Immune-adjuvant loaded Bi₂Se₃ nanocage for photothermal-improved PD-L1 checkpoint blockade immune-tumor metastasis therapy

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ABSTRACT

Checkpoint blockade based immune therapy has shown to be effective but benefit only the minority of patients whose tumors have been pre-infiltrated by T cells. To overcome this obstacles, a PEG-modified Bi₂Se₃ nanocage (NC) loaded with imiquimod (R848), which could efficiently destroy the tumors thus producing enough tumor-associated antigens (TAA) and with the existence of R848, a toll-like-receptor-7 agonist, could generate strong anti-cancer immune responses is reported in this study. Moreover, immunogenic Bi₂Se₃ NC-PEG/R848 mediated photothermal therapy (PTT) sensitizes tumors to checkpoint inhibition mediated by a PD-L1 antibody, not only ablating cancer cells upon NIR laser but also causing strong anti-cancer immunity to suppress distant tumor growth post PTT. Both *in vitro* and *in vivo* experiments demonstrate that the Bi₂Se₃ NC-PEG/R848 could effectively activate a PTT-induced immune response as well as silence immune resistance based on PD-L1 checkpoint blockade to ablate the primary tumor and further inhibit the tumor metastasis. Bi₂Se₃ NC reported here exhibits high photothermal conversion efficiency and stability, as well as competent drug loading capacity with large hollow structures and high surface area. Our study not only provides a facial way to synthesize Bi₂Se₃ NC, but also offers an alternative strategy for tumor metastasis.

KEYWORDS

Bi₂Se₃ nanocage, R848, checkpoint blockade, photothermal-immune therapy, anti-tumor metastasis

1 Introduction

On account of the highly complex biological procedure in cancer formation, tumor metastasis is yet hard to be cured and causes the majority of cancer deaths. Considering the treatment strategies, conventional radio- and chemo-therapy are both suffered by limited efficiency and serious side-effect [1–4]. Thus, it is significant to uncover efficient ways to eliminate and inhibit tumor metastasis.

Due to the stimulation of specific immune-therapy and long-term immune memory capacities [5–7], cancer immune-therapy have drawn great attentions in recent years, particularly for checkpoint blockade therapy and adoptive T cell transfer. Cancer immune-therapy could eliminate the primary tumor, moreover, offer great chances to tumor metastases [8]. Immune checkpoint blockade therapy is a hopeful strategy against tumor metastatic through tumor specific T cells activation, especially tumor-infiltrating cytotoxic T lymphocytes (CTLs) [9–11]. Nevertheless, the activation of anti-cancer immune response and evasion has limited the efficiency of immune checkpoint blockade therapy. Cancer cells could grow against immune response of the host based on a self-protecting mechanism, immune evasion [12]. In the associated processes, programmed death 1 (PD-1) and corresponding receptor (PD-L1) are important immune checkpoint molecules [13, 14]. PD-1 is a type of immunosuppression molecule expressed on the surface of many types of cells, including T cells. After specifically bound with PD-L1, a trans-membrane protein over-expressed in many malignant tumors [15], which may transmit suppressed signal thus suppressing cytokines excretion as well as causing T cells apoptosis [16–18]. Nevertheless, the efficiency

of anti-cancer immune-therapy is yet limited by ineffective immune activation, in spite of efficient decreased immune evasion by PD-L1 checkpoint blockade.

Recently, photothermal-immune therapy has gain lots of attentions due to promising use in PTT-induced anti-cancer immune response activation [19–21]. Through producing hyperthermia, PTT may ablate the cancer cells as well as release tumor-associated antigens (TAA), which will be presented to CTLs and thus activate anti-cancer immunotherapy. Still, immune evasion has negative influence on PTT *in situ* of tumor sites [22, 23]. As a consequence, it is promising to combine PTT and PD-L1 checkpoint blockade immune therapy to increase the efficacy of tumor metastases therapy [21, 23, 24]. Besides, compared with CTLA4, PD-L1 can be expressed on many cell type, including T cells, epithelial cells, endothelial cells, and tumor cells after exposure to the cytokine IFN-γ, produced by activated T cells [25]. This has led to the notion that rather than functioning early in T cell activation, the PD-1/PD-L1 pathway acts to protect cells from T cell attack. Thus, the existence of a T cell infiltrate and select biomarkers, such as expression of PD-L1, which indicate a “hot” tumor microenvironment, does correlate with clinical benefit for patients treated with anti-PD-1 or anti-PD-L1 [13].

Herein, we first introduce a hollow photothermal agent, bismuth selenide nanocage (Bi₂Se₃ NC) based nanomedicine for anti-tumor photothermal-immune therapy, which integrates enhanced immune response activation and PD-L1/PD-1 suppression. As a representative topological insulators, Bi₂Se₃ possess distinguished thermo-electric and photo-electric performance, thus is applying for photothermal and CT contrast agent *in vivo* [26]. Among reported Bi₂Se₃ nanoparticles,

Bi_2Se_3 NC with stronger NIR region optical absorption has not been studied yet. Such high tissue absorption coefficient enables Bi_2Se_3 NC with higher *in vivo* PT conversion efficiency. Meanwhile, compared with other reported Bi_2Se_3 nanoparticles, Bi_2Se_3 NC is a desired drug carrier with larger free volume. Due to these interesting characters, in this study, we synthesized PEG-modified Bi_2Se_3 NC with good bioactivity and biocompatibility to load Resiquimod (R848), a toll-like receptor 7 and 8 (TLR7/TLR8) agonist to enhance immune responses activation [27, 28]. Under NIR laser irradiation, Bi_2Se_3 NC-PEG/R848 induced PTT could ablate primary tumors then expose TAA, which would display vaccine-like properties with the help of nanoparticles loading R848 immune adjuvant. Subsequently, professional antigen presenting cells, such as dendritic cells (DCs) would present these exposed TAA to activate CTLs. In addition, anti-PD-L1 checkpoint blockade could enhance and protect the activity of CTLs. At last, CTLs could move to distant cancer cells without NIR laser irradiation and regulate cell immune response to kill metastasizing cancer cells particularly. Moreover, NC-PEG/R848-based PTT in combination with anti-PD-L1 therapy is able to protect treated mice from tumor re-challenging 40 days after primary tumors ablation, verifying a strong immune-memory effect to save mice from tumor relapse. Thus, we hypothesize that our Bi_2Se_3 NC-PEG/R848 nanoparticle could synergistically inhibit the primary and distant tumor growth through PTT improved PD-L1 immune-therapy.

Scheme 1 Illustration of (a) synthesis procedure of Bi_2Se_3 nanocage (NC)-PEG/R848, (b) Bi_2Se_3 NC-PEG/R848 induced combined photothermal- and immune-therapy.

2 Experimental

2.1 Materials

Oleylamine (OM, 70%, Sigma-Aldrich), oleic acid (OA, 90%, Sigma-Aldrich), 1-octadecene (ODE, > 90%, Sigma-Aldrich), decanoic acid (> 90%, Sigma-Aldrich), 1-dodecanethiol (1-DDT, ≥ 98%, Sigma-Aldrich), $\text{Mn}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$ (99%, Alfa Aesar), Se (> 99.5%, Alfa Aesar), bismuth neodecanoate (98%, Sigma-Aldrich), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride crystalline (EDC) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich), poly(maleic anhydride-alt-1-octadecene) (C18PMH) (Sigma-Aldrich) and mPEG-NH₂ ($M_w = 5\text{K}$) (Biomatrik Co., Ltd). All chemicals were used as received.

2.2 Synthesis of Bi_2Se_3 -PEG/R848 nanoparticles

We first synthesized the MnSe nanocube template based on a reported way [29]. Briefly, we first added manganese acetate tetrahydrate

(22.5 mg) into a mixture of 1-octadecene (ODE, 3.2 mL), oleylamine (OM, 8.75 mL) and OA (1.07 mL) at room temperature. After that, we heated the mixture to 120 °C and maintained this temperature for 60 min, thus producing transparent mixture (solution 1). Afterwards, selenium (40 mg) was dispersed in a solution of OM (3 mL) and 1-dodecanethiol (1-DDT, 0.1 mL), then injected swiftly this mixture into solution 1 at 220 °C. Further kept at 220 °C for 120 min before cooling down to 50 °C.

After MnSe nanocube was cooled down to 50 °C, ODE (2.5 mL) containing bismuth neodecanoate (63 μL) was then injected into the mixture under constant flow of nitrogen. Following, we heated the mixture to 180 °C gradually and then kept for 30 min under vigorous stirring (1,000 rpm). After cooling down to room temperature, Bi_2Se_3 NC was gained by centrifugation (8,000 rpm, 10 min), and then washed twice with ethanol and chloroform [30].

OM ligand coated Bi_2Se_3 NC was then decorated with C18PMH-PEG via an amphiphilic polymer modifying strategy [31]. We dispersed the Bi_2Se_3 NC (20 mg) in chloroform (2 mL) contained C18PMH-PEG (50 mg) and constantly stirred for 18 h. Next, the chloroform was evaporated and then added water (10 mL) into this solution under ultrasonication (15 min). Bi_2Se_3 NC-PEG was thus obtained through centrifugation (12,000 rpm, 10 min).

2.3 R848 loading and releasing

As for R848 loading, 30 μL R848 solutions in DMSO (3 mg/mL) was added to 1 mL Bi_2Se_3 NC-PEG (4 mg/mL). The unloaded R848 was filtered and washed after 24 h constant stirring. For R848 release study, we packed Bi_2Se_3 NC-PEG/R848 (1 mL) and PBS (19 mL) into a dialysis bag (MWCO: 13 kDa). Then we took out the incubation medium (2 mL) at diverse time points and refreshed with new ones. To study R848 release property induced by NIR laser, we adopted an 808 nm laser to irradiate the Bi_2Se_3 NC-PEG/R848 solution at diverse time point.

As for Cy5.5 labeled Bi_2Se_3 NC-PEG/R848, 30 μL R848 solutions in DMSO (3 mg/mL) and 10 μL Cy5.5 solution in DMSO (3 mg/mL) was added to 1 mL Bi_2Se_3 NC-PEG (4 mg/mL). The unloaded R848 and Cy5.5 were filtered and washed after 24 h constant stirring.

2.4 Photothermal performance

We irradiated H₂O (0.5 mL) and various concentrations of Bi_2Se_3 NC/R848 (0.5 mL) with NIR laser (808 nm, 0.8 W/cm², 5 min). We monitored the solution temperature via a digital thermometer at certain time points. The real time thermal images for PBS (0.5 mL) and Bi_2Se_3 NC-PEG/R848 ([NC-PEG] = 80 μg/mL, [Cy5.5] = 6 μg/mL, [R848] = 0.8 μg/mL, 0.5 mL) was taken via an infrared thermal camera as well. In order to calculate the PT conversion efficiency (η), we monitored the temperature changes of Bi_2Se_3 NC-PEG/R848 solution (60 μg/mL, 0.5 mL) upon NIR laser irradiation (808 nm, 0.8 W/cm²) at designed time points. We then calculated the η by the following the formula: $\eta = \frac{hS(T_{\max} - T_{\text{surr}}) - Q_s}{I(1 - 10^{-A_{808}})} \times 100\%$, hS was obtained from Fig. 3(g) [30].

2.5 Cellular experiments

Murine breast cancer 4T1 cells were cultured under recommended conditions with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO₂-containing condition. As for cytotoxicity experiments *in vitro*, 4T1 cells were seeded into 96-well plates at a density of 5×10^4 cells/well until adherent. After incubating with diverse concentrations of Bi_2Se_3 NC-PEG/R84, they were further kept in dark at 37 °C for another 24 h. As for PT treatment group, we irradiated the cells with NIR laser (808 nm, 0.8 W/cm², 3 min) after 6 h incubated. Then we demonstrated the cell viability via the MTT assay.

For Calcein AM/PI co-stained study, we seeded 4T1 cells with a density of 5×10^5 cells per well in CLSM culture dishes and then incubated them in PBS and different formulations. After 6 h, NC- and NC-PEG/R848-treated culture dishes were irradiated with an 808 nm laser (0.8 W/cm², 3 min), with further incubation for 24 h, respectively. After removed the culture medium, we added the AO (10 ng/mL, 1 mL) and PI (10 ng/mL, 1 mL) respectively into culture dishes then further incubated for 20 and 30 min. Finally, we washed the dishes several times with PBS (pH 7.4) and then observed through CLSM.

In order to learn the NC-PEG/R848 cellular uptake profile *in vitro*, we seeded the cells into the CLSM dishes at a density of 1×10^5 cells per well. Post 12 h incubation, we added free Cy5.5 and Cy5.5 labeled-Bi₂Se₃ NC-PEG/R848. After another 4 h incubation, we irradiated the cells under NIR laser (808 nm, 0.8 W/cm², 3 min), followed by washing with PBS and fixing with 4% paraformaldehyde. Next, we stained the nuclei with DAPI (10 µg/mL) and washed cells with PBS to remove unloaded Cy5.5 for CLSM observation.

2.6 Animal models

Balb/c mice were purchased from Huafukang Biological Technology Co., Ltd (Beijing, China). The animal study protocol was approved by the Institutional Animal Care and Use Committee at Tianjin University. To develop the 4T1 tumor model, 4T1 cancer cells (1×10^7) were subcutaneously injected on the outer of each Balb/c mouse.

2.7 In vivo images

For *in vivo* FL images, we i.v. injected free Cy5.5 (100 µL) or Cy5.5 labeled NC-PEG (100 µL, equivalent 100 µM Cy5.5), respectively, into 4T1 tumor-bearing Balb/c nude mice tail veins. The FL images were obtained via a *in vivo* imaging system. Then, we sacrificed the mice post imaged *in vivo* and collected major organs and tumors for quantitative bio-distribution assessment and *ex-vivo* images.

As for *in vivo* CT imaging, 100 µL of NC-PEG/R848 (5 mg/mL) was i.t. injected into 4T1 tumor bearing Balb/c nude mice. We then collected CT imaging before and after injection. The images were taken by micro CT scanner (Quantum FX, PerkinElmer, Hopkinton, MA, USA). Image analysis software: Analyze 12.0 (AnalyzeDirect, Overland Park, KS, USA) was utilized to analyze images. (Main parameters: Scan Time: 4.5 min, field of view (FOV): 73 mm, Current: 180 µA, Voltage: 90 kV.)

To evaluate the quantitative bio-distribution of Bi₂Se₃ NC-PEG/R848, major organs include heart, liver, spleen, lung, kidney and tumors collected from Bi₂Se₃ NC-PEG/R848 treated mice were solubilized for ICP-MS measurement to confirm Bi content after 4, 8, 12 and 24 h.

2.8 In vivo animal model

To develop the 4T1 tumor metastasis model, 4T1 cancer cells (1×10^7) were injected into both left and right flanks of mice, respectively. After the volume of tumors came up to ~ 100 mm³, NC-PEG or NC-PEG/R848 was i.v. injected into mice at day 6 ([NC-PEG] = 80 µg/mL, [Cy5.5] = 6 µg/mL, [R848] = 0.8 µg/mL). And then we exposed the mice to 808 nm laser irradiation (0.8 W/cm², 10 min) in order to kill cancer cells at day 7. Then, we i.v. injected anti-PD-L1 antibody (BioXcell, product number: BE0101, clone number: 10F9G2) into mice from diverse treated groups at a dose of 750 µg/kg at day 8, 9, 10, 11. We measured the mice body weight and tumor size every two days.

2.9 Ex vivo analysis

We evaluated the infiltrated cytotoxic T lymphocytes (CTL) both in distant and primary tumors at day 18 after diverse treatments through flow cytometry post stained with anti-CD8-PE

and anti-CD3-APC (BD Biosciences). Cells were further stained with anti-NKp46 to analyze NK cell via flow cytometry. Secondary tumor cells were further stained with anti-CD3-FITC (eBioscience), anti-CD4-PerCP (Biolegend), and anti-Foxp3-PE (eBioscience) antibodies to analyze CD4⁺ helper T cells. Lymph nodes harvested from mice post different treatment were further stained with anti-CD44-PE (eBioscience), anti-CD62L-APC (eBioscience), anti-CD8-PerCP-Cy5.5 (eBioscience) and anti-CD3-FITC (eBioscience) to analyze memory T cells. Subsequently, we assessed the pro-inflammatory cytokines in sera and DC medium supernatants, such as TNF-α, IFN-γ, and IL-12p40 (eBiosciences) through utilizing ELISA kits under standard protocols.

3 Results and discussion

3.1 Preparation and characterization

The Bi₂Se₃ NCs have been gained via a one-pot synthesis method which is shown in Fig. 1(a). We first synthesized the MnSe template via a hot injection way. The morphology of the obtained MnSe template showed a cubic phase, which was demonstrated through transmission electron microscopy (TEM, Fig. 1(b)), field emission scanning electron microscopy (SEM, Fig. 1(c)) and the powder XRD analysis (Fig. S1 in the Electronic Supplementary Material (ESM)). All these results confirmed that the synthesized MnSe monodisperse possessed a relatively high morphological purity yield with the average particle size of around 36 nm. Then we adopted a cation exchange method to produce Bi₂Se₃ nanocage from pre-made MnSe template by injecting bismuth neodecanoate into the MnSe reaction system at 180 °C. Due to the cation exchange, MnSe@Bi₂Se₃ core-shell structure was produced to further reaction through ions diffusion. The outward diffusion of the core Mn²⁺ was much faster than the inward diffusion of Bi³⁺, as a result, an inward flux of vacancies accompanied the outward Mn²⁺ flux to balance the diffusivity difference. The hollow Bi₂Se₃ structure (Bi₂Se₃ nanocage) was thus formed through coalescence of the vacancies based on the nanoscale Kirkendall effect. The obtained Bi₂Se₃ nanocage (NC) displayed a well-maintained shape of MnSe template with the average size and shell thickness around 37 and 6 nm, respectively. As expected, TEM images (Fig. 1(d) and insert), SEM images (Fig. 1(e)), elemental mapping by the high-angle annular dark-field scanning TEM (HAADF-STEM) (Figs. 1(f)–1(i)) and XRD data (Fig. 2(b)) further demonstrated the successful synthesis of Bi₂Se₃ NC. Besides, the specific surface area of Bi₂Se₃ NC was measured to be 68.3 cm²/g (Fig. 2(c)), which enabled the NC with efficient drug loading capacity.

Considering the bulk Bi₂Se₃ NC dispersion might display slow deposition and oxidation at room temperature over one week, we modified the Bi₂Se₃ NC surface with a PEG grafted amphiphilic polymer. The average hydrodynamic size of coated NC was increased slightly with a relatively low PDI as assessed through dynamic light scattering (Fig. 2(a)), verifying that the NC has been coated with PEG successfully. In addition, compared with the bulk NC, NC-PEG displayed good dispersion and stability in water, which was demonstrated in diverse formulations photos on Day 1 and Day 7 (Figs. S2 and S3 in the ESM). Compared with bulk NC, the appearance of NC-PEG after 7 days (water solution, room temperature) kept unchanged, showing the remarkable stability of the modified nanoparticles, which was promising for *in vivo* biomedical applications.

Moreover, NC-PEG owned strong NIR light absorbance, thereby exhibiting a desirable PT performance upon 808 nm laser irradiation with a relatively low concentration of NC-PEG (80 µg/mL) (Figs. 2(f) and 2(i)). The photothermal conversion efficiency of NC-PEG was calculated to be 36.8% (Fig. 2(g)), which was higher than the normally adopted photothermal agent. The PT stability of NC-PEG

Figure 1 (a) Illustration of the synthesis process of Bi_2Se_3 NC-PEG/R848. (b) TEM image and (c) SEM image of MnSe nanocubes. (d) TEM image and (e) SEM image of Bi_2Se_3 NC ((d) insert: high magnification TEM of the Bi_2Se_3 NC). (f)–(i) Energy dispersive X-ray (EDX) elemental mapping analysis of Bi_2Se_3 NC-PEG/R848 and the corresponding elemental mappings of Bi and Se.

Figure 2 Characterization of Bi_2Se_3 NC-PEG/R848. (a) Size distribution of Bi_2Se_3 NC, Bi_2Se_3 NC-PEG and Bi_2Se_3 NC-PEG/R848. (b) XRD patterns of Bi_2Se_3 NC. (c) N_2 adsorption-desorption isotherms of Bi_2Se_3 NC (inset: pore size distribution). (d) UV-vis absorption spectra of Bi_2Se_3 NC, Bi_2Se_3 NC-PEG and Bi_2Se_3 NC-PEG/R848. (e) Cumulative release of R848 from the Bi_2Se_3 NC-PEG/R848 with or without NIR laser irradiation (0.8 W/cm^2 , 5 min). (f) Temperature curves of different Bi_2Se_3 NC-PEG/R848 concentrations over a period of 5 min exposed to 808 nm laser. (g) Photothermal effect Bi_2Se_3 NC-PEG/R848 with NIR laser irradiation (808 nm , 0.8 W/cm^2). (inset: linear time data versus $-\ln(\theta)$ obtained from the cooling period of (g)). (h) Temperature elevations of Bi_2Se_3 NC-PEG/R848 (80 mg/mL) cycles. (i) Thermographic images of PBS and Bi_2Se_3 NC-PEG/R848 (80 mg/mL) at determined time points (0, 1, 3, and 5 min, respectively).

was also assessed by four laser on/off cycles. As demonstrated in Fig. 2(h), the similar increased temperature of diverse cycles verified excellent PT stability. Results above all verified that the NC-PEG possessed a remarkable PT conversion efficiency and PT stability.

3.2 R848 loading and *in vitro* NIR triggered R848 release profile

Since R848 is an effective immunologic adjuvant, it is important to delivery R848 into tumor sites instead of distributing in whole body. The loading ability of R848 was measured through a UV-vis spectrum, which showed a typical peak at 325 nm (Fig. 2(d)). In order to assess the release behaviors of R848 from NC-PEG based drug delivery systems, the NIR triggered R848 release was investigated *in vitro*. As illustrated in Fig. 2(e), without a 808 nm laser, the amount of R848 released from NC-PEG/R848 showed only 10.7% and 13.1% at 8 and 24 h, respectively. On the contrary, when exposed to 808 nm laser, the release amount reached to 32.1% at the first 8 h, and further enhanced to 59.0% at 24 h, demonstrating that NIR laser could finely controlled the release profile of R848 from NC-PEG/R848, which is promising for decreasing the systemic toxicity.

3.3 *In vitro* cytotoxicity and photothermal effect

We then evaluated the cytotoxicity of diverse formulation to 4T1 cells with various conditions through the MTT assay. As demonstrated

in Fig. 3(a), the cell viability of diverse formulations were over 80% without NIR irradiation, even with the highest concentration (NC-PEG: 80 $\mu\text{g/mL}$ and R848: 0.8 $\mu\text{g/mL}$). However, all of the treatments displayed concentration-dependent cancer cells ablating ability when we exposed the cells to NIR irradiation (Fig. 3(b)).

In anti-tumor therapy, it is important for nanoparticles to be uptake and internalized by cancer cells. Thus, in order to assess the cellular uptake capacity of NC-PEG/R848, we labeled NC-PEG/R848 with an identical Cy5.5 labeled concentration ([NC-PEG] = 80 $\mu\text{g/mL}$, [Cy5.5] = 6 $\mu\text{g/mL}$, [R848] = 0.8 $\mu\text{g/mL}$). 4T1 cells were then treated with diverse conditions. As demonstrated in Fig. 3(c), post 4 h incubation, most Cy5.5 labeled NC-PEG/R848 was distributed in the cytoplasm. The fluorescence intensity of free Cy5.5 was obviously weaker than the final formulation upon the same conditions, demonstrating the remarkable cellular uptake and internalization capacity of our nanoparticles. Moreover, we could see an enhanced Cy5.5 fluorescence intensity inside of the cells under a short time NIR irradiation (808 nm, 0.8 W/cm², 3 min), which might due to the NIR irradiation controlled release profile.

Furthermore, we also adopted fluorescence co-staining of live/dead cells to evaluate therapeutic efficacy of our nanoparticle in Fig. 3(d). Compared with PBS (–NIR), NC-PEG/R848 (–NIR) or NC (+NIR) treated group, the optimal treatment strategy NC-PEG/R848 (+NIR) showed the strongest red color, demonstrating good anti-cancer efficiency of our nanoparticles.

Figure 3 (a) Cell viability of 4T1 cells incubated with diverse concentrations of Bi₂Se₃ NC-PEG, free R848 and Bi₂Se₃ NC-PEG/R848 for dark toxicity. Data were presented as means SD ($n = 5$). (b) Cell viability of 4T1 from various treatment groups after being incubated with diverse concentrations of Bi₂Se₃ NC-PEG, free R848 and Bi₂Se₃ NC-PEG/R848 with 808 nm laser irradiation. Data were presented as means SD ($n = 5$), $*p < 0.05$, $**p < 0.01$. (c) CLSM images of 4T1 cells after incubated with free Cy5.5 or Bi₂Se₃ NC-PEG/R848 for 4 h. NIR means 808 nm (0.8 W/cm²) laser irradiation for 2 min. (d) CLSM images of Calcein AM and PI co-staining 4T1 cells incubated with diverse formulations. Scale bar indicated 100 μm .

3.4 *In vitro* and *in vivo* CT and FL

For evaluating the bio-distribution of our nanoparticle, we adopted Cy5.5 labeled NC-PEG/R848 for FL imaging *in vivo* using a 4T1 tumor-bearing nude mice model. Post i.v. injected free Cy5.5 or Cy5.5 labeled NC-PEG/R848, we recorded the fluorescence signals at 0, 4, 8, 12, and 24 h time intervals, respectively (Figs. 4(a) and 4(b)). At the early period after injection, we observed widely distributed fluorescent signals over the whole body. Continuous accumulation of the Cy5.5 labeled NC-PEG/R848 in the tumor site was observed and achieved the maximum at 8 h compared with any other units of the body (Fig. 4(b), top panel). On the contrary, under the same conditions, the fluorescent signals of free Cy5.5 treated group showed no obvious tumor contrast. We excised the major organs and tumors 24 h post-injection to gain a clearer sight of the bio-distribution. Obviously, most of the Cy5.5 labeled NC-PEG/R848 was accumulated in the tumor site, the majority of free Cy5.5 was distributed in the liver and kidney by contrast (Fig. 4(a), bottom panel). The *ex-vivo* images of different organs further verified higher tumor retention of Cy5.5 labeled NC-PEG/R848 compared with other major organs (Fig. 4(c)).

Encouraged by the high cancer cell uptake of the Cy5.5 labeled NC-PEG/R848 as evaluated by FL images, we further studied the CT images because of the large X-ray attenuation of Bi. We gained phantom images of NC-PEG/R848 with diverse concentration *in vitro* to assess the CT contrast capacity (Fig. 4(d)). We noticed that CT images gradually became brighter with the enhanced concentration and showed a linear increase between the concentration of nanoparticles and the gained CT value (Fig. 4(e)). We then assessed the profile of CT images *in vivo* using a 4T1 tumor-bearing nude mice model. We i.v. injected the NC-PEG/R848 (5 mg/mL, 100 μ L) into the tumor site of the mice to obtain the images via a small animal X-ray CT imaging system at diverse time points. A strong tumor contrast was seen post-injection, compared with the images before injection (Fig. 4(f) and Fig. S4 in the ESM), demonstrating the remarkable CT imaging ability of NC-PEG/R848. All of these results verified that the NC-PEG/R848 could serve as a promising multi-model contrast agents for images *in vivo*, which might be applied to direct the laser irradiation in PTT.

To further confirm tumor uptake of Bi₂Se₃ NC-PEG/R848, we quantitatively measured the biodistribution of the nanoparticles in the mice body. Bi levels in major organs and tumors were measured

Figure 4 Fluorescence images of Balb/c nude mice at diverse time points after administration of (a) free Cy5.5 and (b) Cy5.5 labeled Bi₂Se₃ NC-PEG/R848, the bottom panel shows the *ex vivo* images examined at 8 h post-injection. (c) Average fluorescence signals of tumors at diverse time points after administration of free Cy5.5 and Cy5.5 labeled Bi₂Se₃ NC-PEG/R848. Data were presented as mean $\pm$ SD ($n = 5$), $**p < 0.01$. (d) *In vitro* CT images and (e) corresponding CT intensity of the Bi₂Se₃ NC-PEG/R848 with diverse concentrations. (f) *In vivo* 3D, 2D CT images of Bi₂Se₃ NC-PEG/R848 in the tumor before and after i.t. injection. (g) The biodistribution of Bi₂Se₃ NC-PEG/R848 measured at 4, 8, 12 and 24 h post i.v. injection.

through inductively coupled plasma mass spectrometry (ICP-MS). As Fig. 4(g) demonstrated, high Bi content was detected in tumors at all designed time points, also verifying that Bi_2Se_3 NC-PEG/R848 could be accumulated to, and resided in the tumors. Moreover, post 8 h injection, the content of Bi in the tumors have reached maximum, which was in accordance with FL images results, demonstrating tumor-targeting ability of our nanoparticles.

3.5 Photothermal tumor ablation for immune system activation

Recent years, cancer immune-therapy has shown many exciting clinical results in various cancer treatments [31, 32]. However, the immune-therapy clinical responses are yet limited due to complex tumor microenvironment and heterogeneity. Very recently, some researchers have verified that PTT could stimulate the tumor-specific immune responses through generating TAA from cancer cell residues, which subsequently could be processed by APCs such as DCs and then presented to T cells. Therefore, we experienced PTT could triggered enhanced immunological responses based on NC-PEG/R848. In our *in vivo* experiments, 100 μL of NC-PEG or NC-PEG/R848 ([NC-PEG] = 80 $\mu\text{g}/\text{mL}$, [R848] = 0.8 $\mu\text{g}/\text{mL}$) was respectively i.v. injected into the tail vein of the mice when 4T1 tumors grown on Balb/c mice reached around 100 mm^3 . After 12 h,

we irradiated the tumor sites with 808 nm NIR laser at 0.8 W/cm^2 for 10 min. Five days after PTT (day 12), mice were sacrificed to cut off the draining lymph nodes, which were utilized to analyze DC maturation level via flow cytometry analysis (Figs. 5(b) and 5(c)). It was found that NC-PEG/R848 induced PTT showed a much higher DC maturation level compared with single NC-PEG or NC-PEG/R848 treated group. In conclusion, after the tumor was damaged by PTT, DCs could be recruited to the ablated tumor site as APCs to activate immune responses. In the same time, TAA in tumor debris after PTT could be converted to lymph nodes nearby and then simulated DC maturation, particularly under the assistant of adjuvant nanoparticles.

Cytokines secretion is important in the immune responses as well. In a parallel experiment, various cytokines changes including $\text{TNF-}\alpha$, interferon γ (IFN- γ) and interleukin 12 (IL-12p40, sera from mice of day 12) were studied by ELISA assay. Similarly, although PTT with NC-PEG or NC-PEG/R848 injection alone was able to increase pro-inflammatory cytokines secretion, their secretions induced by NC-PEG/R848 induced PTT were obviously higher, which was favorable for activating anti-tumor immune response (Figs. 5(d)–5(f)). These results demonstrated that NC-PEG/R848 induced PTT could stimulate the immunological system *in vivo*. The *in vivo* adjuvant activities of such nanoparticles combined with

Figure 5 Bi_2Se_3 NC-PEG/R848-based *in vivo* PTT induces DC maturation and activates the pro-inflammatory cytokines expression. (a) Schematic illustration of our experiment design to assess immune responses triggered by Bi_2Se_3 NC-PEG/R848-based PTT. (b) and (c) DC maturation induced by Bi_2Se_3 NC-PEG/R848-based PTT on mice bearing 4T1 tumors. (d)–(f) Cytokine levels of IL-12p40 (d), IFN- γ (e), and TNF- α (f) in sera from mice isolated on day 12. Data were presented as mean $\pm$ SD ($n = 5$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

PTT induced TAA released might act as safe “tumor vaccine”, which was promisingly useful for tumor immune-therapy.

3.6 PTT plus PD-L1 checkpoint blockade to inhibit growth of distant tumors

In this study we further combined NC-PEG/R848 mediate PTT with PD-L1 checkpoint blockade, which could efficiently increase the anti-cancer immune activity of CTLs through preventing their depletion. The method reported here might offer an alternative method to ablate primary tumors and further kill spreading metastatic cancer cells. In this study, Balb/c mice were inoculated with 4T1 cells on the left and right flanks, respectively. The left tumor was selected as primary tumor to be treated with PTT, while right tumor was chosen as distant tumors (1–2 cm away) without direct therapy. Mice were divided into six groups: PBS (Group 1), NC-PEG/R848 (Group 2), PBS + NIR (Group 3), NC-PEG/R848 + anti-PD-L1 (Group 4), NC-PEG/R848 + NIR (Group 5) and NC-PEG + NIR (Group 6), NC-PEG + NIR + anti-PD-L1 (Group 7), NC-PEG/R848 + NIR + anti-PD-L1 (Group 8). After 12 h diverse therapeutic agents i.v. injection, the left tumors of mice from Group 3, Group 5 and Group 6 were exposed to 808 nm laser irradiation (0.8 W/cm², 10 min). At day 8, 9, 10 and 11, we i.v. injected anti-PD-L1 antibody into mice in Group 4 and Group 6 at a dose of 750 µg/kg after laser irradiation (Fig. 6(a)). We found that NC-PEG + NIR + anti-PD-L1 treatment (Group 7) could inhibit the primary tumor growth more effectively than NC-PEG/R848 + NIR (Group 5), whereas NC-PEG/R848 + anti-PD-L1 administration group (Group 4) showed no remarkable therapeutic efficiency at applied anti-PD-L1 dose (Figs. 6(b) and 6(c)), verifying PTT alone could improve PD-L1 immune therapy. Moreover, NC-PEG/R848 + NIR + anti-PD-L1 (Group 8) treated group showed higher anti-tumor efficiency than Group 7, demonstrating the R848 could contributed to improve the PD-L1 therapy combined with PTT.

Additionally, the body weights exhibited no remarkable changes of different groups (Fig. 6(k)). Moreover, 90 percent of mice in Group 6 survived over 40 days after inoculation of tumors (Fig. 6(l)), which was in marked contrast to other treatment groups. All results above indicated that our NC-PEG/R848 induced PTT combined anti-PD-L1 blockade could synergistically cause highly efficient anti-tumor immune responses to both destroy tumors with direct PTT therapy strategy as well as inhibit tumors growth without direct laser irradiation.

3.7 The mechanism study

To study the mechanism of synergistic anti-tumor ability triggered by NC-PEG/R848 mediate PTT plus anti-PD-L1 therapy, NK cells, the subspecies of leukocytes in the distant tumors were studied (Fig. S5 in the ESM). In comparison with the control group (7.02% ± 0.61%), the percentage of NK cells increased to about 51% in NC-PEG/R848 based PTT treated group, which demonstrated that, in comparison with anti-PD-L1 alone, the percentage of NK cells is more affected by NC-PEG/R848 based PTT group. These results suggest that PD-L1 checkpoint blockade plays an important role in promoting the dramatically increased NK cell infiltration and accumulation in the distant tumor sites.

Cytotoxic T lymphocytes (CTL) in tumors were also tested to study the mechanism of PTT combined with anti-PD-L1 therapy. Different from other therapy strategies, only PTT + anti-PD-L1 treatment induced robust CD8⁺ cytotoxic T lymphocytes (CTL) infiltration (over 4 folds than others) in the primary tumor (Figs. 6(d) and 6(e)). Further more we noticed that only PTT plus anti-PD-L1 treatment could inhibit the growth of non-irradiated distant tumors as well, whose progressing was not influenced in any other groups (Figs. 6(g) and 6(h)). In addition, compared to other treated group,

remarkable CTL infiltration increase was also shown in the distant tumors post the combined therapy strategies (Figs. 6(f) and 6(i)). The robust interferon gamma (IFN-γ) production in the serum samples with PTT plus anti-PD-L1 treatment was measured at day 18 post tumor incubation, which demonstrated the highly efficient cellular immune responses mediated by the combined therapy strategy (Fig. 6(j)). However, regulatory T cells (Tregs) could impede efficient anti-tumor immune responses. Thus, Tregs in secondary tumors were also collected for further study post co-staining with CD4 and Foxp3. It was found that the percentage of Tregs was greatly reduced in secondary tumors post PD-L1 blockade therapy (Fig. S6 in the ESM). Moreover, comparing groups 4 and 8 in Fig. S6 in the ESM, PTT combined with anti-PD-L1 could induce the lowest Tregs percentages, which was mainly major responsible for cell immunity in tumor immune-therapy.

3.8 Long-term immune-memory effects

Remembering pathogens for few decades is an essential character of immune systems, which is important for disease prevention. Thus, evaluating immune memory induced by NC-PEG/R848 mediate PTT is of great importance. In this study, the 2nd tumors were inoculated 40 days post surgery or NC-PEG/R848 mediated PTT removing the 1st tumors. Then, mice were i.v. injected with anti-PD-L1 at diverse days (750 µg/kg every time) for two turns of treatment, the first turn was injected right behind the 1st tumor was removed (Day 1 and 5), and then the 2nd turn was injected at Day 41, 44 and 47 (Fig. 7(a)). Effector memory T cells (TEM) locate in non-lymphoid as well as lymphoid tissues, which could induce immediate protection through generating cytokines such as IFN-γ [33–36]. In this case, we analyzed the TEM cells proportion at Day 40 post the 1st tumor removal under various treatments. We noticed that TEM cells percentage in NC-PEG/R848 mediated PTT treated group was much higher (Fig. 7(b)) than other treated groups. Moreover, seven days post the 2nd tumor incubated, we analyzed the cytokines in sera under various treatments by ELISA. It is reported that IFN-γ and TNF-α [37] are cellular immunity typical markers, playing important roles in immune therapy against tumors. The TNF-α and IFN-γ serum levels were obviously increased in NC-PEG/R848 mediated PTT treated group, especially for those under PTT combined the 2nd turn of anti-PD-L1 treatment (post), demonstrating the successful performance of anti-cancer immune responses triggered by the re-challenging of tumor cells 40 days after in this group (Figs. 7(c) and 7(d)).

4 Conclusions

In conclusion, we demonstrated that the multifunctional Bi₂Se₃ NC-PEG/R848 integrating PT agent and immune-adjuvant is able to stimulate vaccine-like immune responses, which could be combined with PD-L1 checkpoint blockade to achieve efficient anti-tumor photothermal-immune therapy. The Bi₂Se₃ NC here with hollow interiors is desirable for stronger NIR region optical absorption and higher tissue penetration. Meanwhile, NC is also a desired drug carrier with larger free volume. Our Bi₂Se₃ NC-PEG/R848 can be utilized for NIR-induced PTT to damage cancer cells directly, as well as trigger the DCs maturation to activate immune responses thus secrete cytokines. In combination with PD-L1 checkpoint blockade strategy to inhibit tumor cells immune escape, such Bi₂Se₃ NC-PEG/R848 based PTT could ablate primary tumors directly and suppress distant tumors via activating strong anti-cancer immune responses. Furthermore, a strong immune-memory effect is observed after NC-PEG/R848 based PTT in combination with anti-PD-L1 therapy could efficiently protect mice from tumor re-challenge.

Figure 6 Anti-tumor metastasis effect of PTT with Bi₂Se₃ NC-PEG/R848 in combination with checkpoint blockade immune-therapy. (a) Schematic illustration of experimental design to combine PTT with anti-PD-L1 therapy. (b) Thermo-graphic images of mice 12 h post i.v. injection of PBS and Bi₂Se₃ NC-PEG/R848 under a 808 nm laser (0.8 W/cm², 5 min). (c)–(e) The tumor growth curves (c), average tumor weights at day 18 (d), and percentages of CTL infiltration at day 18 (e), for primary tumors (left) after various treatments. (f)–(h) The tumor growth curves (f), average tumor weights at day 18 (g), and percentages of CTL infiltration at day 18 (h), for non-irradiated tumors (right) after various treatments. Data were presented as mean $\pm$ SD ($n = 5$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (i) The IFN- γ levels in sera from mice detected at 18 days after various treatments. (j) Changes in body weight of mice during treatment. (k) Percent survival for different treatment groups during 42 days. Data were presented as mean $\pm$ SD ($n = 5$), $^{*}p < 0.01$, $^{***}p < 0.001$.

Figure 7 Long-term immune-memory effects. (a) Schematic illustration of NC-PEG/R848-mediated PTT in combination with anti-PD-L1 therapy to inhibit cancer relapse. (b) Proportions of effector memory T cells (TEM) in the spleen analyzed by flow cytometry before re-challenging mice with 2nd tumor at Day 40. (c) IFN-γ level in sera of mice isolated 7 days post re-challenging with the 2nd tumor (post the 2nd turn of anti-PD-L1 treatment). (d) TNF-α level in sera of mice isolated 7 days post re-challenging with the 2nd tumor (post the 2nd turn of anti-PD-L1 treatment). (Group 1: Surgery; Group 2: Surgery+anti-PD-L1 (pre & post); Group 3: Surgery+NC-PEG/R848+anti-PD-L1 (pre & post); Group 4: NC-PEG/R848+NIR; Group 5: NC-PEG/R848+NIR+anti-PD-L1 (post); Group 6: NC-PEG/R848+NIR+anti-PD-L1 (pre & post)).

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