Gene silencing-mediated immune checkpoint blockade for tumor therapy boosted by dendrimer-entrapped gold nanoparticles

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ABSTRACT Immune checkpoint blockade (ICB) has been regarded as one promising approach for tumor immunotherapy. Here, we report a functional nanoplatform based on generation 5 (G5) poly(amidoamine) (PAMAM) dendrimer-entrapped gold nanoparticles (Au DENPs) as a nonviral vector to deliver programmed death-ligand 1 (PD-L1) small interfering RNA (siPD-L1) for subsequent PD-L1 gene silencing-mediated tumor immunotherapy. In this work, G5 dendrimers with amine termini were partially decorated with methoxy polyethylene glycol (mPEG) on their periphery, entrapped Au NPs within their interiors, and were eventually labeled with fluorescamine. The generated functional Au DENPs possess desired dispersibility in water and colloidal stability, satisfactory cytocompatibility after complexation with siPD-L1, and efficient gene delivery performance. Strikingly, the functional Au DENPs enabled the delivery of siPD-L1 to cancer cells to efficiently knock down the PD-L1 protein expression, thus boosting the ICB-based immunotherapy of a xenografted melanoma mouse tumor model with a tumor inhibition efficiency much higher than the PD-L1 antibody. The immune responses were also well demonstrated by downregulation of PD-L1 protein on the tumor cell surface and abundant distribution of CD8⁺ and CD4⁺ T cells in the infiltrating tumor tissue and spleen organ. The developed functional dendrimer-based nanoplatform may be promising to boost ICB-based immunotherapy of other tumor types.

Keywords: dendrimers, gold nanoparticles, PD-L1 siRNA, immune checkpoint blockade, tumor immunotherapy

INTRODUCTION

The host immune system can recognize and kill tumor cells through the “tumor immune cycle” [1]. However,

malignant tumor cells often express immunosuppressive molecules to avoid the immunological surveillance of the host [2]. In particular, programmed death-ligand 1 (PD-L1), a highly expressed immunosuppressive molecule on the tumor cell surface, is able to be bound by programmed death-1 (PD-1) on the T cell surface, thus restricting the T cell antitumor activity [3,4]. Likewise, tumor cells engaged in the PD-1/PD-L1 binding pathway enabled the creation of immunosuppressive micro-environment, subsequently promoting immune evasion and ultimately leading to tumor recurrence and metastasis [5–7]. Immune checkpoint blockade (ICB)-based therapy has been emerging as a potential strategy for efficient tumor surveillance and inhibition by restoring the T cells’ tumor-killing ability [8,9]. In general, the inhibitors of immune checkpoint (such as PD-L1 or PD-1 monoclonal antibodies) have been employed for ICB-based antitumor immunotherapy [10–12]. However, the immune response rate for different tumor types is quite low (20%–30%), and the treatment cost is quite high, thus limiting their further clinical applications [13–15].

To achieve ICB-based immunotherapy, researchers have attempted to genetically modify either T cells or tumor cells to knock down the expression of immune checkpoint molecules through RNA interference [16–20]. In particular, small interfering RNA (siRNA) has been adopted to silence PD-L1 gene in tumor cells [20–22], which is beneficial for maintaining the long-term ICB to prevent tumor recurrence and metastasis [23]. However, siRNA can be easily degraded by enzymes under physiological circumstances, and its inherent negatively charged nature prevents it from penetrating the cell membrane for efficient transfection. Therefore, it is es-

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essential to deliver siRNA through polycationic nonviral vectors [24]. For instance, polyethylenimine (PEI) polymers functionalized with folic acid have been used to transfect PD-L1 siRNA (for short, siPD-L1) to knock down PD-L1 protein expression on the surface of epithelial ovarian cancer cells with an efficiency of 45% [19]. In another study, Guan *et al.* [23] developed a dual-rebound polyethylene glycol (PEG)[(poly-*L*-glutamate/PEI)/DNA] system to deliver plasmid DNA expressing small hairpin RNA of PD-L1 to achieve efficient ICB-based tumor immunotherapy with a PD-L1 knockdown efficiency of 40% in tumor cells. However, the developed non-viral vector systems either lack good cytocompatibility or controlled size and architecture. It is highly demanded to develop new vector systems with good cytocompatibility and controlled architecture for ICB-based tumor immunotherapy.

Poly(amidoamine) (PAMAM) dendrimers have been well demonstrated as one ideal nonviral vector system that has a superior gene delivery efficiency [25,26]. This should be attributed to their specific characteristics such as possessing a globular shape with highly branched interior, a central core, and abundant surface functional groups and availability from commercial resources. Recently, our group has engaged in the development of functional PAMAM dendrimer-entrapped gold nanoparticles (Au DENPs) as a nonviral vector system for cancer gene therapy [25] and immunotherapy [27]. The beauty of dendrimer vector system is that PAMAM dendrimers can not only be partially modified with PEG [25], acetyl groups [28], zwitterions [29], glycosyl groups [30,31], or targeting ligands [32–34] for improved or serum-enhanced gene delivery applications with low cytotoxicity, but also be incorporated with multiple organic or inorganic components to achieve combination therapy such as gene therapy/chemotherapy [34] and gene therapy/photothermal therapy [35]. Typically, the entrapped Au NPs within the dendrimers help preserve the three-dimensional conformation of the dendrimers, thereby improving the gene compaction ability for enhanced gene delivery applications [26].

Encouraged by earlier successes in the exploration of dendrimer-associated nanosystems [36], here we propose to develop an Au DENP-based vector system to deliver siPD-L1 for ICB-based tumor immunotherapy (Fig. 1). Firstly, generation 5 PAMAM dendrimers with amine termini (G5.NH₂) were partially conjugated with methoxy-polyethylene glycol-succinimidyl carboxymethyl ester (*m*PEG-SCM), loaded with Au NPs within their interiors through sodium borohydride reduction chem-

istry, and finally labeled with fluorescamine to form {(Au⁰)₂₅-G5.NH₂-*m*PEG-F} DENPs. We systematically characterized the vector and investigated its gene delivery efficiency *in vitro* through delivery of plasmid DNA encoding enhanced green fluorescence protein (pDNA-EGFP). Furthermore, the vector was used to deliver siPD-L1 to mouse melanoma cancer cells (B16 cells) *in vitro* and the xenografted B16 tumor model *in vivo* to boost ICB-based tumor immunotherapy. Based on our thorough investigation of literature, we are the first to explore such a dendrimer-based vector system to boost ICB-based tumor immunotherapy.

EXPERIMENTAL SECTION

Synthesis of {(Au⁰)₂₅-G5.NH₂-*m*PEG-F} DENPs

First, *m*PEG-SCM (76.89 mg, 38.45 μmol) with 20 mol equiv. to G5.NH₂ dendrimers was dissolved in water (7.69 mL), dropped into a G5.NH₂ dendrimer solution (50 mg, in 5 mL water), followed by stirring for 4 h according to Ref. [37]. Then, we dialyzed the reaction mixture through a membrane with a molecular weight cut-off (MWCO) of 5000 Da for three days (nine times, 2 L), and lyophilized the dialysis liquid to acquire the G5.NH₂-*m*PEG conjugates.

Next, the created G5.NH₂-*m*PEG conjugates were employed to entrap Au NPs following protocols reported in our earlier work [29]. In brief, the G5.NH₂-*m*PEG conjugates were dissolved in 5 mL of water, dropped with an HAuCl₄·4H₂O solution (687 μL, 30 mg mL⁻¹ in water) at an Au salt/dendrimer molar ratio of 25:1 under strong magnetic stirring for 30 min, and rapidly added with NaBH₄ (5.56 mg, in 1 mL water), followed by stirring for 4 h. We then purified the reaction mixture by dialysis through a 500 MWCO membrane against water for three days (nine times, 2 L), and lyophilized the dialysis liquid to give a solid of {(Au⁰)₂₅-G5.NH₂-*m*PEG} DENPs.

Finally, a fluorescamine solution (4 mg mL⁻¹, in 668 μL acetone) reacted with the {(Au⁰)₂₅-G5.NH₂-*m*PEG} solution (10 mg mL⁻¹, in 12.62 mL phosphate buffer solution, pH 8.0) under vigorous magnetic stirring at 40°C for 30 min in the dark. The reaction mixture was subjected to dialysis and lyophilization according to above procedures to acquire the {(Au⁰)₂₅-G5.NH₂-*m*PEG-F} DENPs.

Preparation of polyplexes

The prepared {(Au⁰)₂₅-G5.NH₂-*m*PEG-F} DENPs were used as a vector to complex pDNA-EGFP or siPD-L1 according to the literature protocols [38]. The {(Au⁰)₂₅-G5.NH₂-*m*PEG-F} DENPs and pDNA-EGFP or siPD-L1

Figure 1 Preparation of $\{(Au^0)_{25}-G5.NH_2-mPEG-F\}$ DENPs/siPD-L1 polyplexes for ICB-based cancer immunotherapy.

were separately dissolved in water and mixed with $1\ \mu\text{g}$ pDNA-EGFP or siPD-L1 at different N/P ratios (the molar ratio of primary amines of the vector to phosphates in the DNA or siRNA backbone). After gentle vortexing and incubation at room temperature for 20 min, the polyplexes were formed for subsequent characterization or transfection.

***In vitro* cell culture assays**

B16 cells were regularly cultured and adopted for *in vitro* cytotoxicity assay of vector, vector/pDNA polyplexes, or vector/siRNA polyplexes, cellular uptake assay of vector/siPD-L1 polyplexes, EGFP gene transfection assay, and gene silencing efficiency test of the vector/siPD-L1 polyplexes.

***In vivo* immunotherapy of tumors**

All animal experiments were carried out according to the protocols approved by the ethical committee of Donghua University for animal care and also in accordance with the policy of the National Ministry of Health. The volumes of tumors, body weights of mice, histological ex-

aminations of tumors and major organs, immunofluorescence staining of tumor sections and spleen, Western blot assay of tumor cells' PD-L1 expression, flow cytometric analysis of T cells extracted from the tumors, and quantification of cytokines in the serum were performed to examine the effect of tumor immunotherapy and immune responses after different treatments.

RESULTS AND DISCUSSION

Synthesis and characterization of functional Au DENPs

G5 PAMAM dendrimers were chosen as a starting platform that was covalently conjugated with *m*PEG-SCM to obtain $G5.NH_2-mPEG$ dendrimers [37]. Then, the $G5.NH_2-mPEG$ dendrimers were loaded with Au NPs through sodium borohydride reduction according to Ref. [39], and labeled with fluorescamine to get the eventual product of $\{(Au^0)_{25}-G5.NH_2-mPEG-F\}$ DENPs (Fig. 1) [40].

The chemical structures of *m*PEG-SCM, fluorescamine and Au DENPs were checked by ^1H NMR spectroscopy

(Fig. S1). As revealed in Fig. S1c, the chemical shift at 3.6 ppm for Au DENPs can be attributed to the $-\text{CH}_2-$ protons of PEG, similar to that for the *m*PEG-SCM (Fig. S1a), suggesting the successful conjugation of *m*PEG-SCM on the G5 dendrimer surface. Meanwhile, the proton peaks at 7.0–7.6 ppm for the Au DENPs can be attributed to the protons of the benzene ring of the fluorescamine, quite similar to those of the free fluorescamine (Fig. S1b), validating the accomplished fluorescamine labeling onto the dendrimer platform. Through the comparison of dendrimer methylene protons in the range of 2.2–3.4 ppm with the associated proton peaks of PEG and fluorescamine, the numbers of *m*PEG-SCM and fluorescamine linked to each G5 dendrimer were estimated to be 17.2 and 3.8, respectively.

The entrapment of Au NPs within the dendrimers was validated by UV-vis spectrometry (Fig. S2). The appearance of a surface plasmon resonance band at about 510 nm suggests the production of Au NPs. We then quantitatively measured the gold content through inductively coupled plasma-optical emission spectroscopy (ICP-OES). The measured practical Au/dendrimer molar ratio is 25:1, which is equal to the initial molar feeding ratio of Au salt/dendrimer. As an interesting note, the selection of the Au salt/dendrimer molar ratio of 25:1 is because Au DENPs formed at this molar ratio display the optimal gene delivery efficiency [26]. The structure of the G5.NH_2 -*m*PEG and $\{(\text{Au}^0)_{25}\text{-G5.NH}_2\text{-mPEG-F}\}$ DENPs were further characterized by Fourier transform infrared (FTIR) spectrometry (Fig. S3). The peak at around 3431 cm^{-1} can be assigned to the $-\text{NH}_2$ groups of G5.NH_2 , and the peak at around 1635 cm^{-1} is assigned to the amide I bond. Meanwhile, the peak at 1101 cm^{-1} could be associated to the C–O bonding of PEG, confirming the successful conjugation of *m*PEG on the surface of G5.NH_2 dendrimers. The FTIR spectra of both G5.NH_2 -*m*PEG and $\{(\text{Au}^0)_{25}\text{-G5.NH}_2\text{-mPEG-F}\}$ DENPs are quite identical, meaning that the entrapment of Au NPs within the dendrimer templates does not seem to appreciably alter the structure of dendrimers.

The conjugation of fluorescamine onto the surface of Au DENPs was next verified through fluorescence spectroscopic assays (Fig. S4). Under the emission wavelength of 485 nm and excitation wavelength of 415 nm, free fluorescamine and the pristine G5.NH_2 dendrimers do not display apparent fluorescence property. In contrast, after the primary amines of Au DENPs were linked with fluorescamine, the functional Au DENPs display the fluorescamine-associated fluorescence emission peak at 485 nm and fluorescence excitation peak at 415 nm, in

agreement with Ref. [41].

The size and shape of the Au core particles for the functional Au DENPs were observed by transmission electron microscopy (TEM, Fig. 2a). The Au cores display a spherical or semi-spherical shape and have a mean diameter of 1.9 nm (Fig. S5). Meanwhile, the colloidal stability of the functional Au DENPs was checked after being stored in different aqueous media such as water, phosphate buffered saline (PBS) and cell culture medium at 4°C for at least two weeks. We did not find any precipitation happened, indicating their desirable stability (Fig. S6a). Moreover, we also monitored their hydrodynamic sizes in water by dynamic light scattering (DLS). Apparently, the Au DENPs do not show any considerable variations in their hydrodynamic size for at least seven days (Fig. S6b). These results together demonstrate that the functional Au DENPs own good colloidal stability. It should be noted that the measured hydrodynamic size of the Au DENPs is much larger than that determined by TEM. This should be attributed to the differences of the measurement mechanism and objective, in consistence with Ref. [29].

Preparation and characterization of vector/pDNA polyplexes for gene delivery

Before the preparation of vector/gene polyplexes, we determined the primary amine density on the surface of Au DENPs by Megazyme's PANOPA Assay Kit. We show that each Au DENP possesses 50 primary amines on the surface, which is much less than that of the pristine G5.NH_2 dendrimer [30]. This should be attributed to the partial PEGylation and fluorescamine modification as well as the consumption of part amines for stabilizing the entrapped Au cores, in agreement with our earlier work [29].

The $\{(\text{Au}^0)_{25}\text{-G5.NH}_2\text{-mPEG-F}\}$ DENPs were subsequently used to complex pDNA-EGFP through electrostatic interaction. The gene compaction ability of the Au DENPs was examined by agarose gel retardation assay (Fig. S7). Apparently, at the N/P ratio of 1 or higher, pDNA-EGFP can be completely compressed by the Au DENPs. Therefore, we chose the vector/pDNA-EGFP polyplexes at an N/P ratio of 1 or higher for subsequent studies.

The zeta potentials and hydrodynamic sizes of the vector/pDNA-EGFP polyplexes under different N/P ratios were next measured. As shown in Fig. S8, at the N/P values of 2, 5, and 10, the zeta potentials of all the polyplexes are in the range of 11–17 mV, and their hydrodynamic sizes are between 213 and 250 nm. Clearly, all

Figure 2 (a) TEM and magnified TEM (inset) images of the $\{Au\}_{25}$ -G5.NH₂-mPEG-F DENPs. (b) Gel retardation assay of siPD-L1 after complexing with the vector at different N/P ratios. Lanes 1–8 represent naked siRNA and N/P ratios of 0.125:1, 0.25:1, 0.5:1, 1:1, 2:1, 3:1 and 4:1, respectively. Zeta potential (c) and hydrodynamic size (d) of vector/siPD-L1 polyplexes under different N/P ratios.

vector/pDNA-EGFP polyplexes possess positive zeta potentials and adequately small sizes, which are essential for gene delivery applications.

Cell Counting Kit-8 (CCK-8) assay was adopted to investigate the cytocompatibility of the vector or the vector/pDNA-EGFP polyplexes (Fig. S9). After treatment with vector or polyplexes for 24 h, B16 cells exhibit decreased viability with the vector concentration. Under the same vector concentrations, the polyplexes exhibit a lower cytotoxicity than vector alone. This should be because the condensation of negatively charged pDNA-EGFP partially neutralizes the positive surface potential of the cationic vector [38]. The cell viability can still maintain more than 72% after treatment with polyplexes at the highest vector concentration tested (3000 nmol L⁻¹) for 24 h, suggesting that the functional Au DENPs can be developed for safe gene delivery.

Fluorescence microscopic observation and flow cytometry assay were next employed to check the gene transfection efficiency of B16 cells after they were transfected with the vector/pDNA-EGFP polyplexes for 4 h and then regularly cultured for 24 h (Figs S10 and S11). Obviously, at the polyplex N/P ratio of 10:1, the cells

exhibit the most prominent green fluorescence signals, verifying the successful transfection of EGFP.

In vitro siPD-L1 delivery

After confirming the feasibility of the Au DENP-mediated pDNA-EGFP delivery, we then tested the potential to use the Au DENPs for siPD-L1 delivery to silence the PD-L1 expression in B16 cells. Gel retardation assay reveals that siPD-L1 can be completely compressed by the functional Au DENPs at an N/P ratio of 1 or greater (Fig. 2b). Next, we checked the zeta potentials and hydrodynamic sizes of the vector/siPD-L1 polyplexes created at different N/P values. As shown in Fig. 2c and d, the zeta potentials and hydrodynamic sizes of all the vector/siPD-L1 polyplexes are in a range of 15–20 mV and 187–228 nm, respectively, which is quite suitable for gene delivery [42]. Furthermore, cytotoxicity assay of the vector/siPD-L1 and vector/scrambled control siRNA (vector/siNC) polyplexes shows that both polyplexes exhibit good cytocompatibility in the vector concentration range of 50–3000 nmol L⁻¹ (Fig. 3a).

We then investigated the cellular uptake of vector/siPD-L1 polyplexes by B16 cells *via* flow cytometry using

Figure 3 (a) Cytotoxicity assay of vector/siPD-L1 and vector/siNC polyplexes after B16 cells were treated with them at different vector concentrations. (b) Relative mean fluorescence of B16 cells transfected with vector/siPD-L1 polyplexes at N/P ratios of 2:1, 5:1, 10:1, and 20:1, respectively, for 4 h was analyzed by flow cytometry. The fluorescence intensity of PBS group was set to be 1.0 (mean $\pm$ SD, $n = 3$). (c) Western blot assay of PD-L1 protein expression (mean $\pm$ SD, $n = 3$). Expression of PD-L1 was set to be 1.0 in cells treated with PBS, and *** represents $p < 0.001$. (d) Confocal microscopic imaging of B16 cells transfected with PBS, free siPD-L1 or vector/siPD-L1 (N/P value = 10) for 4 h. The scale bar represents 20 μ m for each panel.

Cyanine 3 (Cy3)-labeled siPD-L1 (Fig. 3b). Obviously, the treatment of cells with the polyplexes having an N/P value of 10 leads to the highest fluorescence intensity, suggesting the best gene transfection efficiency of the polyplexes at the N/P value of 10. Afterwards, the vector/siPD-L1 polyplexes with the optimal N/P value were chosen for confocal microscopic observation of the intracellular localization of the polyplexes (Fig. 3d). Cells treated with free siPD-L1 and PBS only display green fluorescence associated with the 3,3'-dioctadecyloxa-

carbocyanine perchlorate (DiO)-stained, cell membrane. In sharp contrast, B16 cells transfected with the vector/Cy3-siPD-L1 polyplexes display significant red and blue fluorescent signals in the cytoplasm. The blue fluorescent signals are from the fluorescamine labeled onto the surface of Au DENPs, while the red fluorescent signals are from the Cy3 dye labeled on siPD-L1. The confocal microscopic images confirm that the functional Au DENPs are able to facilitate the cellular internalization of siPD-L1 for subsequent gene transfection.

To confirm the efficiency of polyplex-induced gene silencing of PD-L1 in B16 cells *in vitro*, Western blot analysis was performed after the cells were transfected with the polyplexes (Fig. 3c). Evidently, compared with blank control (PBS), naked siPD-L1 and negative control (vector/siNC), the transfection of vector/siPD-L1 results in the best inhibition of PD-L1 protein expression ($p < 0.001$). Overall, the transfection of vector/siPD-L1 polyplexes can effectively knock down the PD-L1 expression in B16 cells with an efficiency of 47%.

***In vivo* ICB-based tumor immunotherapy**

Based on the above encouraging *in vitro* results, we next validated the ICB-based antitumor immunotherapy effect of the polyplexes using a xenografted tumor model *in vivo* according to the treatment schedule (Fig. 4a). Tumors treated with PBS and vector/siNC exhibit absolutely rapid growth (Fig. 4b). In contrast, the treatments with PD-L1 antibody (for short, a-PD-L1) and vector/siPD-L1 polyplexes are able to inhibit the tumor growth ($p < 0.01$), and the vector/siPD-L1 polyplexes seem to show the best inhibitory effect among all groups ($p < 0.01$). The better immunotherapy effect of vector/siPD-L1 than that of the commercial a-PD-L1 could be because of the high target specificity of siPD-L1 to the tumor cells and the inherent knockdown of the PD-L1, better facilitating the ICB-based tumor immunotherapy than the a-PD-L1. Meanwhile, all groups do not show significantly changed mouse body weights with the time postinjection (Fig. 4c), indicating the good biosafety of vector/siPD-L1 polyplexes. Furthermore, the major organs such as the heart, liver, spleen, lung and kidney of mice after different treatments were also collected on day 14 and stained with hematoxylin and eosin (H&E) (Fig. S12). No apparent abnormality in all major organs are observed when compared with the PBS control, indicating that all treatments do not seem to generate toxicity to the mice and the functional Au DENPs can act as a vector with good biosafety for tumor treatment.

The therapeutic effect of the polyplexes was further examined by H&E and terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) staining of tumor tissues after different treatments (Fig. 4d). The tumors in the vector/siPD-L1 group exhibit the most significant necrotic (H&E) and apoptotic (TUNEL) cell distributions among all groups, in accordance to the above tumor inhibition data. Quantitative tumor cell apoptosis rate evaluated from TUNEL staining images shows that the tumor cell apoptosis rate is in an order of vector/siPD-L1 ($73.61\% \pm 7.34\%$) > a-PD-L1 ($48.29\% \pm$

4.69%) > vector/siNC ($5.52\% \pm 1.85\%$) $\approx$ PBS ($5.48\% \pm 2.37\%$), indicating that the vector/siPD-L1 polyplexes enable the most effective tumor inhibition (Fig. S13).

Antitumor immunotherapy mechanism and immune responses

To delineate the underlining mechanism associated to antitumor immunotherapy, Western blot and immunofluorescence staining were used to determine the PD-L1 protein expression in tumor cells. As shown in Fig. 5a, the vector/siPD-L1 polyplexes enable the most significant knockdown of PD-L1 protein in comparison with other groups ($p < 0.01$). Again, the *in vivo* tumor cell PD-L1 protein knockdown rate in the vector/siPD-L1 group (59%) is significantly higher than that in the a-PD-L1 group (48%), in good agreement with the gene silencing data *in vitro*.

As reported in Ref. [16], PD-1 expressed on the T cell surface can be bound to PD-L1 present on the tumor cells to restrict the T cell antitumor activity. $CD8^+$ and $CD4^+$ T cells are crucial players in the antitumor immunotherapy and adaptive immune responses [43]. In particular, $CD8^+$ T cells are direct tumor killers, while $CD4^+$ T cells as a helper can regulate humoral and cellular immunity. Therefore, we checked the $CD4^+/CD8^+$ T cell distribution in the infiltrating tumor tissues by immunofluorescence staining to prove the PD-L1 gene silencing-induced tumor immunotherapy (Fig. 5b). The PD-L1, CD4 and CD8 proteins in the tumor tissues were labeled with red fluorescent antibodies, and the tumor cell nuclei were stained with blue fluorescent dye of 4',6-diamidino-2-phenylindole. Apparently, tumors treated with PBS and vector/siNC show higher PD-L1 expression and less distribution of $CD8^+$ and $CD4^+$ T cells than those treated with vector/siPD-L1 and a-PD-L1. Compared with tumors treated with the a-PD-L1, the treatment with vector/siPD-L1 polyplexes leads to much more significant reduction of PD-L1 protein expression and increase of $CD8^+/CD4^+$ T cells, further verifying the most effective tumor immunotherapy through PD-L1 gene silencing.

To study the whole body immune response after the treatment, immunofluorescence staining images of the spleen were collected (Fig. S14). The frequency of $CD8^+/CD4^+$ T cell infiltration in the spleen for the vector/siPD-L1 group is the highest, in consistence with the results of T cells in the infiltrating tumor tissues, validating the effective tumor immunotherapy based on gene silencing-mediated ICB. Further, we also attempted to quantify the tumor $CD8^+/CD4^+$ T cells by flow cytometry (Fig. 5c–e). Before this, the purity of T cells extracted from tumor

Figure 4 (a) The timeline used for the tumor immunotherapy *in vivo*. (b) The relative tumor volume changes of the mice in different treatment groups ($n = 6$ for each group, and ** and *** represent $p < 0.01$ and $p < 0.001$, respectively). (c) The relative mouse body weight variations in different treatment groups ($n = 6$ for each group). (d) H&E and TUNEL staining of the tumor sections on the 14th day after different treatments. Scale bar represents 100 μm for each panel.

tissues was tested by flow cytometry (Fig. S15). As a T cell purity marker, CD3 protein expression was analyzed. The percentage of T cells expressing CD3 was tested to be 92.5%, which is adequate for further flow cytometric quantification. As can be seen, the percentages of CD8⁺ T cells in the tumors treated with a-PD-L1 and vector/siPD-L1 are over 6.7-fold and 9.3-fold higher than those treated with PBS or vector/siNC, respectively. Likewise, the percentages of CD4⁺ T cells in the tumors treated with a-PD-L1 and vector/siPD-L1 are over 4.4-fold and 5.8-fold higher than those treated with PBS or vector/siNC, re-

spectively. These results illustrate that the vector/siPD-L1 polyplexes induce the most prominent adaptive immune responses to achieve enduring therapeutic efficacy.

Lastly, we also examined the levels of tumor necrosis factor α (TNF- α), interferon- γ (IFN- γ), and interleukin 6 (IL-6) in the serum of all treatment groups (Fig. 5f–h). These cytokines are key biomarkers released by immune cells that can enhance T cell-mediated antitumor immune responses [44]. Clearly, the concentrations of all cytokines in the vector/siPD-L1 group are much higher than those in the other groups ($p < 0.001$). Again, the vector/siPD-L1

Figure 5 (a) Western blot assay of PD-L1 expression of tumor cells. Expression of PD-L1 in tumor cells treated with PBS was set to be 1.0. (b) The immunofluorescence images of PD-L1, CD4 and CD8 proteins in tumor tissues. (c–e) Flow cytometric assay of CD4⁺/CD8⁺ T cells in the infiltrating tumor tissues (gated on CD3⁺ T cells). (f–h) Contents of the TNF-α, IFN-γ and IL-6 in serum on the 14th day after various treatments. All data are shown as mean ± SD (*n* = 3). In (a) and (d–h), ** and *** represent *p* < 0.01 and *p* < 0.001, respectively.

group shows higher immunotherapy efficiency than the a-PD-L1 group.

CONCLUSIONS

In conclusion, we developed a dendrimer-based delivery platform for gene silencing-mediated ICB to boost tumor immunotherapy. We show that partially PEGylated G5

PAMAM dendrimers can entrap Au NPs within their interiors and be covalently labeled with a blue fluorescent dye for intracellular trafficking purposes. The generated functional Au DENPs with an Au core size of 1.9 nm are colloiddally stable, can compact pDNA or siPD-L1 to form polyplexes with good cytocompatibility, and transfect cancer cells for highly efficient EGFP protein expression

and PD-L1 knockdown on the cell surface. With these outstanding properties, the functional Au DENPs are able to deliver siPD-L1 to knock down PD-L1 in tumors with an efficiency of 59%, thus mediating efficient ICB-based tumor immunotherapy *in vivo* and immune responses as manifested by the increase of CD4⁺/CD8⁺ T cell distribution in the infiltrating tumor tissues and spleen. The developed functional dendrimer platform may be extended to effective immunotherapy of other tumor types or combined with other therapeutic modes for precision or personalized tumor therapy.

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Author contributions Xue X carried out the experiments including synthesis, characterization, and *in vitro* and *in vivo* evaluation of the vector/siPD-L1 polyplexes, and wrote the original draft of the manuscript; Li J and Fan Y carried out part of the material characterization and *in vitro* evaluation of the materials; Shen M performed part of the data analysis and interpretation. Shi X designed and supervised the whole project and did the final manuscript editing.

Conflict of interest The authors declare no competing financial interest.

Supplementary information Supporting data are available in the online version of the paper.

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树状大分子包裹纳米金颗粒用于通过基因沉默介导的肿瘤免疫检查点阻断治疗

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摘要 免疫检查点阻断(ICB)被认为是一种有潜力的肿瘤免疫治疗途径. 本文中, 我们报告了基于第5代(G5)聚酰胺-胺树状大分子包裹金纳米颗粒(Au DENPs)的功能化纳米平台作为非病毒载体传递程序性死亡受体-配体1的小干扰RNA(PD-L1 siRNA, siPD-L1), 用于PD-L1基因沉默介导的肿瘤免疫治疗. 我们在氨基末端的G5树状大分子表面部分修饰甲氧基聚乙二醇, 在其内部空腔包裹金纳米颗粒, 最终标记荧光胺. 制备得到的功能化Au DENPs具有良好的水溶液分散性和胶体稳定性, 与siPD-L1复合后具有良好的细胞相容性和有效的基因传递性能. 通过功能化的Au DENPs将siPD-L1传递至肿瘤细胞可有效沉默PD-L1蛋白的表达, 从而促进基于ICB的异种移植黑色素瘤小鼠肿瘤模型的免疫治疗, 且其肿瘤抑制率远高于PD-L1抗体. 通过肿瘤细胞表面PD-L1蛋白表达的下调以及浸润的肿瘤组织和脾脏中CD8⁺和CD4⁺ T细胞的大量分布, 免疫响应得到了很好的验证. 该功能化树状大分子纳米平台有望用于基于ICB的免疫疗法治疗其他类型肿瘤.