Combining mannose receptor mediated nanovaccines and gene regulated PD-L1 blockade for boosting cancer immunotherapy

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A B S T R A C T

Tumor nanovaccines have potential applications in the prevention and treatment of malignant tumors. However, it remains a longstanding challenge in exploiting efficient nanocarriers for inducing potent specifically cellular immune responses. Toward this objective, we herein explore an intensive tumor immunotherapeutic strategy by combining mannosylated nanovaccines and gene regulated PD-L1 blockade for immune stimulation and killing activity. Here, we fabricate a mannose modified PLL-RT (Man-PLL-RT) mediated nanovaccines with dendritic cells (DCs) targeting capacity. Man-PLL-RT is capable of co-encapsulating with antigen (ovalbumin, OVA) and adjuvant (unmethylated cytosine-phosphate-guanine, CpG) by electrostatic interaction. This positively charged Man-PLL-RT/OVA/CpG nanovaccines can facilitate the endocytosis, maturation and cross presentation in DCs. However, the nanovaccines arouse limited inhibition of tumor growth, which is mainly due to the immunosuppressed microenvironment of tumors. Combining tumor nanovaccines with gene regulated PD-L1 blockade leads to an obvious tumor remission in B16F10 melanoma bearing mice. The collaborative strategy provides essential insights to boost the benefits of tumor vaccines by regulating the checkpoint blockade with gene therapy.

1. Introduction

Tumor immunotherapy is a potential strategy to control and eliminate tumors via restarting and maintaining the tumor-immune circulation and restoring the normal anti-tumor immune response [1]. Including monoclonal antibody class immune checkpoint inhibitors [2], therapeutic antibodies [3], tumor vaccines [4], cell therapy [5] and small molecule inhibitors [6]. Tumor vaccines, including whole tumor cell vaccines, tumor specific/associated antigens, protein or peptide vaccines, DNA/RNA vaccines and so on, demonstrate an enormous potential in tumor immunotherapy [7]. They can activate the patient’s own immune system, so as to achieve the purpose of control or removal of tumors. Among them, protein and polypeptide vaccines have the advantages of high controllability, safe technology, and non-toxic side effects. At present, many kinds of anti-tumor polypeptide vaccines have entered clinical trials [8]. However, the existing problems of protein or peptide vaccines at the present stage are as follows:(1) It is easy to be quickly eliminated by the body and limited antigen cross presentation ability and low immunogenicity to stimulate the body to produce a sustained and strong T-cell immune response [9]. (2) Tumor immune tolerance microenvironment visibly weakens the anti-tumor immune response of the vaccine. Therefore, more effective strategies are urgent needed to improve the responsiveness of tumor vaccines.

It is well known that tumor immunotherapy mainly derives from the
cell-mediated killing effect of CD8+ T cells. The protein and peptide vaccines themselves can only be presented as exogenous antigens by antigen processing cells (APCs) to CD4+ T cells via MHC II pathway [10]. Nanocrystallization of protein or peptide antigens can change their delivery pathway via increasing the connotation of the antigen endosomal escape function, so that the exogenous antigens are transformed into endogenous antigens, subsequently presenting to CD8+ T cells via MHC I pathway and improving antitumor immune response [11]. Nano-vaccines have the following advantages: (1) Protect antigens from degradation during in vivo delivery; (2) Increase the efficiency of vaccine delivery in antigen presenting cells; (3) Improve the antigen cross-presentation of tumor antigens and activate specific anti-tumor T cell immune response. Presently, there are many kinds of vaccine carriers, such as lipid nanoparticles [12], polyethyleneimine (PEI) [10,13], poly(lactic-co-glycolic acid) (PLGA) [14], CaCO3 [7], polymersomes [15], emulsions [16] and so on. In addition, the introduction of APC targeting molecules into the nanovaccines can effectively improve the delivery efficiency of the nano vaccine [17].

Although tumor nanovaccines can effectively activate the body’s T cell immune response, the therapeutic effects are usually still limited, which is mainly due to the multiple immunosuppressive mechanisms in tumor tissues, including the overexpression of immune checkpoint proteins, immunosuppressive factors, and highly infiltration of immunosuppressive cells, including myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) [18]. These immune suppression factors jointly lead to the disability of cytotoxic T lymphocytes (CTL). Among them, immune checkpoint is an immunosuppressive molecule and one of the most crucial reasons for tumor immune tolerance [19]. Immune checkpoint therapy improves the anti-tumor immune response by regulating CTL activity through a series of pathways as co-suppressing or co-stimulation signals. Currently, several monoclonal antibodies targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed death-1 (PD-1), programmed cell death-ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase-1 (IDO-1), have been validated in clinical therapy or clinical trials [20,21]. However, there are still several drawbacks for the antibody treatment. During the development course of tumors, the immune microenvironment of the local tumor tissues greatly changed, while the immunological indexes of other peripheral immune system almost unchanged. In addition, the side effects of systemic “blocking immune suppression” can make patients suffer great pain as well as benefit from therapy. Gene therapy is considered to be one of the most effective and promising therapeutic techniques in the future tumor therapy. Gene therapy has attracted much attention due to its good tolerability, small toxic and side effects [22–25]. Many delivery systems based on nanopolymers have been developed to achieve effective gene therapy [26–29]. However, multiple biological barriers in vivo seriously restrict the therapeutic effect of gene delivery system [30].

We previously prepared a versatile non-viral gene carrier by introducing p-tolualsulfonyl arginine onto the polylysine backbone (PLL-RT), which demonstrated multiple interactions with DNA or cell membrane [31]. In this study, mannose segments that can specifically bind to mannose receptor (MR) overexpressing on APC surface were conjugated on the amino groups of PLL-RT (Man-PLL-RT) to achieve APC-targeting and cross-presentation capacity. Man-PLL-RT could further absorb antigen ovalbumin (OVA) and adjuvant unmethylated cytosine-phosphate-guanine (CpG) via electrostatic adsorption. In addition, regulating tumor immune tolerance microenvironment, we further constructed a short hairpin RNA (shPD-L1) for silencing PD-L1 protein expressing in the membrane of tumor cells and block PD-1/PD-L1 pathway. The gene delivery system (HA/PLL-RT/shPD-L1) consisted of PLL-RT/shPD-L1 complexes shielded by hyaluronic acid. On the one hand, negative hyaluronic acid could counteract the positive charge of PLL-RT/shPD-L1 complexes, improving the in vivo safety and delivery efficiency of gene delivery system. On the other hand, hyaluronic acid can specifically target to CD44 receptor, a type of transmembrane glycoprotein that is overexpressed on the surface of tumor cells [32]. Overall, this strategy possesses (Scheme 1) the following advantages. (1) The nanovaccines are composed of poly-peptides, which are bio-safe and biodegradable; (2) The nanovaccines have APCs target function; (3) Blocking PD-1/PD-L1 through gene therapy can reverse the tumor immune-tolerant microenvironment; (4) The combined strategy can alleviate and inhibit tumor growth, which provides a potentially effective strategy for the clinical treatment of tumors.

2. Materials and methods

2.1. Materials

Hyperbranched PEI-25 k (25 kDa, weight-average molecular weight), ovalbumin (OVA), and hyaluronic acid sodium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). Boc–Arg(Tos)-OH was purchased from GL Biochem Ltd. (Shanghai, China). Dipsopropylethylamine (DEPEA) and 1-hydroxybenzotriazoleleahyndrous (HOBT) were ordered from Dibai Chemical Technology Co., Ltd. (Shanghai, China). N, N-dimethyl formamide (DMF) and diethyl ether were purchased from Xiya Reagent Co., Ltd. (Linyi, China) and harvested through vacuum distillation. α-Mannose and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC·HCl) were purchased from J&K Scientific Ltd. (Beijing, China). Dialysis bag (cut off 3500 Da) was purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China). RPMI 1640, Dulbecco’s Modified Eagle’s medium (DMEM) high glucose and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, USA). Mouse PD-L1 gene was constructed in shRNA expression vector named shPD-L1. Mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were ordered from PeproTech (Rocky Hill, USA). Luciferase plasmid DNA (pGL3), luciferin substrate, and cell lysis buffer were purchased from Promega (Madison, USA). The total RNA extraction kit, reverse transcription kit and RT-qPCR kit were ordered from Tiangen Biotech (Beijing) Co., Ltd. Mouse tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), transforming growth factor-β (TGF-β) and interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience (California, USA).

2.2. Preparation of Man-PLL-RT

PLL-RT was prepared according to our previous study [31]. Briefly, the linear PLL (molecular weight 15 kDa) was dissolved in deionized water and Boc-Arg(Tos)-OH was dissolved in DMF, respectively. Then, EDC·HCl and HOBT were added and activated for 1 h at room temperature, followed by injecting PLL solution and reacting at room temperature for 72 h. After dialysis and lyophilization, the product was reacted with trifluoroacetic acid for 4 h, precipitated by anhydrous ether and vacuum-dried. The white solid product of PLL-RT was harvested after dialyzing and lyophilizing.

The dendritic cells targeted carrier was further modified by condensation reaction of carboxylated mannose on the side chain amino of PLL-RT (Man-PLL-RT). Briefly, 20 mg of PLL-RT was dissolved in 1 mL of NaOH (1 M) and stirred for 30 min 1 mL of chloroacetic acid solution was added to the PLL-RT solution and reacted for 12 h at 60°C. To achieved carboxylated mannose, 250 μL of HCl (1 M) was added to reduce acidity to pH2.5. Afterwards, EDC·HCl and HOBT aqueous solution were added to activate for 30 min. PLL-RT and DEPEA were then added and stirred at 30°C for 48 h. After dialysis and lyophilization, the final product Man-PLL-RT was harvested. The molecular structure of Man-PLL-RT was characterized by 1H NMR spectra on Bruker AV 400 M at 25°C.
2.3. Preparation of the nanovaccines

The Man-PLL-RT/OVA/CpG nanovaccines (abbreviated as NVs) were prepared by electrostatic adsorption. Briefly, Man-PLL-RT, OVA and CpG were diluted in distilled water at the concentrations of 2.5 mg/mL, 1 mg/mL and 0.5 mg/mL. Afterwards, equal volume of them were mixed together and vortexed for 10 s. The nanovaccines were harvested after 20 min incubation.

The loading amounts of OVA and CpG in the NVs were detected. Briefly, Man-PLL-RT/FITC-OVA/Cy5-CpG NVs was prepared by electrostatic adsorption. Man-PLL-RT, FITC-OVA and Cy5-CpG were diluted in distilled water at the concentrations of 2.5 mg/mL, 1 mg/mL and 0.5 mg/mL. Afterwards, equal volume of them were mixed together and vortexed for 10 s. The nanovaccines were harvested after 20 min incubation at room temperature. Distilled water, free FITC-OVA, and Cy5-CpG solution were used as the controls. The samples were centrifuged for 10 min though an ultrafiltration device (MWCO 100 kDa). The filtrate was collected and the amounts of OVA and CpG were determined using fluorescence spectrometry (FITC-OVA: $\lambda_{ex} = 488, \lambda_{em} = 518$; Cy5-CpG: $\lambda_{ex} = 650, \lambda_{em} = 670$) according to the standard curve.

2.4. Preparation of gene delivery system

The gene delivery system was obtained by simple electrostatic interactions. Firstly, PLL-RT/pDNA complexes were prepared by equal volume mixture of 0.5 mg/mL of PLL-RT and 0.2 mg/mL of plasmid DNA. After incubation for 20 min, different amount of hyaluronic acid (HA, 0.05 mg/mL) was added to PLL-RT/pDNA complex solution and incubated for another 20 min. The final HA/PLL-RT/pDNA ternary gene delivery system (NPs) was obtained.

2.5. Particle size, zeta potential and stability

The particle size and zeta potential of Man-PLL-RT/OVA/CpG, PLL-RT/OVA/CpG, PLL-RT/pDNA, and HA/PLL-RT/pDNA nanoparticles were detected by a zeta potential/BI-90Plus particle size analyzer (Brookhaven, USA) at room temperature ($n = 5$). The morphology of Man-PLL-RT/OVA/CpG and HA/PLL-RT/pDNA nanoparticles were observed by field emission scanning electron microscope (Zeiss, Germany). The stability of Man-PLL-RT/OVA/CpG and HA/PLL-RT/pDNA nanoparticles were further determined. Briefly, Man-PLL-RT/OVA/...
CpG or HA/PDLL-RT/pDNA nanoparticles were gently mixed in phosphate-buffered saline (PBS, pH 7.4). The final concentration of nucleic acid was 0.05 mg mL\(^{-1}\). The mean diameters of the nanoparticles were detected once a day for a week. To further stimulate the microenvironment in the body, the stability of HA/PDLL-RT/pDNA nanoparticles was evaluated in bovine serum albumin (FBS). The final concentration of pDNA was 0.05 mg/mL. The mean diameters of the complexes were monitored after different periods of incubation time (1 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h).

### 2.6. Differentiation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were differentiated from the bone marrow cells as described previously [10]. Briefly, C57BL/6 N mice (female, 4–6 weeks) were anesthetized and sacrificed, then the femurs and tibias were harvested under sterile conditions. The bone marrow was rinsed with RPMI 1640 medium and filtered through a 200-mesh filter. The cell suspension was centrifuged at 1500 rpm for 10 min and cultured in a 6-well plate using RPMI 140 medium complete with 10% FBS, granulocyte-macrophage colony stimulating factor (GM-CSF, 10 ng/mL) and interleukin-4 (IL-4, 5 ng/mL). After 48 h incubation, the suspension cells were removed and the adherent cells were cultured for another seven days, during which the medium was half changed every two days. Finally, the highly purified BMDCs were obtained and detected by flow cytometry.

### 2.7. Cytotoxicity assay

The differentiated BMDCs were seeded in a 96-well plate at a density of 1 \(\times\) \(10^4\) cells per well in 200 \(\mu\)L of RPMI 1640 medium complete with 10% FBS. After overnight incubation, 20 \(\mu\)L of OVA/CpG, PEI25k/OVA/CpG, PLL-RT/OVA/CpG and Man-PLL-RT/OVA/CpG nanoparticles was added into the wells. After another 24 h, 10 \(\mu\)L of CCK-8 solution was added gently to avoid bubbles and incubated for 2 h. The absorbance was measured at 450 nm using a microplate reader (Infinite M200, Tecan, Switzerland). The cytotoxicity of various concentrations of each material in gene delivery systems were evaluated after incubation with B16-OVA cells for 24 h. The protocol of subsequent cytotoxicity detection was the same as that of BMDCs.

### 2.8. MR test and intracellular uptake

The mannose receptor expression in BMDCs was tested by incubation with PE-Cd11c and APC-labeled anti-MR antibody for 30 min at 4 °C. The cells were collected and analyzed via a Guava EasyCyte flow cytometer (Guava Technologies, USA). B16-OVA and L929 cells were used as the controls. To evaluate the BMDCs uptake of NVs, the cells were seeded in 24-well plates at 2 \(\times\) \(10^5\) cells/well. After 24 h incubation, FITC-OVA/CpG, PEI25k/FITC-OVA/CpG, PLL-RT/FITC-OVA/CpG, Man-PLL-RT/FITC-OVA/CpG, OVA/FAM-CpG, PEI25k/OVA/FAM-CpG, PLL-RT/OVA/FAM-CpG, and Man-PLL-RT/OVA/FAM-CpG nanoparticles were prepared and added into each well for 4 h. The final CpG concentration was 1 ng/\(\mu\)L. The cells were collected and the intracellular uptake of OVA antigen or CpG adjuvant was analyzed via a flow cytometer. To further verify the specificity of mannose moiety binding to dendritic cells compared to other kinds of cells, the endocytosis experiments of NVs were performed both in B16-OVA and L929 cells.

The endocytosis and co-localization of OVA and CpG were confirmed by confocal laser scanning microscopy (CLSM). Briefly, BMDCs were seeded on coverslips in a 6-well plate at 2 \(\times\) \(10^6\) cells/well for overnight incubation. FITC-OVA/Cy5-CpG, PEI25k/FITC-OVA/Cy5-CpG, PLL-RT/FITC-OVA/Cy5-CpG, Man-PLL-RT/FITC-OVA/Cy5-CpG were added and incubated for another 4 h. The final concentration of FITC-OVA and Cy5-CpG were 2 ng/\(\mu\)L and 1 ng/\(\mu\)L, respectively. The cells were gently washed with cold PBS and the nucleus were stained with DAPI. Finally, the coverslips were observed by a CLSM (ZEISS LSM780, Germany).

The mannose competition experiment was further to evaluate mannose receptor mediated endocytosis. Briefly, 1 \(\times\) \(10^5\) immature BMDCs were seeded in a 6-well plate and incubated overnight. To block MR, BMDCs were incubated with 1 mg/mL mannose in the fresh medium for 2 h at 37 °C prior to addition of PLL-RT/OVA/CpG and Man-PLL-RT/OVA/CpG nanovaccines. After 4 h incubation, the cells were evaluated by flow cytometry according to FITC-OVA fluorescence signals by a flow cytometer.

### 2.9. Activation of BMDCs and cytokine secretion

BMDCs were seeded in a 24-well plate and the density was 5 \(\times\) \(10^5\) cells per well. After 24 h incubation, the cells were stimulated with PEI25k/OVA/CpG, PLL-RT/OVA/CpG, and Man-PLL-RT/OVA/CpG nanoparticles for 24 h and soluble OVA/CpG was used as the control. The cells were washed with cold PBS and labeled with anti-CD11c-FITC, anti-CD80-PE and anti-CD86-APC antibodies (eBioscience, CA, USA) for 30 min at 4 °C. After washing for three times, the expression of surface molecules was analyzed by a Guava EasyCyte flow cytometer. The cytokines in the supernatants were quantified by using mouse TNF-α and IL-12 ELISA kits and calculated according to the standard curve.

To evaluate the in vivo activation after NVs treatment, C57BL/6 N mice (male, 18–20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All the experiments were conducted according to the guidelines of the Animal Care and Use Committee of Jilin University. The mice were random divided into 4 groups (n = 5) and subcutaneous immunized with 50 \(\mu\)L of PBS, OVA/CpG, PLL-RT/OVA/CpG, and Man-PLL-RT/OVA/CpG nanoparticles. The mass ratio of polymers/OVA/CpG was 5:2:1 and the final dose of CpG was 12.5 \(\mu\)g/head/mice. The immunologic protocol was performed once a week for three times and killed for the detection of immune cells in spleens and lymph nodes.

### 2.10. Antigen cross presentation

BMDCs were seeded in a 24-well plate and the density was 5 \(\times\) \(10^5\) cells per well. After 24 h incubation, the cells were stimulated with soluble OVA/CpG, PEI25k/OVA/CpG, PLL-RT/OVA/CpG, and Man-PLL-RT/OVA/CpG nanoparticles for 24 h. After washing with cold PBS, the cells were labeled with CD11c-FITC and SIINFEKL-H2Kb-Cy5 antibodies (eBioscience, CA, USA) for 30 min at 4 °C. Finally, the cells were washed with PBS thrice and the antigen cross-presentation was analyzed by a flow cytometer.

### 2.11. Gene transfection in vitro

Firstly, the optimum transfection activities of the PLL-RT/pDNA complexes were performed at different weight ratios in B16F10 melanoma cancer cells. Briefly, the cells were seeded in a 96-well plate at 0.8 \(\times\) \(10^4\) cells per well overnight. 200 \(\mu\)L of DMEM media containing PLL-RT/pGL3-control (20:1, 10:1, 5:1, 2.5:1, and 1:1, weight ratios) nanoparticles were added and incubated for 48 h. The final amount of pDNA was 0.2 \(\mu\)g per well. The firefly luciferase level was detected using a luminometer (GloMax 20/20, Promega) and protein detection kit according to the manufacturer’s instructions. To further verify the influence after introducing HA, the optimal mass ratio of PLL-RT/pDNA (2.5:1) was chose to form the binary complex. Then, different amount of HA was added to obtain HA/PLL-RT/pDNA ternary complexes. The optimized gene delivery system was screened after 48 h gene transfection in B16F10 cells.

The quantification of PD-L1 mRNA expression was further evaluated with the RT-qPCR. Briefly, 2 \(\times\) \(10^5\) of B16F10 cells were seeded in 6-well plates for 24 h. The fresh DMEM media containing PLL-RT/shPD-L1 or HA/PLL-RT/shPD-L1 nanoparticles were added and incubated for another 24 h. PBS treated group was used as the control. The total RNA was extracted from B16F10 cells using an RNA extracting kit (TIANGEN...
Biotech CO., Ltd, China). After reverse transcription, the mRNA expression of PD-L1 and GAPDH was quantified using the SYBR Green mix kit (TAKALA, Japan) by a RT-qPCR instrument (Mx3000P, Stratagene, USA). The amplification conditions were as follows: 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C for 10 s, 65 °C for 30 s. The primer sequence of the PD-L1 was 5′-CCA GCC ACT TCT GAG CAT GA-3′ (Forward), and the reverse is 5′-CTT CTC TTT CCA CTC ACG GG-3′. The primer of GAPDH was 5′-CTT AGA GGG ATG CTG CC-3′ (Forward), and 5′-TACC GCC CAA ATC CGT TCA-3′ (Reverse).

2.12. In vivo vaccine kinetics of NVs

The local retention and lymphatic tracking ability of NVs was evaluated using an automatic chemiluminescence imaging and analysis system (Tanon 6100, Shanghai, China). Briefly, Man-PLL-RT/Cy5-OVA/CpG NVs was subcutaneous injected into C57BL/6 N mice via their tail base and the dosage of Cy5-OVA was 50 μg per mouse. Soluble Cy5-OVA/CpG was used as the control. The local retention capacity of NVs was monitored by quantifying the fluorescent intensity of the local sites at different time points (0.5 h, 3 h, 6 h, 24 h, 2 d, 3 d, 5 d, and 7 d). The lymphatic tracking of NVs was observed at Day 7 after subcutaneous injection.

2.13. Prophylactic vaccination combined with immunosup Suppress gene

C57BL/6 N mice (female, 16–18 g) were automatically divided into four groups with five heads for each group. The mice were immunized with 25 μg OVA and 12.5 μg CpG in Man-PLL-RT/OVA/CpG NVs thrice with once a week interval. 7 days after the last immunization, 100 μL of 1 × 10^7 B16-OVA cells were subcutaneously inoculated into the back of the mice. After another 7 days, HA/PLL-RT/shPD-L1 NPs (20 μg of shPD-L1 per mouse) were performed every three days via intravenous injection for totally four times. The experimental groups were PBS control, NVs, NPs, and NVs + NPs combination groups. The tumor size and body weight of the mice were monitored during the therapeutic process. Tumor volume was calculated according to the following formula: tumor volume (mm^3) = length × (width^2)/2. Once the tumor volumes of PBS control group exceeded 2000 mm^3 (Day 17), this antitumor study ended and all the mice were euthanized.

2.14. Therapeutic vaccination combined with immunosup Suppress gene

C57BL/6 N mice (female, 18–20 g) were randomly divided into four groups with five heads in each group. 100 μL of 1 × 10^7 B16-OVA cells were subcutaneously injected into the back of the mice on day 0. From the following day, the mice were immunized with Man-PLL-RT/OVA/CpG NVs (25 μg OVA and 12.5 μg CpG) three injections with once week intervals. 9 days after tumor inoculation, HA/PLL-RT/shPD-L1 NPs (20 μg shPD-L1) were intravenous injected every three days for totally four times. The experimental groups were PBS control, NVs, NPs, and NVs + NPs combination groups. The experimental protocols were the same as mentioned above.

2.15. In vivo analysis of immune cells

After the mice were euthanized, the tumors, spleens and inguinal lymph nodes were extracted and dispersed into single-cell suspensions. Afterwards, the cells were filtered using 300-mesh screens and washed by cold PBS. After centrifugation, the cells were resuspended in 90 μL of cold PBS and incubated with 10 μL of fluorescent antibodies at 4 °C for 30 min. Finally, the cells were washed twice and analyzed by a Flow Cytometer (BD-FACS Canto-II, USA). The activated DCs (CD11c+CD86+) in inguinal lymph nodes, memory CD8+ T cells (CD3+CD8+CD44+CD62L-) in spleen, and the intratumoral CD8+ T cells (CD3+CD8+), M2 macrophages (CD11b+CD206+F4/80-), Treg cells (CD3+CD4+FoxP3+), and MDSCs cells (CD11b+Gr1+) were further evaluated.

2.16. In vivo cytokines detection

The serum was extracted after antitumor treatment and centrifuged at 8000×g for 15 min. Afterwards, the supernatants were harvested and quantified by using mouse TNF-α and IFN-γ ELISA kits according to the manufacturer’s protocols and quantified according to the standard curves.

2.17. Safety evaluation

To confirm the in vivo safety of antitumor therapy, the liver and renal toxicity were evaluated by analyzing the biochemical indexes, including alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) for liver function, urea nitrogen (BUN), creatinine (CRE), and uric acid (UA) for kidney function. The samples were measured using mouse ELISA kits according to the manufacturer’s protocols and detected by an automatic microplate reader (Infinite M200, Tecan, Switzerland).

2.18. Histological and immunofluorescence staining analyses

To analyze the pathological features, the tumors and main organs (heart, liver, spleen, lung, and kidney) were extracted after euthanizing the mice. The tissue sections were further stained with hematoxylin and eosin (H&E) and observed by light microscopy (Nikon Y-TV55, Japan) for histopathology analysis. The tumor infiltrating CD8+ T cells and PD-L1 expression in tumors were observed by immunofluorescence staining. Briefly, the paraffin-embedded coverslips were treated by deparaffinization, antigen retrieval, and blocking in 5% goat serum at 37 °C for 30 min. Afterwards, the samples were co-cultured with rabbit-anti-mouse CD8 or PD-L1 antibody at 4 °C overnight, then incubated with Cy3 conjugated goat-anti-rabbit secondary antibody at 37 °C for 1 h. After washing with cold PBS for three times, the nuclei were stained with DAPI. Finally, the coverslips were observed by a CLSM.

2.19. Statistical analysis

GraphPad Prism 8.0 and FlowJo v10 softwares were used for constructing graphs and analyzing flow cytometry data, respectively. The statistical significance of the data was calculated by Student’s t-test.

3. Results and discussion

3.1. Synthesis and characterization of nanocarriers

Soluble antigens are rapidly eliminated in the body and cannot produce an effective immune response. In addition, as the exogenous antigens, they are hard to be cross-presented by DCs, thus activating cell-specific immunity to tumor cells [33]. However, nanoparticle-based vaccines can enable a sustained antigen exposure to DCs, enhanced antigen uptake, and effective antigen cross presentation, thus promoting long-term antitumor immunotherapy [11]. In this study, we constructed tosyl protected arginine group grafted polylysine (PLL-RT), which was synthesized by condensation reaction of PLL and Boc-Arg(Tos) (Fig. S1). To further promote immunological response, a DCs specific targeting mannose molecule was conjugated to PLL-RT (Man-PLL-RT) (Fig. 1).

The optimized degrees of polymerization of PLL and the grafting amount of mannose segments was 7.2 (Fig. S2).
3.2. Preparation and immune activation of nanovaccines

Man-PLL-RT/OVA/CpG nanovaccines (NVs) were constructed via a facile electrostatic interaction of positive charged Man-PLL-RT and negative charged antigen OVA and CpG agonist (Fig. 2(A)). The actual loading amount of either OVA or CpG in the NVs was nearly 100%. The electrification performance of NVs was evaluated by zeta potential assay. As shown in Fig. 2(B), Man-PLL-RT/OVA/CpG nanovaccines demonstrated a positive charge of 17.8 mV, which was slightly decreased compared with that of PLL-RT/OVA/CpG. Generally, there are negative charges on the cell membranes of BMDCs cells [34]. This is mainly due to the phospholipid bilayer structure of the cell membrane, with hydrophilic negatively charged phosphate groups on the outside and hydrophobic fatty acids on the inside. In addition to phospholipids, the carboxyl group of sialic acid on the glycoprotein is also an important cause of the negative charge. Under the same conditions, nanoparticles with positive charge had higher endocytosis efficiency than those with neutral or negative charge. Thus, the positively charged NVs are more conducive to the endocytosis of BMDCs via electrostatic interaction with DCs, thus increasing the intracellular uptake.

The bottleneck restricting the efficacy of tumor vaccines lies on the low uptake and presentation efficiency of antigen presenting cells, which makes it difficult to fully activate T cells that can specifically kill tumors [35]. Nanoscale vaccines offer a promising solution to this issue. Suitable particle size is the prerequisite for effective delivery of tumor antigens and adjuvants to DCs, thus triggering powerful antitumor immune responses [36]. Man-PLL-RT/OVA/CpG nanovaccines exhibited an average particle size of 80.1 nm (PDI: 0.121), displaying efficient capacity to encapsulate antigens and agonists. Moreover, the morphology of NVs was observed by SEM. As shown in Fig. 2(Ca), the NVs exhibited uniform spherical structures, which was consistent with the previous hydrate particle size. The stability tests also exhibited that the particle size of the NVs showed no significant change in PBS buffer for one week, proving their good stability (Fig. S3). Man-PLL-RT/OVA/CpG nanovaccines with suitable zeta potential and particles size indicated their potential capacity for DCs’ engulfment and

![Synthetic route of Man-PLL-RT](image)

![Preparation strategy and characterization of Man-PLL-RT/OVA/CpG nanovaccines (NVs)](image)

Fig. 1. Synthetic route of Man-PLL-RT.

Fig. 2. Preparation strategy and characterization of Man-PLL-RT/OVA/CpG nanovaccines (NVs). (A) Schematic illustration of the fabrication of NVs. Zeta potential (B) and Particle size (C) of NVs. The morphology of Man-PLL-RT/OVA/CpG NVs was observed by SEM (Ca). Scale bar = 200 nm.
activation of the specific antitumor immune responses.

3.3. Cytotoxicity, intracellular uptake and BMDCs activation of NVs

For cationic nanoparticles, cytotoxicity is a double-edged sword. Increasing the concentration of nanoparticles can improve the endocytosis efficiency of target cells, and at the same time, high concentration of nanoparticles can intensively interact with the electronegative cell membrane, thereby destroying the structure of cell membrane and causing cell death [37]. As shown in Fig. S4, OVA and CpG exhibited no toxicity on BMDCs. Obvious cytotoxicity was observed for PEI25k when the concentration is over 5 μg/mL, which was due to the high density of positive charges on its surface. For PLL-RT and Man-PLL-RT groups, they showed a similar cytotoxic trend. They exhibited a mild toxicity when their concentrations were higher than 40 μg/mL. To further verify the effect of mannose modification, we evaluated the cytotoxicity of PLL-RT/OVA/CpG and Man-PLL-RT/OVA/CpG NVs under the condition of fixed mass ratio. The results indicated that the introduction of mannose had no effect on the cytotoxicity of the NVs (Fig. S5). Normally, for cationic polymers, high concentration can intensively interact with the electronegative cell membrane, thereby destroying the structure of cell membrane and causing cell death. While in this study, the material concentration was equivalent to 5 μg/mL, so it is safe and non-toxic. To verify the loading ratio of the prepared NVs on cytotoxicity, we evaluated the cytotoxicity of PLL-RT/OVA/CpG and Man-PLL-RT/OVA/CpG NPs under different conditions of loading ratios. In this study, the concentrations of OVA and CpG were fixed at 2 μg/mL and 1 μg/mL, respectively. As shown in Fig. S6, the loading ratio had no effect on cytotoxicity under our experimental conditions. The cytotoxicity of the NVs mainly comes from the surface charge of the cationic polymers.

Fig. 3. Intracellular uptake and stimulation of BMDCs by various formulations of NVs in vitro. Intracellular uptake of FITC-OVA (A) and FAM-CpG (B) by BMDCs. (C) CLSM images of BMDCs uptake with different formulations (2 μg/mL equivalent OVA) after incubation for 4 h. Scale bar = 20 μm. (D) BMDCs maturation induced by different formulations for 24 h. TNF-α (E) and IL-12p70 (F) secretion in culture medium after stimulation for 24 h. (G) Expression of SIINFEKL-MHC I signals on BMDCs after 24 h incubation with different formulations.
While in this study, the final concentration of Man-PLL-RT was 5 μg/mL, indicating a safe and non-toxic feature. It is mainly owned to the biodegradable amino acid skeleton structure of nanovaccines.

The effective endocytosis of nanovaccines in DCs is a prerequisite for immune activation. However, many reported nanovaccines were non-specific uptake by immature DCs before antigen presentation. Therefore, it is still the key to improve the anti-tumor effect of vaccine and has become a new trend in the development of cancer vaccines to develop new antigen-peptide targeting transport carriers. MR is an important pattern recognition receptor and endocytic receptor in the innate immune system. It mainly exists on the cell membrane surface of macrophages and dendritic cells. MR has multiple extracellular domains and can recognize and bind a wide range of endogenous and exogenous ligands. MR is capable of binding carbohydrate structures such as fucose and mannose. It plays an important role in maintaining homeostasis, identifying pathogens, inducing cytokines and antigen presentation [38–42]. Therefore, we evaluated the MR expression in BMDCs. As shown in Fig. S7A, BMDCs expressed significant levels of MR protein on their cell surface, indicating the specific mannose targeting ability. While for B16-OVA (Fig. S7B) and L929 cells (Fig. S7C), there was no significant difference between the expression of MR and the isotype control. The endocytosis efficiency of target Man-PLL-RT/OVA/CpG NVs in BMDCs was further measured. The optimal ratio of Man-PLL-RT/OVA/CpG nanovaccines was at the mass ratio of 5:2:1 by quantitative fluorescence labeling OVA (Fig. S8) and CpG (Fig. S9). As shown in Fig. 3(A and B), PEI25k could efficiently deliver OVA antigen and CpG agonist into BMDCs compared with soluble OVA/CpG. While PLL-RT/OVA/CpG achieved only a little incremental delivery efficiency. When mannose targeting ligands were introduced, the delivery efficiency of antigens and agonists was significantly increased, indicating that mannose receptor-mediated intracellular uptake greatly elevated internalization of NVs. To further verify the specificity of mannose moiety binding to dendritic cells compared to other kinds of cells, the endocytosis experiments of NVs were performed both in B16-OVA and L929 cells. As shown in Fig. S10, PLL-RT/OVA/CpG and Man-PLL-RT/OVA/CpG exhibited comparable endocytosis efficiency both in B16-OVA and L929 cells, further indicating the specific effect of mannose moiety in BMDCs. Next, we further verify the MR mediated endocytosis via a mannose competition experiment. The results indicated that BMDCs targeted Man-PLL-RT/OVA/CpG NVs achieved obvious enhanced antigen endocytosis compared with non-targeted NVs. When mannose was previously introduced to block the MR of BMDCs membrane, the endocytosis efficiency of FITC-OVA by targeted NVs group was obviously reduced, while that of non-targeted NVs group showed no significant differences (Fig. S11). These results could further verify the specificity of mannose moiety binding to dendritic cells.

The intracellular uptake was further evaluated with CLSM. As shown in Fig. 3(C), several weak green fluorescence of FITC-OVA and Cy5-CpG for OVA/CpG control group could be observed after 4 h incubation. While for PEI25k/OVA/CpG and PLL-RT/OVA/CpG groups, significantly stronger intracellular fluorescence signals were observed in BMDCs. It was encouraging that Man-PLL-RT/OVA/CpG nanovaccines demonstrated the maximum amounts of fluorescent signals. Furthermore, the colocalization of green and red signals demonstrated the highly efficient co-delivery of antigens and adjuvants to the same BMDCs, facilitating to induce tumor specific T cell immune responses.

Efficient endocytosis is a prerequisite for BMDCs activation. The cells surface receptors of mature BMDCs were detected by flow cytometry after incubating with NVs for 24 h. The mature BMDCs were gated by CD11c+ and CD86+ and soluble OVA/CpG group showed no evident BMDCs activation (Fig. 3(D)). As expected, all NVs exhibited upregulated the expression of CD86 compared with free OVA-agonists group. Among them, Man-PLL-RT/OVA/CpG nanovaccines demonstrated the most optimal activation efficiency of BMDCs, which was mainly attributed to the efficient co-endocytosis of antigens and adjuvants. Furthermore, the cytokines of the supernatants were measured by mouse TNF-α and IL-12p70 ELISA kits after 24 h incubation with NVs. Man-PLL-RT/OVA/CpG nanovaccines group greatly increased the secretion of TNF-α and IL-12p70, which was consistent to the BMDCs maturation results (Fig. 3(E and F)). More importantly, these BMDCs expressed approximately fivefold increase of OVA-specific SIINFEKL-MHC I complexes than that of soluble OVA/CpG (Fig. 3(G)). All these results suggested that Man-PLL-RT/OVA/CpG nanovaccines could induce the most activation and antigen processing via the MHC I pathway.

3.4. In vitro evaluation of HA/PLL-RT/pDNA delivery system

Efficient gene delivery system is the key to successful gene therapy. In this study, PLL-RT was used as a core gene delivery system, which proved excellent transfection performance in several kinds of tumor cells [31]. The optimized polymerization degree of PLL was 120 and the grafting amount of RT was 64. To further improve the precise delivery to the tumor tissues, negative hyaluronic acid (HA) was introduced to shielding the positive charge of PLL-RT/pDNA complexes. The optimal mass ratio of PLL-RT/pDNA was 2.5:1 confirmed by in vitro transfection assay (Fig. S12). Furthermore, HA could specifically target to CD44 receptor that overexpressed on tumor cells. The electrification performance of HA/PLL-RT/pDNA nanoparticles (NPs, mass ratio of 0.25:2:5:1) was evaluated by zeta potential assay. As shown in Fig. 4(A), HA/PLL-RT/pDNA nanoparticles demonstrated a positive charge of about 13.5 mV, which was slightly decreased compared with that of PLL-RT/pDNA complex. The decreased charge was due to the introduction of the negatively charged shielding molecular HA. The size of NPs exhibited an average particle size of 142.3 nm (PDI: 0.112), displaying efficient capacity to encapsulate plasmid DNA (Fig. 4(B)). Moreover, the morphology of NPs was observed by SEM. As shown in Fig. 4(C), the NPs exhibited uniform spherical structures, which was consistent with the previous hydrate particle size. The stability tests also exhibited that the particle size of the NPs showed no significant change in PBS buffer for seven days, proving their good stability in vitro (Fig. S13). While in simulated humoral conditions, the size of HA/PLL-RT/pDNA nanoparticles increased from 143.2 nm to 201.4 nm, which was mainly due to the formation of crowns in FBS. In addition, HA/PLL-RT/pDNA nanoparticles also showed good stability with a slightly increment in size (Fig. 4(D)), indicating their potential in vivo application. HA/PLL-RT/pDNA nanoparticles with suitable zeta potential and particles size indicated their potential capacity for tumor cell engulfment.

The cytotoxicity of NPs in B16F10 cells was evaluated by CCK-8 assay. As shown in Fig. S14, pDNA and HA exhibited no toxicity in B16-OVA cells. For PLL-RT and HA/PLL-RT/pDNA groups, a similar cytotoxic trend was observed. They exhibited a mild toxicity when their concentrations were higher than 40 μg/mL. Overall, the cytotoxicity is mainly due to the concentration of cationic gene delivery system, while the toxicity is not affected by the polymer itself, the formation of nanoparticle or various loading ratios. In this study, the final concentration of PLL-RT is 2.5 μg/mL, a safe and non-toxic dose. All the treated groups demonstrated negligible cytotoxicity compared with PBS control group, indicating the superior biocompatibility of the gene delivery system (Fig. 5(A)).

The protein expression level of CD44 was monitored in tumor cells by flow cytometry. As shown in Fig. S15, B16-OVA cells expressed significantly high levels of CD44 on their cell surface compared with the isotype control group, indicating the CD44 molecule mediated endocytosis. To evaluate the influence of endocytosis efficiency after introducing HA, different amount of HA was added into PLL-RT/pDNA complex (mass ratio of 2:5:1). The endocytosis efficiency was measured with flow cytometry. The delivery efficiency of DNA showed an observable improvement (Fig. 5(B)), indicating the HA receptor-mediated intracellular uptake. The intracellular uptake was further evaluated with CLSM. As shown in Fig. 5(C), significantly stronger intracellular fluorescence signals were observed in B16F10 cells for both
Fig. 4. Characterization of HA/PLL-RT/pDNA nanoparticles (NPs). Zeta potential (A), Particle size (B), SEM (C), and stability of NPs in FBS (D) at different incubation time (0 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h).

Fig. 5. Characterization of cytotoxicity, endocytosis and transfection efficiency of HA/PLL-RT/pDNA nanoparticles in B16F10 cells. (A) Cytotoxicity of pDNA, PLL-RT, HA, PLL-RT/pDNA and HA/PLL-RT/pDNA nanoparticles. (B) Flow cytometry of PLL-RT/FAM-DNA nanoparticles after introducing different amount of HA. (C) Intracellular uptake of PLL-RT/FAM-DNA nanoparticles by CLSM after 4 h incubation. Scale bar = 20 μm. (D) In vitro gene transfection efficiency of HA/PLL-RT/pGL3-control at various HA contents. (E) PD-L1 mRNA expression after treating with different formulations for 24 h.
PLLA-RT/pDNA and HA/PLL-RT/pDNA nanoparticles.

The transfection activities of HA/PLL-RT/pGL3-control nanoparticles were evaluated at various weight ratios and the optimized transfection ratio was 2.5:1 (Fig. S12). Compared with PLL-RT/pGL3-control and other commercial groups, HA/PLL-RT/pGL3-control nanoparticles significantly improved the transfection efficiency in B16F10 cells (Fig. 5(D)), which was mainly due to the targeted recognition induced high endocytosis (Fig. 3(D and E)).

To further evaluate the biological significance of tumor targeting therapy, the transfection efficiency of HA/PLL-RT/shPD-L1 nanoparticles was investigated by PD-L1 expression after 24 h using RT-qPCR assay. B16F10 cells treated with PBS were used as the control. As shown in Fig. 5(E), HA/PLL-RT/shPD-L1 nanoparticles induced 68.5% down-regulation of PD-L1. This result was consistent with prior gene transfection experiment.

3.5. Retention effect and activation of nanovaccines in vivo

Increasing the residence time of vaccines in organisms can effectively improve the immune activation of the vaccines [17]. Thus, we investigated the retention capacity of nanovaccines using an automatic chemiluminescence imaging and analysis system (Tanon 6100, Shanghai, China). Fig. 6(A) demonstrated the presence of Cy5-OVA signals at the injection sites at different time points. The fluorescence signals of soluble Cy5-OVA/CpG group weakened rapidly and almost disappeared at 48 h post-injection. As expected, NVs group showed a significant antigen retention effect and gradual clearance with obvious signals at the injection sites at different time points. The fluorescence signals were observed for soluble Cy5-OVA/CpG group, whereas much stronger signals were achieved for NVs-treated mice. All these results demonstrated that NVs could effectively improve the antigen retention ability to inguinal lymph nodes, thus inducing the specific immune responses.

Encouraged by the long-term retention of NVs, we investigated their immune responses in vivo. The C57BL/6 N mice were randomly divided into three groups (n = 5) and received three subcutaneous immunizations once a week. The immune cells of inguinal lymph nodes and spleens were detected at 7 days after last immunization. Compared with PBS or soluble OVA/CpG group, NVs could stimulate more mature DCs in LNs (66.5%), which was due to their long-term retention and co-delivery of antigens and agonists (Fig. 6(C)). In addition, the amount of CD8+ T cells and Treg cells in spleens significantly increased after NVs immunization (Fig. 6(D and E)), confirming their strong cellular immunity and immune memory effect.

3.6. In vivo antitumor efficacy

Encouraged by the previous results, we next evaluated the anticancer efficiency by prophylactic tumor NVs combined with immune checkpoint blocking gene therapy strategy in B16F10 tumor model. The therapeutic protocol was shown in Fig. 7(A). The mice were immunized with Man-PLL-RT/OVA/CpG NVs thrice with weekly intervals. 7 days after the last immunization, B16-OVA cells were subcutaneously inoculated into the back of the mice. From day 7, HA/PLL-RT/shPD-L1 NPs were performed via intravenous injection every three days for totally four times. The tumor size and body weight of the mice were monitored during the therapeutic process. The subcutaneous administration of NVs slightly delayed tumor growth (Fig. 7(B)), reflecting poor immunogenicity. This was probably due to the weak CD8+ T cells infiltration from immunosuppressive microenvironment in tumors [1]. The PD-L1/PD-L1 blockade by systemic gene therapy could effectively inhibit tumor growth via downloading the expression of PD-L1 molecules on tumor cells. However, both monotherapy strategies still failed to achieve satisfactory results. Intriguingly, the tumor growth was obviously delayed in mice treated with both NVs and NPs (Fig. 7(B)). The tumors were extracted from the mice after B16F10 cells were inoculated for 17 days. Afterwards, tumor photographs (Fig. 7(C)) and weights (Fig. 7(D)) further verified the antitumor effect of combined tumor vaccines and gene-regulated immune checkpoint blocking therapy. There was no obvious difference in body weight for all the treatment groups (Fig. 7(E)), indicating the safety of the antitumor strategy. In addition, histological analysis was carried out to further evaluate the antitumor effect. Large areas of necrotic tumor cells were observed in the combined treated group compared with other groups (Fig. 7(F)).
We further performed antitumor treatment by combing tumor therapeu- tic vaccines with gene-regulated immune checkpoint blockade. The therapeutic protocol was shown in Fig. S15. The mice were subcutaneous- ously inoculated with B16-OVA cells and immunized with Man-PLL-RT/ OVA/CpG NVs thrice with weekly intervals one day after inoculating tumor cells. From day 9, HA/PLL-RT/shPD-L1 NPs were injected via intravenous injection every three days for totally four times. The tumor size and body weight of the mice were monitored during the therapeutic process. The results were similar to those of the prophylactic vaccines, the combination of NVs and NPs induced the optimist antitumor responses (Fig. S16-S20).

3.7. Immunological evaluation after combined treatment

To deeply analyze the antitumor mechanism, the immunological evaluation of the tumors, LNS and spleens was performed after combined treatment. As shown in Fig. 8(A), either NVs monotherapy or combined treatment markedly increased the proportion of CD11c⁺CD86⁺ mature DCs in inguinal lymph nodes compared with PBS group. While there was no significant difference between the gene-regulated immune checkpoint blockade group and PBS group. The increased cell maturation rate was mainly due to the immune activation of NVs. The intratumoral immune cells were further detected by flow cytometry after isolating the single cells. It was found that the intratumoral CD3⁺CD8⁺ T cells (Fig. 8(B and C)) and NK (Fig. 8(D)) cells were greatly promoted after NVs combined gene therapy based immune checkpoint blockade (ICB) compared with each monotherapy group. The improvement of these immune-activated cells was attributed to the dual immune activation induced by targeted NVs and ICB. While the immune-suppressive cells of MDSCs (Fig. 8(E)), Tregs (Fig. 8(F)) and M2 macrophages (Fig. 8(G)), were obviously increased after NVs mono-therapy, which could be the key factor leading to poor treatment results.

Fig. 7. In vivo antitumor therapy with prophylactic vaccines combined with gene regulated PD-L1 blockade on B16F10 tumor model. (A) Treatment scheme of various formulations. (B) Tumor growth curves during treatment. (C) Tumor photos and (D) Tumor weight after various treatment formulations. (E) Body weight during treatment. (F) H&E staining after various treatment formulations. Scale bar = 100 μm.
When combined with anti-PD-L1 treatment, these cell components decreased to the comparable levels of PBS group or even lower, alleviating the immunosuppressive microenvironment of tumor tissues. In addition, anti-PD-L1 therapy was further verified by immunofluorescence analysis. As shown in Fig. S21, a great amount of fluorescent signals were observed both in PBS and NVs groups, indicating the highly expressed PD-L1 protein in tumors. While for NPs and NVs + NPs groups, PD-L1 signals were significantly weakened, declaring valid gene silencing effect and potential antitumor contributions. Furthermore, the immune memory function can promote and enhance the long-lasting antitumor responses, which is closely related to tumor metastasis and recurrence. To verify the antitumor immune memory responses, we collected the spleens and analyze the effector memory T cells (T_{EM}) after various treatments (Fig. 8(H)). Encouragingly, the combined therapy exhibited the most significant increasement of T_{EM} compared with other groups, facilitating immune surveillance of tumor metastasis and recurrence. Furthermore, the pro-inflammatory factors in serum, including TNF-α (Fig. 8(I)) and IFN-γ (Fig. 8(J)), were upregulated after combined therapy, which facilitated the recruitment and maturation of antigen presenting cells and further activated the immune killing capacity of CTL cells. Generally, the changes of immune cells and cytokines induced by the combined therapy were conductive to the specific killing effect of the immune system on tumors.

3.8. Security analysis

Biosafety of nanoparticles is a prerequisite for the clinical application. In this study, we further evaluated the safety of the implemented antitumor treatment strategies. The serum liver function markers (alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST)) (Fig. S22) and kidney function markers (blood urea nitrogen (BUN), lactate dehydrogenase (LDH), and uric acid (UA)) (Fig. S23) in all treated groups were within the normal ranges, indicating the absent systemic toxicity of the combined strategy. In addition, H&E staining was introduced to evaluate the security analysis. No obvious inflammation or abnormality were achieved among the histological sections of the main organs (Fig. S24), which further confirmed the safety and good biocompatibility of the combined anti-tumor strategy. All these results demonstrated the valuable killing ability against tumors for the combined treatment without significant toxicity and side effects.

4. Conclusion

At present, although tumor vaccines can effectively activate the body’s T cell immune response, the therapeutic effects are usually still limited due to the multiple immunosuppressive microenvironment in tumor tissues. Thus, reversing immunosuppressive microenvironment combined with tumor vaccines would benefit for curing the tumors. In this study, we successfully prepared an APC-targeting nanovaccines based on Man-PLL-RT by absorbing antigen OVA and adjuvant CpG via electrostatic adsorption. Taking advantage of the versatile chemical components and physicochemical properties of Man-PLL-RT, Man-PLL-RT/OVA/CpG nanovaccines could effectively deliver antigens and
adjuvants to BMDCs, induce cell maturation, and achieve efficient antigen cross presentation. Additionally, the NVs were able to greatly improve the retention time of antigens in the injection sites and the capacity to target lymph nodes, thus activating the cellular immune function of the bodies. Moreover, the tumor immune tolerance microenvironment was relieved by ICB achieved by targeted gene therapy of HA/PLL-RT/shPD-L1 nanoparticles. Encouragingly, the enhanced antitumor immune responses were achieved by combined therapy with NVs and ICB. The changes of immune cells and cytokines induced by the combined therapy were conductive to the specific killing effect of the immune system on tumors. We believe that the combined strategy here can alleviate and inhibit tumor growth, providing a potentially effective strategy for the future clinical treatment of tumors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jie Chen: Methodology, Investigation, Experiments, Writing – original draft. Huapan Fang: Methodology, Investigation, Experiments, Writing – original draft. Yingying Hu: BMDCs extraction, In vitro experiments. Jiayuan Wu: Animal experiments, Data curation. Yuanjing Feng: BMDCs extraction, In vitro experiments, Animal experiments, Data curation. Lin Lin: Animal experiments, Data curation. Huayu Tian: Conceptualization, Project administration, Funding acquisition, Writing – review & editing. Xuesi Chen: Resources, Supervision, Funding acquisition.

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Appendix A. Supplementary data

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[33] S. Foster, C.L. Duvall, E.F. Crownover, A.S. Hoffman, P.S. Stayton, Intracellular delivery of a protein antigen with an endosomal-releasing polymer enhances CD8 T-cell production and prophylactic vaccine efficacy, Bioconjugate Chem. 21 (12) (2010) 2205–2212.

[34] E.S. N, E. Korotchenko, S. Scheibholfer, R. Weiss, M. Schneider, Functionalized multifunctional nanovaccine for targeting dendritic cells and modulation of immune response, Int. J. Pharm. 593 (2021) 120123.

[35] Y. Qian, H.L. Jin, S. Qiao, Y.F. Dai, C. Huang, L.S. Lu, Q.M. Luo, Z.H. Zhang, Targeting dendritic cells in lymph node with an antigen peptide-based nanovaccine for cancer immunotherapy, Biomaterials 98 (2016) 171–183.

[36] J. Xu, H. Wang, L.G. Xu, Y. Zhao, C.Y. Wang, X. Han, Z.L. Dong, H. Chang, R. Peng, Y.Y. Cheng, Z. Liu, Nanovaccine based on a protein-delivering dendrimer for effective antigen cross-presentation and cancer immunotherapy, Biomaterials 207 (2019) 1–9.

[37] J. Chen, K. Wang, J.Y. Wu, H.Y. Tian, X.S. Chen, Polycations for gene delivery: dilemmas and solutions, Bioconjugate Chem. 30 (2) (2019) 338–349.

[38] J.H. Zhu, F.H. Qin, Z.H. Ji, W.D. Fei, Z. Tan, Y. Hu, C.H. Zheng, Mannose-modified PLGA nanoparticles for sustained and targeted delivery in Hepatitis B virus immunoprophylaxis, Immunoprophylaxis 21 (2020) 13.

[39] J.F. Liu, J. Wang, Q.Q. Zhu, C.Q. Yu, J.R. Yin, L.P. Zheng, A. Li, Mannosylated PEGylated polyethyleneimine as efficient CpG oligodeoxynucleotide carriers for efficient dendritic cell targeting delivery and activation, J. Biomed. Nanotechnol. 15 (2019) 1454–1467.

[40] G.N. Shi, C.N. Zhang, R. Xu, J.F. Niu, H.J. Song, X.Y. Zhang, W.W. Wang, Y. M. Wang, C. Li, X.Q. Wei, D.L. Kong, Enhanced antitumor immunity by targeting dendritic cells with tumor cell lysate-loaded chitosan nanoparticles vaccine, Biomaterials 113 (2017) 191–202.

[41] P. Li, S.M. Chen, Y.H. Jiang, J.Y. Jiang, Z.R. Zhang, X. Sun, Dendritic cell targeted liposomes-protamine-DNA complexes mediated by synthetic mannosylated cholesterol as a potential carrier for DNA vaccine, Nanotechnology 24 (2013) 295101.

[42] S.K. Singh, I. Streng-Ouwehand, M. Litjens, H. Kalay, S. Burgdorf, E. Saeland, C. Kurts, W.W. Unger, Y.V. Kooyk, Design of neo-glycoconjugates that target the mannose receptor and enhance TLR-independent cross-presentation and Th1 polarization, Eur. J. Immunol. 41 (4) (2011) 916–925.