was able to achieve higher tumor killing compared to laser+SWNT [25,19].

Due to the unique combination of SWNT and GC, they can target the tumor cells simultaneously. First, SWNT selectively absorbs laser to induce the tumor cell death. Second, GC could enhance the tumor immunogenicity and induce immune cells activation, which resulted in the combined effect of an induced immunity. Therefore, the advantage of SWNT-GC in cancer phototherapy depends on the synergistic effect of SWNT and GC.

Previous research by our research team confirmed that SWNT-GC has similar photothermal conversion capability to SWNT [19]. Due to the photothermal conversion effects, SWNT-GC can trigger effective photothermal interaction in combination with NIR laser to achieve local ablation in deeper tumors. SWNT-GC significantly enhanced the temperature in tumors under irradiation of a 1064-nm laser. Due to the heat intolerance of tumor cells, the higher temperatures lead to better local therapeutic effects with suppressed tumor growth. Simultaneously, dead or dying tumor cells release antigens, which are important to stimulate tumor-specific immune response [26]. The ability of DCs to induce and regulate the host's innate and adaptive immune responses plays an important role in anti-tumor immune responses. DCs are important regulators of T lymphocyte and B lymphocyte immunity and they are the most important antigen presenting cells (APCs) [27]. CD40 and CD80 are the important costimulatory molecules that indicate mature DCs. The expression of CD40 on DCs indicates their ability to present antigens and activate MHC class II (MHC-II) restricted T cells [28]. T cells activate the costimulatory activity of CD80 by binding to CD40, and CD40 signaling enhances APC-activated T cell efficiency, characterized by a marked increase in CD80 expression [29, 30]. Our experimental results showed that the laser treatment can increase the expression of CD40 and CD80. However, the expression of CD40 and CD80 in DCs was even further increased under the condition of combined use of laser and SWNT-GC.

Using non-metastatic EMT6 murine breast model, the combination of SWNT-GC with 980-nm laser irradiation could not only eradicate the treated primary tumor but also induce abscopal effects by triggering a systemic antitumor immunity. The previous results confirmed that laser+SWNT-GC treated tumor cells stimulated macrophages to secrete more IFN- γ and significantly enhanced the activation and phagocytosis of DC compared to laser or laser+SWNT alone [19]. Here, we further demonstrated the systemic antitumor effects by laser+SWNT-GC using a metastatic 4T1 murine breast model. After the treatment on primary tumors using a 1064-nm laser, lungs from treated mice showed no significant difference in terms of the number of lung metastases between laser group and control group. However, in the group of laser+SWNT-GC, the number of lung metastases was significantly decreased. Since laser+SWNT-GC can enhance the local therapeutic effect of primary tumors and inhibit lung metastasis, the survival of 4T1 tumor-bearing mice were significantly prolonged. These results further confirmed that SWNT-GC can stimulate the body's anti-tumor immune response, thus controlling metastatic tumors.

The purpose of anti-CTLA therapy is to block the CTLA-4 signaling pathway, thereby reducing the immunosuppressive effects of malignant tumors and improving the body's own

anti-tumor immune response [31]. In recent years, CTLA-4 inhibition therapy has been approved by the FDA as a clinical treatment for advanced malignant tumors, and satisfactory treatment effects have been obtained in some patients [32]. In contrast, the primary reason why many patients fail to benefit from anti-CTLA therapy is thought to be the depletion of immune cells in patients with advanced malignancies, resulting in a lack of local infiltration of immune cells. The results of our study confirmed that laser+SWNT-GC can promote the number and quality of immune cells in mice. Therefore, laser+SWNT-GC can theoretically be an effective complement to anti-CTLA therapy. The effect of stimulating the infiltration of T cells by laser+SWNT-GC and reducing immunosuppression by anti-CTLA therapy can produce a synergistic therapeutic effect [31].

Our experimental result demonstrated that laser+SWNT-GC+anti-CTLA could further prolong the survival of tumor-bearing mice compared to laser+SWNT-GC treatment alone. The results of immunostimulation experiments showed that Laser+SWNT-GC combined with anti-CTLA treatment can significantly increase IFN- γ secretion from spleen cells and induce CD8⁺ T cell infiltration in tumor tissues. Cytotoxic T cells with CD8⁺ are important effector cells of the body's immune response [33]. Previous studies have shown that antigen-specific cytotoxic T cells (CTLs) lead to the emergence of resistant cancer cell clones in the presence of IFN- γ in the tumor microenvironment [34]. IFN- γ has also been shown to be a key requirement in the process of inducing T cell migration to tumor sites [35]. The above results proved that the combination of laser+SWNT-GC+anti-CTLA can further enhance the effect of immunotherapy by stimulating the systemic anti-tumor immune response.

5. Conclusion

In summary, we designed an effective treatment strategy consisting of PTT combined with SWNT-GC and checkpoint inhibitors for the treatment of metastatic cancers. PTT releases antigens into the local microenvironment by killing tumor cells, and the composite nanomaterial SWNT-GC stimulates an antitumor immune response while enhancing local tumor killing effects. The checkpoint inhibition treatment significantly enhances the antitumor immune response. The therapeutic strategies of PTT combined with SWNT-GC and checkpoint inhibitor have showed a high efficacy in the controlling primary tumors and metastases in the 4T1 breast cancer model. This combination therapy provides a novel treatment strategy for comprehensive treatment of metastatic tumors.

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References

- [1]. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* 2018; 68(1):7–30. [PubMed: 29313949]
- [2]. Yousefi M, Nosrati R, Salmaninejad A, et al. Organ-specific metastasis of breast cancer: molecular and cellular mechanisms underlying lung metastasis. *Cell Oncol (Dordr).* 2018; doi: 10.1007.

- [3]. Mariotto AB, Etzioni R, Hurlbert M, et al. Estimation of the Number of Women Living with Metastatic Breast Cancer in the United States. *Cancer Epidemiol Biomarkers Prev.* 2017; 26(6):809–815. [PubMed: 28522448]
- [4]. Xu HM. Th1 cytokine-based immunotherapy for cancer. *Hepatobiliary Pancreat Dis Int.* 2014; 13(5):482–494. [PubMed: 25308358]
- [5]. Al-Khami AA, Mehrotra S, Nishimura MI. Adoptive immunotherapy of cancer: Gene transfer of T cell specificity. *Self Nonself.* 2011; 2(2):80–84. [PubMed: 22299059]
- [6]. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* 2018; 359(6382):1350–1355. [PubMed: 29567705]
- [7]. Sahin U, Tureci O. Personalized vaccines for cancer immunotherapy. *Science.* 2018; 359(6382):1355–1360. [PubMed: 29567706]
- [8]. Ciccicarese C, Alfieri S, Santoni M, et al. New toxicity profile for novel immunotherapy agents: focus on immune-checkpoint inhibitors. *Expert Opin Drug Metab Toxicol.* 2016; 12(1):57–75. [PubMed: 26565919]
- [9]. Maeng H, Terabe M, Berzofsky JA. Cancer vaccines: translation from mice to human clinical trials. *Curr Opin Immunol.* 2018; 51:111–122. [PubMed: 29554495]
- [10]. Lollini PL, Cavallo F, Nanni P, et al. Vaccines for tumour prevention. *Nat Rev Cancer.* 2006; 6(3):204–216. [PubMed: 16498443]
- [11]. Finn OJ. The dawn of vaccines for cancer prevention. *Nat Rev Immunol.* 2018; 18(3):183–194. [PubMed: 29279613]
- [12]. de Melo-Diogo D, Pais-Silva C, Dias DR, et al. Strategies to Improve Cancer Photothermal Therapy Mediated by Nanomaterials. *Adv Healthc Mater.* 2017; 6(10): doi: 10.1002.
- [13]. Huang XH, El-Sayed IH, Qian W, et al. Cancer Cell Imaging and Photothermal Therapy in the Near-Infrared Region by Using Gold Nanorods. *J. Am. Chem. Soc* 2006; 128(6): 2115–2120. [PubMed: 16464114]
- [14]. Chen J, Glaus C, Laforest R, et al. Gold nanocages as photothermal transducers for cancer treatment. *Small.* 2010; 6(7): 811–817. [PubMed: 20225187]
- [15]. Ming Z, Wentao W, Fan W, et al. Magnetic and fluorescent carbon nanotubes for dual modal imaging and photothermal and chemo-therapy of cancer cells in living mice. *Carbon.* 2017; 123:70–83.
- [16]. Yang C, Yuliang G, Pomchol H, et al. Graphitic carbon nanocages as new photothermal agent and drug Carrier for 980-nm-laser-driven cancer therapy. *Carbon.* 2018; 136:234–247.
- [17]. Chen WR, Korbelik M, Bartels KE, et al. Enhancement of 1136 aser cancer treatment by a chitosan-derived immunoadjuvant. *Photochem Photobiol.* 2005; 81(1):190–195. [PubMed: 15535737]
- [18]. Yao HJ, Sun L, Liu Y, et al. Monodistearoylphosphatidylethanolamine-hyaluronic acid functionalization of single-walled carbon nanotubes for targeting intracellular drug delivery to overcome multidrug resistance of cancer cells. *Carbon* 2016; 96:362–376.
- [19]. Zhou F, Wu S, Song S, et al. Antitumor immunologically modified carbon nanotubes for photothermal therapy. *Biomaterials.* 2012; 33(11):3235–3242. [PubMed: 22296829]
- [20]. Buchbinder E, Hodi FS. Cytotoxic T lymphocyte antigen-4 and immune checkpoint blockade. *J Clin Invest.* 2015; 125(9):3377–3383. [PubMed: 26325034]
- [21]. Kitiyanan B, Alvarez WE, Harwell JH, et al. Controlled production of single-wall carbon nanotubes by catalytic decomposition of CO on bimetallic Co-Mo catalysts. *Chem Phys Lett.* 2000; 317(3–5):497–503.
- [22]. Bachilo SM, Balzano L, Herrera JE, et al. Narrow (n,m)-distribution of single-walled carbon nanotubes grown using a solid supported catalyst. *J Am Chem Soc.* 2003; 125(37):11186–11187. [PubMed: 16220926]
- [23]. Saha LC, Nag OK, Doughty A, et al. An Immunologically Modified Nanosystem Based on Noncovalent Binding Between Single-Walled Carbon Nanotubes and Glycated Chitosan. *Technol Cancer Res Treat.* 2018 1 1;17:1533033818802313. [PubMed: 30261832]
- [24]. Scully OJ, Bay BH, Yip G, et al. Breast cancer metastasis. *Cancer Genomics Proteomics.* 2012; 9(5):311–320. [PubMed: 22990110]

- [25]. Zhou F , Xing D , Ou Z , et al. Cancer photothermal therapy in the near-infrared region by using single-walled carbon nanotubes. *J Biomed Opt* 2009 Mar-Apr (2): 021009. [PubMed: 19405722]
- [26]. Zhou F, Xing D, Chen WR. Regulation of HSP70 on activating macrophages using PDT-induced apoptotic cells. *Int J Cancer*. 2009; 125(6):1380–1389. [PubMed: 19533746]
- [27]. Tacke PJ, de Vries IJ, Torensma R, et al. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. *Nat Rev Immunol*. 2007; 7(10):790–802. [PubMed: 17853902]
- [28]. Grewal IS, Foellmer HG, Grewal KD, et al. Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science*. 1996; 273(5283): 1864–1867. [PubMed: 8791592]
- [29]. Clatza A, Bonifaz LC, Vignali DA, et al. CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. *J Immunol*. 2003; 171(12):6478–6487. [PubMed: 14662847]
- [30]. Jia R, Li X, Yu C, et al. The splicing factor hnRNP C regulates expression of co-stimulatory molecules CD80 and CD40 in dendritic cells. *Immunol Lett*. 2013; 153(1-2):27–32. [PubMed: 23831410]
- [31]. Kvistborg P, Philips D, Kelderman S, et al. Anti-CTLA-4 therapy broadens the melanoma-reactive CD8⁺ T cell response. *Sci Transl Med*. 2014; 6(254):254ral28.
- [32]. Finn L, Markovic SN, Joseph RW. Therapy for metastatic melanoma: the past, present, and future. *BMC Med*. 2012; 10:23. [PubMed: 22385436]
- [33]. Mittrücker HW, Visekruna A, Huber M. Heterogeneity in the differentiation and function of CD8⁺T cells. *Arch Immunol Ther Exp (Warsz)*. 2014 12;62(6):449–458. [PubMed: 24879097]
- [34]. Takeda K, Nakayama M, Hayakawa Y, et al. IFN- γ is required for cytotoxic T cell-dependent cancer genome immunoediting. *Nat Commun*. 2017 2 24;8:14607. [PubMed: 28233863]
- [35]. Nakajima C, Uekusa Y, Iwasaki M, et al. A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. *Cancer Res*. 2001 4 15;61(8):3399–3405. [PubMed: 11309299]

Fig. 1. Characteristics of SWNT-GC.

A. Absorption spectra of SWNT-GC solution (100 µg/ml). **B.** The images of SWNT-GC detected by transmission electron microscope. **C.** Temperature increase of SWNT-GC solution (100 µg/ml) under irradiation of a 1064-nm laser at different doses. **D.** Temperature increase of SWNT-GC solution at different concentrations under laser irradiation (1 W/cm²). **E.** The temperature curve of SWNT-GC solution (100 µg/ml) which was repeatedly irradiated by a 1064 nm laser (1 W/cm²).

Fig. 2. Effects of SWNT-GC on 4T1 tumor cells

A. Fluorescent images of treated cells stained with CM-H₂DCFDA (indicator of ROS) and CMTMRos (indicator of Mφ). Bar graphs show results of densitometry analysis of indicated fluorescence intensity. (n=5, ** $P < 0.01$). **B.** The images of scratch (wound) at 0 h and 12 h in different groups. Bar graph shows result of migration distance of cells. (n=5, * $P < 0.05$). **C.** Images of treated cells stained with Calcein-AM (green, for live cells) and PI (red, for dead cells) **D.** Cell viability of treated cells determined by Trypan blue staining. (n=5).

Fig. 3. Cytotoxic effects of SWNT-GC on 4T1 tumor cells combined with laser.

A. Temperature increase of 4T1 cells under different treatments. Optical image of the treatment of laser irradiation and the typical thermographic image of tumor cells following laser treatment. **B.** Viability of cells 3 h, 6h and 12h after different treatments, determined by trypan blue staining ($n = 5$, ** $P < 0.01$). **C.** ATP release from cells 3 h after different treatments ($n = 5$, * $P < 0.05$). **D.** Images of cells 3h after different treatments, stained with Calcein-AM (green, for live cells) and PI (red, for dead cells).

Fig. 4. Immunological effects of SWNT-GC on DCs.

A. CD40 and CD80 expression on DCs stimulated by treated 4T1 cells. **B.** The percentage of CD40⁺/CD80⁺ cells under different treatments, (n = 5, * $P < 0.05$). **C.** TNFα secretion by DCs stimulated by treated 4T1 cells, (n = 5, * $P < 0.05$).

Fig. 5. Therapeutic effects of SWNT-GC combined with laser on 4T1 tumors

A. Thermographic images of tumor bearing mice under laser irradiation with or without SWNT-GC. Target spot indicated the treated tumors. **B.** Temperature increase on the surface of tumor tissue under laser irradiation with or without SWNT-GC. **C.** The diameter of tumors 15 days after different treatments, ($n = 5$, $*P < 0.05$). **D.** Representative images of lung tissue with H&E staining in different treatment groups. Bar graph shows the number of lung metastases 15 days after different treatments ($n = 5$, $*P < 0.05$). **E.** Survival rate of 4T1 tumor bearing mice in the indicated treatment groups up to 50 days after the initial tumor

inoculation, (n =10, laser+SWNT-GC group vs. control group, $P < 0.05$; laser+SWNT-GC group vs. laser group, $P < 0.05$). **F.** Survival rate of 4T1 tumor bearing mice in the indicated treatment groups up to 60 days after the initial tumor inoculation, (n =6, laser+SWNT-GC +IgG group vs. IgG group, $P < 0.05$; laser+SWNT-GC+IgG group vs. α -CTLA-4 group, $P < 0.05$; laser+SWNT-GC+ α -CTLA-4 group vs. IgG group, $P < 0.05$; laser+SWNT-GC+ α -CTLA-4 group vs. α -CTLA-4 group, $P < 0.05$; laser+SWNT-GC+ α -CTLA-4 group vs. laser +SWNT-GC+IgG group, $P < 0.05$).

Fig. 6. *In vivo* immunostimulatory effect of Laser+SWNT-GC combined with α-CTLA
A. Representative images of blue spots that indicated IFN- γ secretion of splenocytes harvested from treated mice. Bar graph shows the number of spots in different treatment groups. (n=6 * P < 0.05). **B.** Representative images of tumor sections with immunofluorescent staining. Bar graph shows the proportion of CD8+ cells in tumor tissue. (n=4 * P < 0.05).