Hyperthermia based individual in situ recombinant vaccine enhances lymph nodes drainage for de novo antitumor immunity

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Abstract The continuing challenges that limit effectiveness of tumor therapeutic vaccines were high heterogeneity of tumor immunogenicity, low bioactivity of antigens, as well as insufficient lymph nodes (LNs) drainage of antigens and adjuvants. Transportation of in situ neoantigens and adjuvants to LNs may be an effective approach to solve the abovementioned problems. Therefore, an FA-TSL/AuNCs/SV nanoplatforam was constructed by integrating simvastatin (SV) adjuvant loaded Au nanocages (AuNCs) as cores (AuNCs/SV) and folic acid modified thermal-sensitive liposomes (FA-TSL) as shells to enhance de novo antitumor immunity. After accumulation in tumor guided by FA, AuNCs mediated photothermal therapy (PTT) induced the release of tumor-derived protein antigens (TDPAs) and the shedding of FA-TSL. Exposed AuNCs/SV soon captured TDPAs to form in situ recombinant vaccine (AuNCs/SV/TDPAs). Subsequently, AuNCs/SV/TDPAs could efficiently transport to draining LNs owing to the hyperthermia induced vasodilation effect and small particle size, achieving co-delivery of antigens and adjuvant for initiation of specific T cell response. In melanoma bearing mice, FA-TSL/AuNCs/SV and laser irradiation effectively ablated primary tumor, against metastatic tumors and induced immunological...
1. Introduction

Vaccines have protected countless people from various diseases and recently made some achievements in the fight against cancer. Despite taking a number of efforts to develop cancer vaccines, efficacious therapies still remain challenging due to high heterogeneity of tumor immunogenicity. In recent years, personalized vaccines targeting cancer-specific neoantigens have proposed to elicit individual antitumor immunity. However, the effectiveness of personalized vaccines is limited by various hurdles, such as costly and time-consuming procurement process of neoantigens, low immunogenicity of single neoantigen peptides, rapid clearance of free neoantigens, as well as insufficient transport of antigens and adjuvants to lymph nodes (LNs). Herein, it is urgent to develop a versatile vaccination strategy that can synergistically modulate multiple aspects for enhancing antitumor immunity.

LNs are the critical therapeutic target for various vaccines, which contain a large number of immature dendritic cells (DCs) presenting neoantigens to initiate strong T cell responses for adaptive immunity. Peptide and protein antigens are broadly applied in cancer vaccines due to excellent immune activation effect as well as lower toxicity. However, neoantigen proteins/peptides are always cleared which result in low immunogenicity, further limiting optimal antigen presentation. Encouragingly, nanovaccines have been designed to overcome this obstacle. More interestingly, these nano-vaccines with an intermediate size (20–70 nm) prefer to drain to LNs and retain here. Therefore, vesicles with appropriate physical-biochemical properties are good candidates for vaccines construction.

A myriad of Au nanomaterials exhibits outstanding light-to-heat conversion efficiency due to the localized surface plasmon resonance, making them great potentials for cancer photothermal therapy (PTT). Interestingly, PTT not only achieved tumor ablation via hyperthermia effect, but also enhanced nanodrugs accumulation in tumor site as well as antitumor immunity. Among various Au nanomaterials, AuNCs with particle size of 40–100 nm could induce the release of tumor-derived protein antigens (TDPAs), such as damage-associated molecular patterns (DAMPs) and neoantigens. It is noteworthy that Au nanomaterials with special surface can directly capture proteins through some forces such as Au–S bond, electrostatic interactions and hydrophobic interactions. So, AuNCs could not only convert cancer into a nidus for presentation of cancer antigens, but also capture these TDPAs to construct an \textit{in situ} recombinant vaccine. Interestingly, negative surface and suitable particle size endow AuNCs based vaccine with excellent LNs drainage ability which could be further promoted by vasodilation effect under hyperthermia. This approach could avoid complicated preparation process and promote LNs drainage of personalized vaccine.

In addition to the above advantages, AuNCs with hollow mesoporous structure are in favour of the encapsulation of adjuvant that is essential to enhance antitumor immune responses. It has been reported that simvastatin (SV) exhibited vaccine adjuvant activities by blocking mevalonate (MVA) pathway. SV can arrest endosomal maturation, prolong antigen presentation during endocytosis, increase antigen presentation to both CD4+ and CD8+ T cells, thus displaying excellent immune-boosting effects. Therefore, we are encouraged to encapsulate SV into AuNCs (AuNCs/SV) for the construction of \textit{in situ} personalized cancer vaccine.

In light of these considerations, a folic acid modified thermal-sensitive liposomes (FA-TSL) shell was engineered onto the surface of AuNCs/SV to construct an FA-TSL/AuNCs/SV nanoplatfor for de novo antitumor immunity (Fig. 1). FA-TSL shells could protect AuNCs/SV from interference of non-tumor-related proteins in circulation and guide the nanoplatfor to tumor. After irradiation with 808 nm laser in tumor site, AuNCs mediated photothermal therapy (PTT) induce the release of TDPAs and the shedding of FA-TSL. Immediately, exposed AuNCs/SV capture TDPAs to form an FA-TSL/AuNCs/SV nanoplatform for in situ personalized cancer vaccine (AuNCs/SV/TDPAs), which could efficiently transport to draining LNs due to hyperthermia induced vasodilation effect, negatively charged surface and appropriate particle size. Once in contact with DCs, the recombinant vaccine facilitated cross presentation of TDPAs to stimulate antitumor immune responses. This nanoplatform seeks to convert irradiated tumor into a nidus for presentation of TDPAs and transport these TDPAs to LNs, thus facilitating personalized \textit{de novo} antitumor immunity.

2. Materials and methods

2.1. Materials

Silver nitrate (AgNO₃), sodium borohydride (NaBH₄), tripotassium citrate dihydrate (C₉H₂₅Na₂O₇·2H₂O) and ovalbumin (OVA) were obtained from Shanghai Sinopharm Chemical Reagent Co., Ltd. (China). Polyvinylpyrrolidone (PVP), 3-[(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), hydrogen tetrachloroaurate tetrahydrate (HAuCl₄·4H₂O) and ovalbumin (OVA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, 99.0%) were got from CorpernDerma Biotech, Inc. (Shanghai, China). Flourescein 5 (6)-isothiocyanate (FITC) was purchased from Aladdin (Shanghai, China). Cy5 dye was got from Beijing Solebo Technology Co., Ltd. (Beijing, China). All the other chemical reagents were obtained from Sigma–Aldrich. Anti-CD40-PE, anti-CD86-APC, anti-CD45-APC-Cy7, anti-CD8a-PerCP-Cy5.5, anti-CD3-BV510 and anti-CD4-PE were purchased from BD biosciences (New Jersey, USA). Anti-CD11c-BV605, anti-CD44-PE, anti-CD62L-APC, anti-SIINFEKL-MHC-I-APC-R700 and anti-MHC-II-PE were purchased from BioLegend (CA, USA).

The mice melanoma B16F10 cell line in this study was from the China Center for Type Culture Collection at Wuhan University.
B16F10 cells were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) in 5% CO$_2$ at 37 °C. DCs were isolated from the C57BL/6J mice bone marrow. Female C57BL/6J mice (six weeks old) were purchased from Center for Experimental Animals, Henan University. All animal studies were conducted in accordance with the guidelines of the National Regulation of China for Care and Use of Laboratory Animals.

2.2. Synthesis of AuNCs

28.5 nm silver nanoparticles (AgNPs) were prepared according to a previous report. Then, the 28.5 nm AgNPs were added into PVP solution that had been pre-stirred and heated at 90 °C for 1 h. After heating for 2 min, 0.1 mmol/L HAuCl$_4$·4H$_2$O solution was added at 0.7 mL per minute, and a series of color change could be observed. When the solution turned blue, addition of HAuCl$_4$·4H$_2$O solution was stopped. When color of mixed solution was no longer changed, the reaction system was stopped and cooled down to room temperature. Then, AuNCs were collected and stored at 4 °C for further use.

2.3. Synthesis of FA-TSL/AuNCs/SV

To prepare AuNCs/SV NPs, SV (2 mg/mL) in methanol solution was mixed with AuNCs (2 mg/mL) in aqueous solution (2:1, v/v) and kept stirring for 24 h. Then, the mixture was centrifuged (12,000 rpm, 5 min, TGL-16G, Shanghai Anting Scientific Instrument Factory, Shanghai, China) and washed three times with deionized water/methanol (1:1, v/v) to remove free SV. AuNCs/SV were collected and re-dispersed in deionized water.

Subsequently, FA-TSL/AuNCs/SV were prepared by thin-film hydration method. Briefly, DPPC, cholesterol, DSPE-PEG2000-FA (7:1:0.5, mol/mol) were dissolved in chloroform. A lipid film was formed when chloroform was removed by using a rotary evaporator (RE-52AA, Shanghai, China) and washed three times with deionized water/methanol (1:1, v/v) to remove free SV. AuNCs/SV were collected and re-dispersed in deionized water.

Figure 1  Schematic diagram of FA-TSL/AuNCs/SV with assistance of 808 nm laser to improve cancer immunotherapy.
intracellular high mobility group box 1 (HMGB1).

2.8. Immune response examination in vitro

For immunogenic cell death (ICD) detection, B16F10 cells were treated with different formulations. Then, cells were collected to detect calreticulin (CRT) exposure by using anti-CRT-FITC antibody. Cell culture supernatant was collected to examine released intracellular high mobility group box 1 (HMGB1). For DCs maturation measurement, BMDCs were incubated with antigens-NPs which were separated from NPs-containing cell lysate. 24 h later, DCs were marked with anti-CD86-FITC and anti-CD80-APC antibodies and analyzed by flow cytometry (Accuri C6, BD, USA).

2.9. In vivo distribution

Because fluorescence signals in tumor site of C57BL/6 mice were invisibility, BALB/c mice (female, 6-8 weeks) were used to detect in vivo biodistribution of the nanoplatform. 4T1-bearing mice were intravenous injection (i.v.) injected with IR780-labeled FA-TSL and FA-TSL/AuNCs, respectively. IR780 signal was acquired by an ivis spectrum in vivo imaging system (IVIS, PerkinElmer, USA) at scheduled time points.

2.10. In vivo antitumor activity

B16F10 tumor-bearing C57BL/6 mice were unconsciously divided into six groups (n = 6). Mice were i.v. injected with PBS, SV, FA-TSL/AuNCs, FA-TSL/AuNCs + laser, FA-TSL/AuNCs/SV and FA-TSL/AuNCs/SV + laser every 2 days for five times. Mice received different formulations at SV dose of 0.625 mg/kg and AuNCs dose of 0.625 mg/kg (Au concentration). The irradiation groups were irradiated with 808 nm laser (0.75 W/cm², 3 min) at 4 h post injection. Tumor size and body weight were recorded during treatment. Tumor size was calculated according to the following Eq. (1):

\[
\text{Volume} = \text{Length} \times \text{Width}^2/2 
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Then, main organs were collected from sacrificed mice for hematoxylin and eosin (H&E) staining. Tumors were removed for H&E, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), CRT and HMGB1 staining.

2.11. Antitumor immunity

B16F10 tumor-bearing mice were i.v. administered different formulations to investigate antitumor immunity. Tumor, LNs and spleens were harvested and digested to single-cell suspension, which was stained with fluorescent-labeled antibody. Flow cytometry (BD FACS Canto, USA) was used to detect the percentage of matured DCs (CD45+CD11c+CD40+CD86+) and T cells (CD45+CD3+CD4+CD8+) in tumors, matured DCs, T cells, MHCI (CD45+CD11c+SIINFEKL-MHC-I+) and MHC II (CD45+CD11c+MHC II+) in LNs, and effector memory T cells (TEM cells (CD45+CD3+CD8+CD44+CD62L-) in spleen. IFN-γ, TNF-α and IL-6 cytokines in serum were detected by ELISA kits (Multi-Sciences Biotech, China).

2.12. Abscopal effect

To study abscopal effect, B16F10 cells were (1 × 10^6 cells per side) subcutaneously injected in right flank of C57BL/6J mice on Day 0 (primary tumors) and left flank on Day 5 (secondary tumors). When the right tumor reached ~100 mm³, the mice were treated with drugs similar to those in vivo.
2.13. Investigation of lung metastasis

Anti-metastasis effectiveness of nanoplatfrom was investigated in a lung metastasis models. B16F10 cells were i.v. injected into C57BL/6 mice which received various treatments. On Day 21, whole lungs were excised and stained with H&E for quantification of metastasis area.

2.14. Statistical analysis

The data were presented as the mean ± standard deviation (SD). All statistical analysis were processed with Graph Pad Prism 8.0 (La Jolla, CA, USA). The differences between two groups and multiple groups were analyzed by one-way ANOVA and two-way ANOVA, respectively. The level of significance was set at probabilities: *p < 0.05, **p < 0.01, ***p < 0.001.

3. Results and discussion

3.1. Synthesis and characterization of FA-TSL/AuNCs/SV

AuNCs were prepared by employing Ag NPs as the sacrificial templates. TEM results indicated that AuNCs with average diameter of ~40 nm showed hollow mesoporous structure (Fig. 2A). Moreover, elemental mapping (Fig. 2B) and EDS (Fig. 2C) analysis demonstrated the presence of Au element. The diffraction pattern in Fig. 2D showed the peaks appeared at 38.3°, 44.5°, 64.7°, 77.7° and 82.3°, which were assigned to crystalline planes of gold nanostructure (JCPDS 04-0874), indicating the existence of gold crystalline phases. After encapsulation of SV, AuNCs/SV showed no obvious difference in particle size (Supporting Information Fig. S2), demonstrating successful loading of SV. Afterward, FA-TSL was coated onto AuNCs/SV surface to obtain FA-TSL/AuNCs/SV. DLS displayed a particle size of 55.3 ± 0.80 nm and a zeta potential of -18.9 ± 0.79 mV for FA-TSL/AuNCs/SV (Fig. S1). Then, the existence of FA-TSL was further confirmed by TEM (Fig. 2F), which revealed an obvious core-shell structure. Above results validated the construction of FA-TSL/AuNCs/SV.

Then, FA-TSL/AuNCs/SV were resuspended in 1640 medium containing 10% fetal bovine serums to test the stability. As indicated in Supporting Information Fig. S3, particle size and zeta potential were changed obviously and in line with that of AuNCs/SV (Supporting Information Fig. S6). In addition, FA-TSL/AuNCs/SV exhibited spherical core-shell structure, while naked AuNCs/SV were observed by TEM (Fig. 2H). Above results suggested the shedding of FA-TSL, which is necessary for AuNCs/SV exposure and antigen absorption.

3.2. Construction of recombinant vaccine

B16F10 melanoma cells were incubated with FA-TSL/AuNCs/SV and treated with 808 nm laser irradiation for in vitro recombinant vaccine construction (Fig. 2I). As shown in Fig. 2J, a TDPAs layer could be obviously observed on the surface of AuNCs. Then, the total amount of absorbed antigens was quantified using bicinchoninic acid (BCA) assay, which showed significant Au concentration-dependence (Fig. 2K). Moreover, TDPAs were quite different from raw cell lysate of untreated B16F10 cells, as SDS-PAGE indicated (Fig. 2L). To determine whether AuNCs/SV captured proteins contained neo-antigens expressed by B16F10 cells, proteins types of TDPAs were identified by using mass spectrometry. As Fig. 2M displayed, the recombinant vaccine contained neoantigens and DAMPs. In addition, tumor specific antigens, such as ACTN4, TNPO3, GP100 and MAKT3, were further determined by western blotting (Fig. 2N). The above results demonstrated that TDPAs produced by tumor cells during hyperthermia therapy could be used to construct recombinant vaccine for specific antitumor immunotherapy.

3.3. Cells experiments on B16F10

Cellular uptake experiment was next investigated in B16F10 cells. Fig. 3A and Supporting Information Fig. S7 showed that FA-TSL/AuNCs/SV were more internalized into tumor cells than naked AuNCs/SV/FITC-OVA and AuNCs/SV/FITC-OVA by BMDCs was examined, promoted by recombinant vaccine. Firstly, the internalization of FITC-OVA and AuNCs/SV/FITC-OVA by BMDCs was examined, respectively. According to Supporting Information Fig. S8 fluorescence intensity in AuNCs/SV/FITC-OVA group gradually increased with extended time and reached highest level at 8 h, which was significantly stronger than that in FITC-OVA group (Fig. 3G). Next, intracellular fate of AuNCs/SV/FITC-OVA was evaluated using CLSM. The result in Fig. 3H indicated that lysosomes signal (red) and NPs signal (green) completely overlapped after 4 h incubation. With the extension of incubation time,
green fluorescence was overflowed from red lysotracker at 8 h, indicating that these NPs escaped from endo/lysosomes, which was important for SV to exert its adjuvant effect. It is well known that Rab5 expressed early endosomes mature through acidification and material exchange to form Rab7a expressed late endosomes. After treatment with AuNCs/TDPAs and AuNCs/SV/TDPAs, the expression of Rab5 and Rab7a were observed by CLSM. As displayed in Supporting Information Fig. S9, the transition from an Rab5-positive to an Rab7a-positive endosome was significantly slowed down by AuNCs/SV/TDPAs. Meanwhile, AuNCs/SV/TDPAs treatment dramatically increased the retention time of model antigen OVA in BMDCs (Supporting Information Fig. S10), which was meaningful for antigen presentation and immune activation.

To verify immune response activation, the effects of AuNCs/SV/TDPAs and other formulations on BMDCs maturation were further studied. After different treatments, flow cytometry was used to quantify the frequency of DCs maturation. As shown in Fig. 3I and Supporting Information Fig. S11, TDPAs, SV, AuNCs, AuNCs/TDPAs, AuNCs/SV and AuNCs/SV/TDPAs groups
exhibited around 23.62%, 13.40%, 10.87%, 33.55%, 23.82% and 41.85% of DCs maturation, respectively. Therefore, the recombinant vaccine was able to bring out an enhanced immunotherapy by boosting immune response.

### 3.5. Tumor targeting and LNs drainage ability assessment

Afterward, IR780-labeled nanoplatform was used to investigate tumor targeting and LNs drainage ability of FA-TSL/AuNCs/SV. In detail, FA-TSL/AuNCs/IR780 were administrated by i.v., and then their biodistribution in vivo was detected by a near-infrared fluorescence imaging system. The experimental results in Supporting Information Fig. S12 revealed that IR780 fluorescence showed effective accumulation in tumors after injection of FA-TSL/AuNCs/IR780 compared with free IR780, demonstrating good tumor-targeting effect of this nanoplatform. Next, LNs migration ability of recombinant vaccine was also evaluated. The results of fluorescence reflectance imaging (FRI, Fig. 3J) showed that more IR780-labeled nanoplatform transport to lymphatic system with assistance of 808 nm laser irradiation, which mainly attributed to small size of AuNCs and photothermal induced vasodilation effect45,46. The axillary and inguinal LNs

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**Figure 3** (A) Cellular uptake (scale bar = 100 μm). (B) Cytotoxicity of B16F10 cells. (C) Cytotoxicity of B16F10 cells with 808 nm laser irradiation (1.5 W/cm², 2 min). (D) Calcein-AM/PI staining (scale bar = 100 μm). (E) Surface CRT expression. (F) Extracellular release of HMGB1. (G) The quantitative analysis of DCs uptake of FITC-OVA. (H) Lysosomal escape test (scale bar = 10 μm). (I) DCs maturation measurement. (J) FRI of mice received different formulations. (K) FRI of LNs harvested from mice at 36 h. (L) Quantification of fluorescence intensity in axillary and inguinal LNs. The results of significant difference analysis were compared with FA-TSL/AuNCs/IR780 + laser group. The data are presented as mean ± SD (n = 3), ***P < 0.001.
were collected from sacrificed mice at 36 h. As displayed in Fig. S3K and L, the intensity of IR780 signal in axillary and inguinal LNs was significantly higher after treated with 808 nm laser.

To verify the drainage of recombinant vaccine via lymphatic vessels, FITC-labelled AuNCs were administrated by i.v. The tumor tissue was collected at 4 h post laser irradiation and stained with podoplanin which is expressed specifically in lymphatic endothelium [27,28]. As shown in Supporting Information Fig. S13, the green fluorescence of FITC could be clearly observed in lymphatic vessels. Moreover, large amounts of AuNCs/FITC accumulated around lymphatic vessels, further confirmed the lymphatic drainage pathway of the recombinant vaccines. All above results proved that FA-TSL/AuNCs/IR780 and laser irradiation could enhance LNs-drainage effect of the recombinant vaccine, which could further improve the antitumor immunotherapy.

3.6. In vivo antitumor effect induced by FA-TSL/AuNCs/SV + laser

To assess in vivo PTT and therapeutic efficacy of FA-TSL/AuNCs/SV, tumor-bearing mouse model was constructed by subcutaneous injection of B16F10 cells into right axilla of C57BL/6J mice. The mice were injected with different preparations and irradiated with 808 nm (0.75 W/cm², 3 min) laser at 4 h post injection. Tumor thermal images were recorded by infrared (IR) thermal image instrument. After irradiation for 5 min, temperature-rise curves and IR images displayed that tumor temperature of the mice treated with FA-TSL/AuNCs/SV and laser irradiation reached to ~52.3 °C (Supporting Information Fig. S14). Such superior in vivo photothermal effect of FA-TSL/AuNCs/SV was ascribed to their enhanced tumor accumulation and high photothermal conversion efficiency.

Then, in vivo antitumor effect was evaluated and experiment schedule was designed in Fig. 4A. Tumor-bearing mice were unconsciously divided into six groups and treated with Saline (as control group), SV, FA-TSL/AuNCs, FA-TSL/AuNCs + laser, FA-TSL/AuNCs/SV, and FA-TSL/AuNCs/SV + laser. As shown in Fig. 4B, FA-TSL/AuNCs + laser treatment only slightly inhibited tumor growth in the absence of SV adjuvant, while FA-TSL/AuNCs/SV + laser could significantly delay tumor growth. After treatment with FA-TSL/AuNCs/SV + laser, 50% of the animals were still alive at 50 days, and two out of six were tumor free at the end of experiment (Fig. 4C). Efficient immune activation induced by co-delivery of TDPAs and SV might account for this phenomenon. Moreover, immunohistochemical and immunofluorescent examinations were used to analyze tumor tissues after various treatments (Fig. 4D). H&E-stained image significantly enlarged apoptosis or necrosis areas in tumor tissues after treatment with FA-TSL/AuNCs/SV + laser. TUNEL staining also proved the excellent anticancer effect of FA-TSL/AuNCs/SV complex with laser irradiation. In addition, all tumor-bearing mice did not show significant weight loss during treatments (Supporting Information Fig. S15). H&E staining showed that no inflammation or necrosis was observed in main organs of mice treated with different formulations and there were no significant differences in all groups (Supporting Information Fig. S16). Furthermore, safety profile of the formulations was evaluated by blood routine, liver function and kidney function examination. The parameters of hepatic and renal function including alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA) and urea showed no significant difference between healthy and FA-TSL/AuNCs/SV treated mice.

Similarly, same phenomenon was observed in the detection of white blood cells (WBC), red blood cells (RBC), blood platelet (PLT) and hemoglobin (HGB) (Supporting Information Fig. S17). Thus, these data demonstrated the excellent tumor suppressive ability and good biocompatibility of FA-TSL/AuNCs/SV.

3.7. Antitumor immune responses

To further elucidate potential antitumor mechanism of FA-TSL/AuNCs/SV, mice immune status was subsequently analyzed by immunofluorescent examination and flow cytometry. As shown in Fig. 4E and F and Supporting Information Fig. S18, FA-TSL/AuNCs + laser and FA-TSL/AuNCs/SV + laser could significantly increase CRT expression and HMGB1 level in tumor tissue compared with un-irradiation groups, indicating that FA-TSL/AuNCs based PTT might significantly increase tumor immunogenicity by inducing tumor ICD.

DCs, as a kind of APCs, are key to innate and adaptive immunity. The maturation of DCs promoted the binding of major histocompatibility complex (MHC) peptide and T cell receptor (TCR) for T cell activation [30–34]. The ability of FA-TSL/AuNCs/SV to recruit DCs via photothermal effect and activate DCs by delivered AuNCs/SV/TDPAs were estimated by measuring mature DCs (CD11c⁺ CD40⁺ CD80⁺ CD86⁺) in tumor tissue. After treated with FA-TSL/AuNCs/SV + laser, the frequency of mature DCs increased from 8.23% to 62.96% compared to untreated group (Supporting Information Fig. S19A). DCs maturation ratio in FA-TSL/AuNCs/SV + laser group was around 1.8- and 3.9-fold compared to that of FA-TSL/AuNCs + laser and FA-TSL/AuNCs/SV groups respectively, suggesting that adjuvant was necessary for enhancement of antigen presentation [35]. Correspondingly, highest percentage of tumor-infiltrating cytotoxic T cell (CTLs) (CD³⁺ CD8⁺) was observed in FA-TSL/AuNCs/SV + laser group (Fig. S19B), implying that FA-TSL/AuNCs/SV combined with laser irradiation strategy could evoke T cell-mediated antitumor immunity.

To verify systemic immune activation, tumor draining LNs and spleen were collected and analyzed by flow cytometry. According to Fig. 4G, CD40⁺ CD86⁺ DCs population was only increased in FA-TSL/AuNCs/SV with laser irradiation treatment group. These results demonstrated that the absence antigen or adjuvant could not induce efficient immune response, which was confirmed by low CD40⁺ CD86⁺ frequency in FA-TSL/AuNCs/SV and FA-TSL/AuNCs + laser groups. A major function of SV adjuvant is promotion of antigen presentation, in which process MHC played a key role [36]. Flow cytometry results suggested that FA-TSL/AuNCs/SV + laser treatment could significantly increase CD11c⁺ MHC-I (12.03%) and CD11c⁺ MHC-II (7.49%) DCs populations (Fig. 4H and I). In contrast, relatively few MHC⁺ DCs populations were detected in FA-TSL/AuNCs group, further confirming that AuNCs/SV/TDPAs had major effect on antigen presentation and immune activation.

After antigen presentation, naive CD8⁺ and CD4⁺ T cells were activated and gained their cytotoxic capabilities and helper functions. As shown in Fig. 4J and K, FA-TSL/AuNCs/SV with 808 nm laser treatment increased the helper CD4⁺ and CTLs in LNs and the frequency of CD4⁺ and CD8⁺ T cells were 2.57- and 2.59-fold compared to control group respectively. Subsequently, long-term antitumor immune response was examined by testing memory T cells (TEM) in spleen. FA-TSL/AuNCs/SV + laser group significantly increased TEM frequency in spleen, whereas FA-TSL/AuNCs/SV and FA-TSL/AuNCs + laser showed moderate efficacy to generate TEM.
Furthermore, we analyzed anti-tumor immunity cytokines with immune capabilities. As shown in Fig. 4L, compared with other groups, FA-TSL/AuNCs/SV + laser group expressed highest level of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in serum. Collectively, these results suggested that PTT-mediated recombinant vaccine based

**Figure 4** (A) Schematic illustration of experiment design. (B) In vivo tumor growth curve. (C) Mice survival curve. (D) H&E and TUNEL staining. (E) CRT expression in tumor tissue. (F) HMGB1 level in tumor tissue. The data are presented as mean ± SD (n = 6). (G) Mature DCs in draining LNs. (H) MHC I molecules in draining LNs. (I) MHC II molecules in draining LNs. (J) CD3⁺ CD8⁺ T cells and (K) CD3⁺ CD4⁺ T cells in draining LNs. (L) Cytokine levels of IL-6, TNF-α, IFN-γ in serum. The data are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

(Supporting Information Fig. S20).
on AuNCs successfully induced a strong innate and adaptive immune response in vivo.

3.8. Abscopal effect and anti-metastasis evaluation

System antitumor immune response has been found to facilitate abscopal effect. In order to further verify FA-TSL/AuNCs/SV with laser irradiation treatment induced antitumor immunity, we assessed abscopal effect of different treatments by measuring growth rate of the secondary tumors. A bilateral B16F10 subcutaneous tumor model was established and the schedule of animal experiments was revealed in Fig. 5A. Tumor in right axillary was defined as a primary tumor and distant tumor was in left axillary. As presented in Fig. 5B–D, FA-TSL/AuNCs/SV with laser irradiation treatment could significantly suppress primary tumors and distant tumor compared with other groups. Moreover, there was no significant change in body weight (Fig. 5E). In addition, FA-TSL/AuNCs/SV with laser irradiation treatment had a 100% survival rate for 30 days and 40% for 50 days (Fig. 5F), consistently confirming excellent antitumor efficacy of this therapy regimen. Simultaneously, we constructed a melanoma metastatic tumor model to study the anti-metastasis effect of different preparations (Fig. 5G). As shown in Fig. 5H, FA-TSL/AuNCs/SV with laser irradiation treatment had almost no metastatic nodules. On the
whole, the nano-system could inhibit both distant and metastatic tumor by activating systemic immune responses effectively.

4. Conclusions

In summary, this work first describes that a personalized in situ recombinant vaccine is drained to lymph nodes by hyperthermia to drive specific antitumor immunity. FA-TSL/AuNCs/SV + laser treatment turn tumors into a nidus of TDPAs to construct a recombinant antitumor vaccine in vivo. Impressively, the recombinant vaccine could efficiently flow into LNs owing to its particle size of ≈ 50 nm and the hyperthermia induced vasodilatation effect. And it increased the number of mature DCs in tumor site and draining LNs due to the co-delivery and synergism of TDPAs and SV, resulting in the strong activation of CD4⁺ and CD8⁺ T cells. By using bilateral subcutaneous melanoma tumor model, our results indicated that FA-TSL/AuNCs/SV combined laser irradiation treatment induce a systemic immune response, resulting in delayed tumor growth of primary and distant, as well as prevented tumor metastasis. Potentially, this approach might offer a new strategy to induce robust personalized vaccination.

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Author contributions

Lei Wang and Qianhua Feng designed the research. Cuixia Zheng and Xinxin Liu carried out the experiments and performed data analysis. Yueyue Kong, Lei Zhang, Qingling Song, Hongjuan Zhao, Lu Han and Jiannan Jiao participated a part of the experiments. Lei Wang and Qianhua Feng provided experimental drugs and quality control. Cuixia Zheng and Xinxin Liu wrote the manuscript. Cuixia Zheng, Xinxin Liu and Yueyue Kong revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

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References

1. Saxena M, van der Burg SH, Melief CJM, Bhardwaj N. Therapeutic cancer vaccines. Nat Rev Cancer 2021;21:360–78.
2. Banchereau J, Palucka K. Immunotherapy: cancer vaccines on the move. Nat Rev Clin Oncol 2018;15:9–10.
3. Sahin U, Tureci O. Personalized vaccines for cancer immunotherapy. Science 2018;359:1535–60.
4. Zhao H, Xu J, Li Y, Guan XX, Han X, Xu Y, et al. Nanoscale co-ordination polymer based nanovaccine for tumor immunotherapy. ACS Nano 2019;13:13127–35.
5. Li AW, Sobral MC, Badrinath S, Choi Y, Graveline A, Stafford AG, et al. A facile approach to enhance antigen response for personalized cancer vaccination. Nat Mater 2018;17:528–34.
6. Patel RB, Ye M, Carlson PM, Jaquish A, Zangl L, Ma B, et al. Development of an in situ cancer vaccine via combinational radiation and bacterial-membrane-coated nanoparticles. Adv Mater 2019;31:e1902626.
7. Blass E, Ott PA. Advances in the development of personalized neoantigen-based therapeutic cancer vaccines. Nat Rev Clin Oncol 2021;18:215–29.
8. Li WH, Li YM. Chemical strategies to boost cancer vaccines. Chem Rev 2018;120:11420–78.
9. Yu X, Dai YF, Zhao YF, Qi SH, Liu L, Lu LS, et al. Melittin-lipid nanoparticles target to lymph nodes and elicit a systemic anti-tumor immune response. Nat Commun 2020;11:1110–3.
10. Hong XY, Zhong XF, Du GS, Hou YY, Zhang YT, Zhang ZR, et al. The pore size of mesoporous silica nanoparticles regulates their antigen delivery efficiency. Sci Adv 2020;6:eaaaz446.
11. Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nat Immunol 2011;12:509–17.
12. Qian Y, Jin HL, Qiao S, Dai YF, Huang C, Lu LS, et al. Targeting dendritic cells in lymph node with an antigen peptide-based nano-vaccine for cancer immunotherapy. Biomaterials 2016;98:171–83.
13. Min Y, Roche KC, Tian S, Eblan MF, McKinnon KP, Caster JM, et al. Antigen-capturing nanoparticles improve the abscopal effect and cancer immunotherapy. Nat Nanotechnol 2017;12:877–82.
14. Zeng LM, Liao ZL, Li WW, Yuan QJ, Wu P, Gu ZP, et al. Non-covalent glycosylated gold nanoparticles/peptides nanovaccine as potential cancer vaccines. Chin Chem Lett 2020;31:1162–4.
15. Kang YM, Kang S, Shin H, Kim T, Yu B, Kim J, et al. Sequential and timely combination of a cancer nanovaccine with immune checkpoint blockade effectively inhibits tumor growth and relapse. Angew Chem Int Ed Engl 2020;59:14628–38.
16. Fan Y, Moon JJ. Nanoparticle drug delivery systems designed to improve cancer vaccines and immunotherapy. Vaccines (Basel) 2015;3:662–85.
17. Zhao HJ, Zhao BB, Wu LX, Xiao HF, Ding KL, Zheng CX, et al. Amplified cancer immunotherapy of a surface-engineered antigen microparticle vaccine by synergistically modulating tumor microenvironment. ACS Nano 2019;13:12553–66.
18. Kim SY, Noh YW, Kang TH, Kim JE, Kim S, Um SH, et al. Synthetic vaccine nanoparticles target to lymph node triggering enhanced innate and adaptive antitumor immunity. Biomaterials 2017;130:56–66.
19. Wang BH, An JY, Zhang SF, Zhang HF, Wang L, et al. Personalized cancer immunotherapy via transporting endogenous tumor antigens to lymph nodes mediated by nano Fe₃O₄. Small 2018;14:e1801372.
20. Thomas SN, Vokali E, Lund AW, Hubbell JA, Swartz MA. Targeting the tumor-draining lymph node with adjuvanted nanoparticles reshapes the anti-tumor immune response. Biomaterials 2014;35:814–24.
21. Zhang AM, Hai L, Wang TZ, Cheng H, Li M, He XX, et al. NIR-triggered drug delivery system based on phospholipid coated ordered mesoporous carbon for synergistic chemo-photothermal therapy of cancer cells. Chin Chem Lett 2020;31:5156–61.
22. Huang SN, Duan SF, Wang J, Bao SJ, Qiu XJ, Li CM, et al. Folic-acid-mediated functionalized gold nanocages for targeted delivery of anti-miR-181b in combination of gene therapy and photothermal therapy against hepatocellular carcinoma. Adv Funct Mater 2016;26:2532–44.
23. Hu YY, Lin L, Gao ZP, Chen I, Manuyama A, Tian HY, et al. In situ vaccination and gene-mediated PD-L1 blockade for enhanced tumor immunotherapy. Chin Chem Lett 2021;32:1770–4.
24. Zhao XY, Han Y, Sun Y, Feng W, Liu JG, Li DS, et al. Combining photothermal ablation-based vaccine with immune checkpoint blockade for synergistic osteosarcoma immunotherapy. Mater Design 2021;198:190311.
25. Yang SQ, Zhou LZ, Su Y, Zhang R, Dong CM. One-pot photoreduction to prepare NIR-absorbing plasmonic gold nanoparticles tethered by amphiphilic polysaccharide copolymer for synergistic photothermal-chemotherapy. Chin Chem Lett 2019;30:187–91.
26. Chen JY, Glaus C, Laforest R, Zhang Q, Yang MX, Gidding M, et al. Gold nanocages as photothermal transducers for cancer treatment. Small 2010;6:811–7.

27. Dong LY, Li Y, Li Z, Xu N, Liu P, Du HY, et al. Au nanocage-strengthened dissolving microneedles for chemo-photothermal combined therapy of superficial skin tumors. ACS Appl Mater Interfaces 2018;10:9247–56.

28. Mosquera J, García I, Henriksen-Lacey M, Martínez-Calvo M, Dhanjani M, Mascaréhas JL, et al. Reversible control of protein corona formation on gold nanoparticles using host-guest interactions. ACS Nano 2020;14:5382–91.

29. Kang S, Ahn S, Lee J, Kim JY, Choi M, Gujrati V, et al. Effects of gold nanoparticle-based vaccine size on lymph node delivery and cytotoxic T-lymphocyte responses. J Control Release 2017;256:56–67.

30. Liu J, Li HJ, Luo YL, Xu CF, Du XJ, Du JZ, et al. Enhanced primary tumor penetration facilitates nanoparticle draining into lymph nodes after systemic injection for tumor metastasis inhibition. ACS Nano 2019;13:8648–58.

31. Liptrott NJ, Kendall E, Nieves DJ, Farrell J, Rannard S, Fernig DG, et al. Partial mitigation of gold nanoparticle interactions with human lymphocytes by surface functionalization with a ‘mixed matrix. Nanomedicine 2014;9:2467–79.

32. Xia Y, Xie YH, Yu ZS, Xiao HY, Jiang GM, Zhou XY, et al. The mevalonate pathway is a druggable target for vaccine adjuvant discovery. Cell 2018;175:1059–73.

33. Wan Y, Guo ZR, Jiang XL, Fang K, Lu X, Zhang Y, et al. Quasi-spherical silver nanoparticles: aqueous synthesis and size control by the seed-mediated Lee-Meisel method. J Colloid Interface Sci 2013;394:263–8.

34. Xia YN, Li WY, Cobley CM, Chen JY, Xia XY, Zhang Q, et al. Gold nanocages: from synthesis to theranostic applications. Acc Chem Res 2011;44:914–24.

35. You J, Zhang GD, Li C. Exceptionally high payload of doxorubicin in hollow gold nanospheres for near-infrared light-triggered drug release. ACS Nano 2010;4:1033–41.

36. Ray S, Cheng CA, Chen W, Li Z, Zink JI, Lin YY. Magnetic heating stimulated cargo release with dose control using multifunctional MR and thermosensitive liposome. Nanotheranostics 2019;3:166–78.

37. Au L, Zheng DS, Zhou F, Li ZY, Li XD, Xia YN. A quantitative study on the photothermal effect of immuno gold nanocages targeted to breast cancer cells. ACS Nano 2008;2:1645–52.

38. Skrabalak SE, Au L, Li XD, Xia YN. Facile synthesis of Ag nanocubes and Au nanocages. Nat Protoc 2007;2:2182–90.

39. Daemi S, Ashkarran AA, Bahari A, Ghasemi S. Gold nanocages decorated biocompatible amine functionalized graphene as an efficient dopamine sensor platform. J Colloid Interface Sci 2017;494:290–9.

40. Jia YP, Shi K, Yang F, Liao JF, Han RX, Yuan LP, et al. Multifunctional nanoparticle loaded injectable thermoresponsive hydrogel as NIR controlled release platform for local photothermal immunotherapy to prevent breast cancer postoperative recurrence and metastases. Adv Funct Mater 2020;30:2001059.

41. Sweeney EE, Cano-Mejia J, Fernandes R. Photothermal therapy generates a thermal window of immunogenic cell death in neuroblastoma. Small 2018;14:e1800678.

42. Wei JI, Long Y, Guo R, Liu XL, Tang X, Rao JD, et al. Multifunctional polymeric micelle-based chemo-immunotherapy with immune checkpoint blockade for efficient treatment of orthotopic and metastatic breast cancer. Acta Pharm Sin B 2019;9:819–31.

43. Margiotta A, Frei DM, Sendstad IH, Janssen L, Neeffjes J, Bakke O. Invariant chain regulates endosomal fusion and maturation through an interaction with the SNARE Vti1b. J Cell Sci 2020;133:244624.

44. Skjeldal FM, Haugen LH, Mateus D, Frei DM, Rodseth AV, Hu X, et al. De novo formation of early endosomes during Rab 5-to-Rab7 transition. J Cell Sci 2021;134:254185.

45. Chen Q, Hu QY, Dukhovlinova E, Chen GJ, Ahn S, Wang C, et al. Photothermal therapy promotes tumor infiltration and antitumor activity of CAR T cells. Adv Mater 2019;31:e1900192.

46. Zhang XY, Zhang XZ, Zhang YQ, Liu MY, Jin J, Yan J, et al. Mitochondrial uncoupler triclosan induces vasorelaxation of rat arteries. Acta Pharm Sin B 2017;7:623–9.

47. Fankhauser M, Broggi MAS, Potin L, Bordry N, Jeanbart L, Lund AW, et al. Tumor lymphangiogenesis promotes T cell infiltration and potentiates immunotherapy in melanoma. Sci Transl Med 2017;9:eaal4712.

48. Sasso MS, Mitroussis N, Wang Y, Briquez PS, Hauert S, Ishihara T, et al. Lymphangiogenesis-inducing vaccines elicit potent and long-lasting T cell immunity against melanomas. Sci Adv 2021;7:eabe4362.

49. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol 2012;12:557–69.

50. Yang JX, Zhang CF. Regulation of cancer-immunity cycle and tumor microenvironment by nanobiomaterials to enhance tumor immunotherapy. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2020;12:e1612.

51. Shen LJ, Zhou TJ, Fan YT, Chang X, Wang Y, Sun JG, et al. Recent progress in tumor photodynamic immunotherapy. Chin Chem Lett 2020;31:1709–16.