TLR7/8-agonist loaded M1 Macrophage-derived nanovesicles promote the polarization of macrophages to enhance bladder cancer immunotherapy

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Abstract

Immune checkpoint inhibitors (ICIs), such as PD-1/PD-L1 antibodies, have been successfully used to modulate the cancer killing function of immune cells in the tumor microenvironment (TME). However, in bladder cancer, the level of the proinflammatory M1 macrophages were significantly low than the immunosuppressive M2 macrophages. The abundant of M2 macrophages released cytokines to induce the resistance of ICIs treatment and alleviate immune cell attack, finally resulting in promoting tumor growth. To enhance the bladder cancer immunotherapy, nanovesicles derived from M1 macrophages (M1 NV) was applied to load the TLR7/8 agonist of R848, a potent driver of M1 macrophages, for constructing M1 NV-R848. The results revealed M1 NV-R848 could more effectively induce polarization of M2 macrophages into M1 macrophages when compared to M1 NV or R848 alone both *in vitro* and *in vivo*. In addition, intravenous injection of M1 NV-R848 would improve the immunosuppressive TME by upregulation of proinflammatory M1 macrophages, improvement of CD8+T cells infiltration, finally enhancing the immunotherapy of PD-L1 antibodies with good biocompatibility in bladder cancer bearing mouse model. Thus, our study demonstrates that M1 NV-R848 could serve as a potential strategy to improve the polarization of M1 macrophages in bladder cancer, which might be further used for other cancer with the immunosuppressive TME.

Introduction

Bladder cancer (BC) is the 11th most common malignant tumor worldwide^[1], which has a significant impact on the mortality, quality of life as well as economic burden of patients^[2]. At the time of diagnosis of BC, about 75% cases are non-muscle invasive BC (NMIBC), and other patients are muscle invasive (MIBC) ^[3,4]. Recently, surgical resection is one of the main treatments for BC. However, the prognosis of most BC patients, especially those with muscle invasion remains suboptimal^[5]. Therefore, new therapeutical strategies for enhancing the efficacy of BC are needed.

Immunotherapy is a therapeutic approach used to activate immune cells, such as T cells, B cells, and macrophages, to induce anticancer immune responses. It has been reported that BC is a type of immunogenic tumor, which has been treated clinically with intravesical immunotherapy using Bacillus Calmette–Guérin (BCG) in BC patients for decades^[6]. Recently, the advent of immune checkpoint inhibitors (ICIs), particularly those with antibodies against programmed cell death protein 1 (PD-1) or its ligand (PD-L1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), has improved the treatment of metastatic BC^[7, 8]. However, present research found that the immunosuppressive tumor microenvironment (TME) in BC would weaken the activation of effector T cell, which would hinder the ICI efficacy.

Tumor-associated macrophages (TAMs) are the main macrophage type involved in the formation of TME. Due to upregulated expression of IL-4, IL-10, TGF- β 1 and lactate in the TME, TAMs would be polarized into immunosuppressive M2 macrophages but not proinflammatory M1-type macrophages ^[9]. It has been reported that M2 macrophages have an important role in promoting tumor growth, metastasis and drug resistance^[10], while M1 macrophages could inhibit tumor growth by releasing proinflammatory factors, inducing stromal destruction, and normalizing the tumor vasculature. Macrophages are the main cell type interacting with cytotoxic T cells in TME, relevant studies for targeting macrophages to modulate cancer immunotherapy have carried out in recent years^[10-12]. It was found that the antitumor activation of T cells by PD-L1 antibody (aPD-L1) may be restrained by M2 macrophages, which would be due to recruitment of regulatory T cells and release of immunosuppressive cytokines, resulting in suppressing the function of T cells ^[13, 14]. In contrast, many studies reported that classically activated M1 macrophages in TME could enhance the function of T cells in malignant tumors ^[15].

It is reported that re-education strategies not only eliminate the tumor-supporting function of TAMs, but also improve the anti-tumor immune function, and will be more effective when combination with ICIs^[16, 17]. R848, a hydrophobic

imidazoquinoline-like molecule that binds to TLR-7/8, is an immunomodulator approved by the US FDA for the treatment of skin lesions and skin cancer^[18]. However, the poor water solubility, unfavorable pharmacokinetics and systemic side effects make it unable to be widely used in cancer immunotherapy^[19]. Recently, it was shown that R848 encapsulated in β -cyclodextrin nanoparticles promoted the delivery of R848 to TAMs *in vivo* and improved the immunotherapeutic response when combined with ICIs aPD-1^[20]. Moreover, many studies have used nanovesicles from M1 macrophages (M1 NV) to repolarize M2 macrophages into M1 macrophages, which release proinflammatory cytokines and induce antitumor immune responses, enhancing the anticancer efficacy of aPD-L1^[21]. In this study, we utilized M1 NV as a drug delivery vector to carry R848, thus constructing M1 NV-R848, to enhance the bladder cancer immunotherapy of aPD-L1. M1 NV-R848, which contains an intracellular component of M1 macrophages and TLR7/8-agonist R848, would induce repolarization of immunosuppressive M2 macrophages to proinflammatory M1 macrophages in TME, increase CD8⁺ T cell infiltration, thereby improved the anti-tumor efficacy of aPD-L1 *in vivo* (Scheme 1).

Materials And Methods

Materials:

R848 was purchased from DC Chemicals (Shanghai, China). LPS were obtained from Sigma-Aldrich (Dorset, UK). IFN- γ and IL-4 were purchased from Invitrogen (CA, USA). Anti-CD86, anti-CD206, anti-MHC and anti-CD8A antibodies for flow cytometry were obtained from eBioscience (San Diego, CA, USA).

Cell Culture: The macrophage cell line RAW264.7 and the bladder cancer cell line MB49 were purchased from the American Type Culture Collection (Rockville, MD, USA). RAW264.7 cells were cultured in DMEM and MB49 cells were cultured in RPMI-1640. All media supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical, Tokyo, Japan), at 37 °C in a humidified 5% CO₂ atmosphere. M1 macrophages were induced by the addition of 100 ng/mL lipopolysaccharide (LPS) and 20 ng/ml IFN- γ . M2 macrophages were induced by the addition of 20 ng/mL IL-4 (Invitrogen, CA, USA).

Preparation of M0 NV, M1 NV and M1 NV-R848: To obtain M0 NV or M1 NV, we suspended RAW264.7 cells (M0 macrophages) or LPS/IFN- γ -treated RAW264.7 cells (M1 macrophages) in PBS at a concentration of 5×10^6 cells/mL. Nanometer-sized extracellular vesicles were obtained by successively extruding 11 times through polycarbonate membrane filters (Whatman) with pore sizes of 1 μ m, 400 nm, and 200 nm using a microextruder (AvantiPolarLipids). The extracellular vesicles were then ultracentrifuged at 100,000 g for 2 h at 4 °C in a density gradient formed by 10 and 50% OptiPrep layers, and the extracellular vesicles obtained from the interface of the layers were further ultracentrifuged at 100,000 g for 2 h at 4 °C to obtain M0 NV or M1 NV. To obtain M1 NV-R848, 10 mg M1 NV (10 mL in PBS) was incubated with 4 mg R848 (1 mL in DMSO) and mixed for 12 h at 37 °C on a rotator. Unencapsulated R848 was removed and M1 NV-R848 was concentrated using a 30 kDa centrifugal filter. The collected M1 NV-R848 were rinsed by centrifugal filtration with an equal volume of PBS 3 times. The amount of free R848 that remained in the dialysis water was analyzed by high performance liquid chromatography (HPLC) to obtain the loading content (LC) and loading efficiency (LE) of M1 NV-R848. The drug LC and LE for M1NV-R848 were calculated using the following equations: $LC(\%) = (\text{total amount of R848 added} - \text{free R848}) / \text{amount of M1NV} \times 100\%$, $LC(\%) = (\text{total amount of R848 added} - \text{free R848}) / \text{total amount of R848 added} \times 100\%$.

Characterization of M0 NV, M1 NV and M1 NV-R848: The morphology and particle size of M0 NV, M1 NV and M1 NV-R848 were measured by transmission electron microscopy (TEM) (Hitachi H-7650). The particle size of the M0 NV, M1 NV and M1 NV-R848 was detected by dynamic light scattering (DLS) (Brookhaven Instruments, USA). The particle concentration of M0 NV, M1 NV and M1 NV-R848 was also measured by the Nanoparticle Tracking Analysis (NTA). The colloidal stability of M1 NV-R848 was investigated in PBS and 10% serum at 37 °C by measuring the mean diameter with DLS. The amount of R848 loaded in M1 NV-R848 was determined by HPLC according to previous report^[22]. The release of R848 from M1 NV-R848 were evaluated by dialysis method (20 kDa cassettes, Thermo Scientific). M1 NV-R848 were suspended in 10 mL of PBS at 37 °C. The release system was maintained at 37 °C under shaking. The release medium (0.5 mL) was withdrawn each time and HPLC was used to determine the percentage of released R848. The release of R848 from M1 NV-R848 were calculated according to a formula, accumulative release (%) = M_r/M_t , where M_r is the amount of released R848, and M_t is the total amount of loaded R848.

***In vitro* cellular uptake of NVs by M2 macrophages:** RAW264.7 cells was applied to construct M2 macrophages by incubating with IL-4^[23]. To investigate the cellular uptake of NVs by M2 macrophages, M2 macrophages were incubated in culture plates with 10% (v/v) FBS medium for 24 h. Subsequently, the fluorescent probe (DiR)-labeled M0 NV, M1 NV and M1 NV-R848 were added into M2 macrophages at a concentration of 50 µg/mL DiR for incubating 4 h. The cellular uptake of NVs by M2 macrophages was evaluated using fluorescence microscopy and flow cytometry. Three samples were analyzed per group.

***In Vitro* Analyses of Macrophages Polarization:** M2 macrophages were treated with M0 NV, M1 NV, R848 and M1 NV-R848 for 24 h. Subsequently, cells were washed with PBS, marked by PE-conjugated anti-mouse CD86 (eBioscience, San Diego, CA, USA) for 30 min at 4°C and analyzed by flow cytometry. Moreover, qRT-PCR was performed to determine the relative expression of M1 macrophages markers (iNOS, IL-6 and TNF-α) and M2 macrophages markers (IL-10, Fizz-1 and IL-4) after treated with M0 NV, M1 NV, R848 and M1 NV-R848 for 24 h. Each group was repeated three times. Murine primers for iNOS, IL-6, TNF-α, IL-10, Fizz-1, IL-4 and b-actin are listed as follows:

*iNOS*F: 5'-GTTCTCAGCCCAACAATACAAGA-3'

*iNOS*R: 5'-GTGGACGGGTCGATGTCAC-3'

*IL-6*F: 5'-TAGTCCTTCCTACCCCAATTTCC-3'

*IL-6*R: 5'-TTGGTCCTTAGCCACTCCTTC-3'

*TNF-α*F: 5'-CCCTCACACTCAGATCATCTTCT-3'

*TNF-α*R: 5'-GCTACGACGTGGGCTACAG-3'

*IL-10*F: 5'-GCTCTTACTGACTGGCATGAG-3'

*IL-10*R: 5'-CGCAGCTCTAGGAGCATGTG-3'

*Fizz-1*F: 5'-CCAATCCAGCTAACTATCCCTCC-3'

Fizz-1-R: 5'-ACCCAGTAGCAGTCATCCCA-3'

IL-4-F: 5'-GGTCTCAACCCCCAGCTAGT-3'

IL-4-R: 5'-GCCGATGATCTCTCTCAAGTGAT-3'

b-actin-F: GGCTGTATTCCCCTCCATCG

b-actin-R: CCAGTTGGTAACAATGCCATGT

The establishment of orthotopic bladder cancer bearing model: Female C57BL/6 mice (8 weeks old, weight 18-20 g) were purchased from GemPharmatech Co., Ltd. (Nanjing, China). All animal experiments were approved by the Institutional Animal Care Committee of Jiangsu Province and the Ethics Committee of Nanjing Drum Tower Hospital, Medical school of Nanjing University. An orthotopic bladder cancer bearing model was established in C57BL/6 mice with a minimally invasive method based on our previously published article^[24]. Briefly, we made a longitudinal small incision (approximately 2 mm) on the skin of the lower abdomen of the mice, found the bladder, and carefully clamped the bladder using smooth forceps. MB49^{luc} cells were injected into the bladder wall using a 1 mL syringe, the bladder was returned to its original position, and the abdominal incision was closed. One week later, tumor in the bladder was monitored using an IVIS Spectrum computed tomography system (PerkinElmer, Waltham, MA, U.S.).

The biodistribution of M1 NV-R848 in orthotopic bladder cancer bearing model: The biodistributions of DiR labelled M0 NV, M1 NV and M1 NV-R848 were investigated using an IVIS Spectrum computed tomography system (PerkinElmer, Waltham, MA, U.S.) after intravenous injection into orthotopic bladder cancer bearing model. After 24 h post-injection, the mice were sacrificed, and NIR fluorescence images of the major organs (heart, liver, spleen, lung, and kidney) and bladder with tumor were acquired at a 748 nm excitation wavelength and a 780 nm emission wavelength. Then, the relative fluorescence intensities of the organs and bladder with tumor were quantified using the software Living Image 3.1.

The function of macrophage polarization induced by M1 NV-R848 *in vivo*: The orthotopic bladder cancer bearing model was established in C57BL/6 mice. One week later, the bladder tumor was confirmed using an IVIS Spectrum computed tomography system. Mice were intravenously injected with PBS, M0 NV (5 mg/kg), M1 NV (5 mg/kg), R848 (0.5 mg/kg) and M1 NV-R848 (M1 NV:5 mg/kg, R848 0.5 mg/kg) at day 0, day 3 and day 6. At day 20, tumors were surgically excised and frozen sections of tumors were collected. IHC staining for M1 and M2 macrophages markers was performed. Sections were treated with sodium citrate buffer (10 mM Sodium citrate and 0.05% Tween 20, pH 6.0) for 10 min at 85°C for antigen retrieval after hydration. Prior to staining, the tissue sections on slides were blocked with 5% (v/v) normal goat serum (Gibco) and incubated with primary antibodies against iNOS (18985-1-AP, Proteintech, China), and arginase 1 (#93668, Cell Signaling Technology) for 18 h at 4°C. The sections were washed three times with PBS and incubated for 1 h with rhodamine-conjugated secondary antibodies. After washing with PBS, the slides were mounted with a mounting medium (VectaMount mounting medium, Vector Labs Inc., Burlingame, CA, USA). For flow cytometry, tumor tissues were mechanically cut into small pieces and enzymatically digested (DMEM containing 1.5 mg/mL collagenase I, 125 units/mL hyaluronidase, 0.1 mg/mL DNAase I) to generate single-cell suspension. Then, single-cell suspension was incubated with anti-CD206 and anti-MHC antibodies for 30 min on ice and detected by flow cytometry.

The therapeutic efficacy of M1 NV-R848 with or without aPD-L1 in subcutaneous xenograft model of bladder

cancer: Female C57BL/6 Mice were anesthetized and MB49 cells (5×10^6 cells in 100 μ L PBS per mouse) were subcutaneously injected into a flank of the mice. The tumor volume (V) was estimated according to an ellipsoidal calculation, whereby $V = a \times b^2 \times 0.5$, where a is the largest and b is the smallest diameters of the tumor ellipsoid. When the tumor size reached 70 mm³, either M0 NV (5 mg/kg), M1 NV (5 mg/kg), R848 (0.5 mg/kg), M1 NV-R848 (M1 NV: 5 mg/kg, R848: 0.5 mg/kg) and anti-PD-L1 mAb (5 mg/kg) (aPD-L1, BioXcell) only, or a combination of M1 NV (5 mg/kg) and aPD-L1 (5 mg/kg), R848 (0.5 mg/kg) and aPD-L1 (5 mg/kg), M1 NV-R848 (M1 NV: 5 mg/kg, R848: 0.5 mg/kg) and aPD-L1 (5 mg/kg) were injected intravenously at day 0, day 3 and day 6. The tumor volume was determined every 5 days. At day 20, tumors were removed from euthanized mice and weighted. The body weight of mice was measured during the treatment.

M1 NV-R848 potentiate antitumor effects of PD-L1 inhibitors (aPD-L1) in orthotopic bladder cancer bearing model: The orthotopic bladder cancer bearing model was established in C57BL/6 mice. One week later, the bladder tumor was confirmed using an IVIS Spectrum computed tomography system. Mice were intravenously injected with PBS, aPD-L1 (5 mg/kg), M1 NV-R848 (M1 NV: 5 mg/kg, R848: 0.5 mg/kg) and aPD-L1 (5 mg/kg) combined with M1 NV-R848 (M1 NV: 5 mg/kg, R848: 0.5 mg/kg) at day 0, day 3 and day 6. At day 20, tumor tissues were removed from euthanized mice and were homogenized after grinding with a scalpel. For IHC and H&E staining, tumor tissues were fixed with formaldehyde, embedded in paraffin, and sliced at a thickness of 4 μ m. The sections of the tumor tissues were stained with H&E and were examined using an optical microscope (Olympus, Tokyo, Japan). IHC staining with Ki67 was performed. Tumor tissues were enzymatically digested to generate single-cell suspensions. Then, the single-tumor cell suspensions were incubated with anti-CD8A antibody for 30 min on ice followed by flow cytometry.

Statistical Analyses: Data were presented as mean $\pm$ standard deviation (SD). Statistical comparisons were performed using unpaired Student's t-test for two group comparisons, one way analysis of variance (ANOVA) for comparisons of more than three groups using Prism 8 (GraphPad software). Differences were considered statistically significant when $p < 0.05$.

Results And Discussions

Characterization of M1 NVs.

M0 NV and M1 NV were obtained from RAW264.7 cells (M0 macrophages) and LPS-treated RAW264.7 cells (M1 macrophages) by serial extrusion through polycarbonate membranes with pore sizes of 1 μ m, 400 nm, and 200 nm, respectively. The M1 NV-R848 was prepared by co-incubation of M1 NV with R848 for 12 h. The DLS results showed that the particle size of M0 NV, M1 NV and M1 NV-R848 was about 161.8 ± 7.1 nm, 163.4 ± 5.4 nm and 164.3 ± 9.1 nm, respectively, with good polydispersity (Fig 1a). Meanwhile, the TEM images revealed that the above NVs showed subspherical morphology with diameter like that of DLS (Fig 1a). Similar particle concentration was found in M0 NV, M1 NV and M1 NV-R848 when detected by NTA (Fig 1b). In addition, the loading content of R848 in M1 NV-R848 was about 10% and the loading efficiency of R848 in M1 NV-R848 was about 25% when detected by HPLC. Besides, M1 NV-R848 could maintain good colloidal stability in the PBS and serum and no significant difference in hydrodynamic diameter was found over 72 h (Fig 1c). Furthermore, the release profiles of R848 from M1 NV-R848 showed that M1 NV-R848

possessed sustained release behavior and the cumulative release of R848 from M1 NV-R848 was about 78.6% after 72 h post-incubation (Fig 1d).

The polarization of M2 to M1 macrophages induced by the NVs treatment *in vitro*.

According to the previous reports, M2 macrophages could be obtained by incubating RAW264.7 with IL-4 (Fig 2a). To investigate the uptake capacity of M2 macrophages for NVs *in vitro*, M2 macrophages was incubated with NVs labeled with fluorescent dye (DiR), and then analyzed by fluorescence imaging and flow cytometry. The results showed that M2 macrophages could effectively internalize M0 NV, M1 NV, and M1 NV-R848 and no significant difference could be found between different groups (Fig S1a and S1b). As showed in Fig 2a, different treatment was evaluated for the function of polarization of M2 to M1 macrophages. The flow cytometric analysis was applied to investigate the change of CD86 (M1 macrophages marker) in M2 macrophages after treated with M0 NV, M1 NV, R848, or M1 NV-R848. The results showed that R848 and M1 NV could significantly increase the expression of CD86 in M2 macrophages (Fig 2b and 2c), which was similar to previous reports^[20, 21]. In addition, the expression of CD86 in M2 macrophages could be further enhanced after incubated with M1 NV-R848 when compared to R848 and M1 NV (Fig 2b and 2c), indicated that M1 NV-R848 exhibit the combined effect of R848 and M1 NV, and polarizing more M2 to M1 macrophages. To further evaluate the function of NVs to polarize M2 macrophages into M1 macrophages, the changes of mRNA levels for M1 macrophages markers (iNOS, IL-6 and TNF- α) and M2 macrophages markers (IL-10, Fizz-1 and IL-4) in M2 macrophages after treated with M0 NV, M1 NV, R848, or M1 NV-R848 was analyzed. As showed in Fig 2d, e and f, the relative mRNA levels of M1 macrophages markers were significantly increased, while the relative mRNA levels of M2 macrophages markers were significantly down regulation after treated with M1 NV, R848, or M1 NV-R848 (Fig 2g, h and i). The enhanced polarization of M2 macrophages to M1 macrophages by M1 NV-R848 would improve the immunosuppressive TME, and finally resulting in improving anticancer effect *in vivo*.

The biodistribution and macrophages polarization in orthotopic bladder cancer bearing mouse model after intravenous injection of M1 NV-R848

Previous studies demonstrated that cell-derived NVs including RAW264.7 cells were biocompatible and retained the membrane topology of the cells and stability in serum, which could be used to enhance the drug delivery properties^[25-28]. To assess the biodistribution of M1 NV-R848, we established the MB49 orthotopic bladder cancer bearing mice firstly and verified by H&E staining (Fig S2). The biodistribution of DiR-labeled M0 NV, M1 NV, and M1 NV-R848 in tumor-bearing mice was evaluate after intravenous injection. The results showed that M0 NV, M1 NV and M1 NV-R848 could effectively accumulated in the bladder tumor and no significant difference on fluorescence signal was found between each group (Fig 3a and 3b). The bladder tumor targeted effect of M0 NV, M1 NV and M1 NV-R848 would be due to the enhanced permeability and retention (EPR) effect to the bladder tumor. In addition, the NVs derived from the macrophages would exhibit the active targeting effect based on the expression of chemokine receptors, which would specially recognize and bind to the chemokines expressed in the bladder tumor^[21].

Previous studies have shown that the increase of M1 macrophages in the TME could enhance the efficacy of ICIs therapy^[29]. To determine whether M2 macrophages polarization to M1 macrophages by M1 NV-R848 *in vivo*, we compared the protein expression of the M2 macrophages marker of Arg-1 and the M1 macrophages marker of iNOS in bladder tumor tissue after treated with M0 NV, R848, M1 NV and M1 NV-R848, respectively. The results of IHC indicated that both of R848, M1 NV and M1 NV-R848 could increase the expression of iNOS and decrease the expression of Arg1 in bladder cancer tissues when compared with PBS and M0 NV (Fig 3c, 3e, and 3f). In addition, from the results of single-cell suspension of bladder tumor detected by flow cytometry, the ratio of M1 macrophages (MHC II⁺ CD206⁻ cells) was increased in bladder tumor tissues after treated with R848, M1 NV and M1 NV-R848 (Fig 3d and 3g). It is reported that R848 and M1 NV could be used to increase M1 macrophages polarization in tumor tissue^[20, 21]. Moreover, in this

study we found that the combination of R848 and M1 NV showed more effectively to enhance M1 macrophages polarization in the bladder cancer.

The antitumor effects of M1 NV-R848 combined with PD-L1 inhibitors (aPD-L1) in bladder cancer mouse model.

To investigate whether M1 NV-R848 could enhance the antitumor efficacy of ICIs, we first established the MB49 xenograft bladder cancer bearing mice. When the tumor size reached to 70 mm³ approximately (day 0), the mice were intravenously injected with PBS, M0 NV, M1 NV, R848, M1 NV-R848, aPD-L1, aPD-L1 combined with M1 NV, aPD-L1 combined with R848 or aPD-L1 combined with M1 NV-R848. As shown in Fig 4, treatment with M1 NV, R848, M1 NV-R848 and aPD-L1 alone could inhibit the growth of bladder tumor when compared with PBS and M0 NV. Treated with M1 NV-R848 showed more effectively to hinder the growth of bladder tumor when compared with M1 NV and R848 alone. In addition, aPD-L1 combined with M1 NV, R848 or M1 NV-R848 could further enhance the antitumor effect when compared to aPD-L1 (Fig 4). Due to the strongest polarization of M1 macrophages by M1 NV-R848, aPD-L1 combined with M1 NV-R848 also resulted in the strongest bladder tumor growth inhibition. Moreover, there is no significant difference in the body weight of mice between the different treatment groups (Fig 4b), suggesting that M1 NV-R848 possessed good biological safety *in vivo*.

Next, we subsequently investigated whether M1 NV-R848 had the antitumor efficacy for enhancing the ICIs of aPD-L1 in MB-49^{luc} orthotopic bladder cancer bearing models. As shown in Fig 5a and 5c, the orthotopic bladder cancer bearing mouse models were confirmed by *in vivo* imaging and similar bioluminescence signal was found before treatment. Then, the mice were intravenously injected with PBS, aPD-L1, the combination of aPD-L1 and M1 NV, or the combination of aPD-L1 and M1 NV-R848. The H&E staining of the bladder tumor showed that both of aPD-L1 alone and aPD-L1 combined with M1 NV or M1 NV-R848 could significantly inhibit the growth of bladder tumors at day 20 (Fig 5b and 5d). Moreover, aPD-L1 combined with M1 NV or M1 NV-R848 could further improve the antitumor effect when compared to aPD-L1 alone, through polarization of M1 macrophage by M1 NV or M1 NV-R848. Like the results of xenograft bladder cancer bearing model in our study, aPD-L1 combined with M1 NV-R848 could almost completely inhibit the progression of bladder tumor. In addition, the results of Ki67 expression of bladder cancer showed that the combination treatment with M1 NV-R848 and aPD-L1 significantly decreased the proliferative capacity of bladder cancer (Fig 5e and 5f). It had been reported that repolarization of M2 macrophages into M1 macrophages would upregulate the proinflammatory cytokines and induce antitumor immune responses by recruitment of CD8⁺ T cells^[30]. To further illustrate the enhanced inhibitory effect of aPD-L1 combined with M1 NV-R848, we evaluate the level of CD8⁺ T cells in bladder cancer. The results indicated that combination treatment of M1 NV-R848 and aPD-L1 could significantly increase the infiltration of CD8⁺ T cells in bladder cancer (Fig 5g and 5h).

Conclusion

In this study, M1 NV-R848 could effectively induce polarization of M2 macrophages to M1 macrophages when compared to M1 NV or R848 alone *in vitro* and *in vivo*. In addition, M1 NV-R848 combined with aPD-L1 treatment significantly suppress the growth of bladder cancer, by repolarization of M2 to M1 macrophages, improvement of CD 8⁺ T cells infiltration. This study suggests that M1 NV-R848 can be used as an immune regulator in the TME to potentiate ICIs therapies for bladder cancer.

Declarations

Ethics approval

The study was approved by the Ethical Committee of Nanjing Drum Tower Hospital, Medical School of Nanjing University.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no competing interest exists.

Authors' contributions

HQG, QZ and WLZ conceived and designed the experiments. QZ, WLZ, XH, YZD, TSL, WFL, WMC, and WC participated in the experiments and drafted the manuscript. TZW, WFL, and XFW contributed to the sample collection and interpretation of the data. All authors read and approved the final manuscript.

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Disclosure

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Figures

Figure 1

Characterization of NVs

Figure 2

The polarization of M2 macrophages to M1 macrophages induced by the NVs treatment *in vitro*

Figure 3

The biodistribution and macrophages polarization in orthotopic bladder cancer bearing model after intravenous injection of M1 NV-R848

Figure 4

Therapeutic efficacy of M1 NV-R848 with or without aPD-L1 in xenograft bladder cancer bearing mouse model

Figure 5

M1 NV-R848 potentiates antitumor effects of PD-L1 inhibitors (aPD-L1) in orthotopic bladder cancer bearing mouse model

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