Nanovaccines integrating endogenous antigens and pathogenic adjuvants elicit potent antitumor immunity

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ABSTRACT

Cancer nanovaccines have the ability to train the immune system to specifically recognize and eliminate tumor cells. However, the vaccine’s efficacy is hampered by limited access to draining lymph nodes, poor recognition and internalization by dendritic cells (DCs), as well as insufficient immunogenicity to elicit the DC maturation. To overcome these challenges, we developed potent vesicular cancer nanovaccines by fusing bacterial outer membrane vesicles (OMVs) and tumor cell membranes (TCMs), namely BTs. The bacteria-derived pathogenic adjuvants in BTs enabled DC targeting, promoted full maturation of DCs and antigen presentation by DCs. The TCMs in BTs assembled a multitude of diverse tumor endogenous antigens as training cues to generate multiantigenic antitumor immunity. Integrating the two components via a lipid bilayer assembly avoids the premature separation in the biological environment and enables the high spatial and temporal delivery of the antigens and adjuvants into the same DCs. Immunization with BTs containing as low as 1 μg of OMVs confers an excellent tumor prophylactic effect with ~30 % of treated mice maintaining a tumor-free status for over 100 days. Moreover, the nanovaccines exhibit a remarkable therapeutic effect in treating established solid tumors and potentiate immune checkpoint blockade therapy. Overall, the BTs are promising as personalized cancer nanovaccines and carry no systemic side effects.

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Introduction

Formulated nanoparticle-based vaccines, known as nanovaccines, have drawn great attention in the arena of cancer immunotherapy. By co-delivering tumor antigens and immunostimulatory adjuvants into antigen presenting cells (APCs), nanovaccines can simultaneously induce the maturation of APCs and antigen presentation, two requirements for eliciting the antigen-specific T cell immune response [1–4]. However, a fundamental challenge for cancer nanovaccines lies in an inadequate capacity to induce a full maturation of dendritic cells (DCs), the most potent APCs. Derivatives of native or synthetic pathogen-associated molecular patterns (PAMPs) have been utilized as adjuvants of nanovaccines to elicit a stronger immune response [5]. However, most nanovaccines generally adopt a single type of PAMPs as the adjuvant and are not as effective as the counterparts of whole pathogens, which contain multiple types of PAMPs to synergistically activate a broad repertoire of toll-like receptors (TLRs). While pathogenic adjuvants using whole pathogens can activate multiple subsets of DCs by stimulating multiple TLRs and thus generate potent immune synergy, their clinical translation is hampered by their high toxicity profiles [6–9].

Currently, the therapeutic outcomes of nanovaccines are also impeded by their poor recognition and internalization by DCs and by a limited access to draining lymph nodes, where the efficient priming and amplification of T cells are initiated. Lymphatic and DC targeting can be achieved by surface decorating antibodies or ligands, such as mannos, to promote the delivery efficacy of nanovaccines [10–12]. However, the targeting efficiency can be low given the heterogeneous receptor expression profiles among different DC subsets [13,14]. In addition, such targeting ability can easily decline or even disappear in biological system due to the formation of surface protein corona [15].
In nature, invading bacteria are recognized as non-self and then are phagocytosed by DCs and phagocytes [16]. The recognition is mediated by the interactions between native PAMPs on bacteria and pattern recognition receptors (PRRs) on DCs. Based on the efficient recognition and phagocytosis of bacteria by DCs, we thus hypothesize that engineering nanovaccines composed of bacteria-derived components will facilitate their targeting to DCs. With biosafety concerns, bacteria-derived outer membrane vesicles (OMVs) are more promising without toxicity profiles due to their non-replicating nature [17]. In addition, as OMVs inherit multiple PAMPs from parental bacteria, they hold more promise for eliciting effective immune synergy as compared to the whole pathogen adjuvants [18-20].

Herein, we established a vesicular platform as potent cancer nanovaccines, which were fabricated by fusing bacterial outer membranes with tumor cell membranes (TCMs), hereafter denoted as BTs (Scheme 1). Moreover, we optimized a nanoparticle core as the component of the nanovaccine system to further enhance the effect of BTs. Specifically, on the one hand, the bacteria-derived pathogenic adjuvants in BTs reinforced DC targeting, maturation, and antigen presentation. On the other hand, TCMs in BTs assembled a multitude of diverse tumor endogenous antigens as training cues to create a diverse T cell repertoire by amplifying the processing and presentation of tumor-specific antigens [10,21–24].

The adjuvants and tumor antigens were assembled in meta-stable cell membranes, thus avoiding premature separation in the biological environment [25]. The integrated polymeric core composed of poly(lactic-co-glycolic acid) (PLGA) into the BTs could support the vesicular structure, improve the display of multivalent epitopes, and facilitate immune recognition [26]. The design of BTs exploits the synergy between bacterial OMVs and TCMs to realize the similar function of presenting tumor antigens by bacteria to APCs, thus generating robust antitumor immunity. BTs containing as low as 1 μg of OMVs significantly enhance the particle uptake, DC maturation, antigen presentation, and the eliciting of antigen-specific T cell responses. Immunization with BTs confers

![Scheme 1](image-url)
an excellent tumor prophylactic effect with ~30% of treated mice maintaining a tumor-free status for over 100 days. Moreover, BTs show remarkable therapeutic effect in treating established solid tumors and potentiate immune checkpoint blockade therapy.

**Results and discussion**

**Preparation of BTs via the fusion of TCMs and OMVs**

TCMs were prepared from murine B16F10 melanoma cells via hypotension disruption, sonication, and centrifugation. OMVs were extracted from the culture supernatant of *Escherichia coli* (*E. coli*). Both TCMs and OMVs contain conical phospholipids, such as phosphatidylethanolamine, which possess negative spontaneous curvature and can facilitate bilayer disruption and fusion of the membranes [27]. Furthermore, we applied mechanical forces by sonication and membrane extrusion to bring initially separated TCMs and OMVs into close juxtaposition, thus promoting inter-membrane fusion [28,29]. To verify the fusion, the isolated TCMs and OMVs were pre-labelled with lipophilic fluorescent dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) and 3,3′-dioctadecylxocarbocyanine perchlorate (DiO), respectively (Fig. 1A). Upon sonication and repeated extrusion, the two types of cell membranes were well-fused, as evidenced by the highly co-localized and homogeneous fluorescence signals of BTs compared to that of the mixture of free TCMs and OMVs (Fig. 1B, C). Quantifications of the Pearson correlation coefficients confirmed the high co-localization of TCMs and OMVs in
BTs (Pearson’s r value: 0.80 for the BTs group versus 0.10 for the TCMs + OMVs group) (Fig. 1D). SDS-PAGE analyses revealed that the BTs preserved the proteins from the parental TCMs and OMVs (Supplementary Fig. S1). Moreover, the presence of TCMs-derived membrane-bound melanoma-specific antigens, such as gp100 and TRP2, and OMVs-derived LPS in BTs were further confirmed by western blotting (Supplementary Fig. S2). Thus, the BTs enable the stable integration of multiple natural pathogenic PAMPs and diverse tumor endogenous antigens.

**BTs induced maturation of DCs**

We next evaluated whether the internalized BTs could induce the maturation of BMDCs in vitro. We detected the expression of co-stimulatory molecules and the secretion of cytokines by BMDCs, which are essential to mount a strong antigen-specific cytotoxic T cell lymphocyte (CTL) response by DCs after vaccine immunization. To rule out the endotoxin contamination in TCMs-induced DC maturation, we detected the endotoxin levels of TCMs. It was found that the endotoxin levels were less than 0.002 EU per 10 μg TCMs (Supplementary Fig. S5), excluding the possibility of endotoxin contamination-induced DC maturation. After incubation for 18 h, both BTs and OMVs could significantly induce the up-regulated expression of CD40, CD80, CD86, and MHC-II, consistent with the fact that sensing infection through recognition of PAMPs can induce the DC maturation (Fig. 2A-D) [30,31]. By contrast, TCMs without OMVs showed significantly decreased ability on the induction of DC maturation. Pro-inflammatory cytokines are a third signal required to elicit T cell response by DCs, along with antigen and co-stimulation signals [31]. Determining the secretion of pro-inflammatory cytokines IL-12p40 and IL-6 by the BMDCs revealed analogous results, further proving the ability of OMVs to promote DC maturation (Fig. 2E, F). We next screened an optimal ratio of TCMs to OMVs in BTs. Considering the demand of simultaneously activating DCs and inducing antigen presentation, but without causing potential toxicity to mice, the ratio of 100:1 is selected and adopted for further studies (Supplementary Fig. S6).
Collectively, these data indicated that BTs could promote the uptake of tumor antigens by DCs and elicit enhanced DC maturation. Moreover, adopting OMVs as pathogenic adjuvants avoids the screen of the optimal combination of PAMPs as effective adjuvants.

**Enhanced uptake of BTs@PLGA by DCs**

Adding a solid core with suitable size into soft nanoparticles is known to affect their physicochemical properties and interactions with biological systems [32]. To support the vesicular structure of BTs, we next selected three commonly used nanoparticles composed of polymer PLGA, silicon dioxide (SiO₂), and colloidal gold (Au) as the core materials of BTs (denoted as BTs@PLGA, BTs@SiO₂, and BTs@Au, respectively) (Supplementary Fig. S7). Transmission electron microscopy (TEM) revealed a clear core-shell structure, indicating the successful coating of membranes on the core nanoparticles (Fig. 3A). The successful coating of BTs on PLGA NPs was also confirmed by confocal microscope (Supplementary Fig. S8). Dynamic light scattering (DLS) analysis using number distribution showed that all the BTs had a diameter of ~100 nm, consistent with the TEM observation (Supplementary Fig. S9). The BTs had a zeta potential of ~15 ~25 mV, similar to that of TCMs. The addition of core materials to BTs improved the stability of free BTs probably by preventing vesicle fusions (Supplementary Fig. S10).

First, the uptake profile of BTs with different cores by BMDCs was assessed by flow cytometry. As shown in Fig. 3B, C, adding a nanosized core to the BTs enhanced their cellular uptake by BMDCs, consistent with previous reports that too soft NPs were unfavorable for cellular uptake [33–35]. Among the three types of cores, the PLGA core showed the strongest ability to enhance the uptake of BTs by DCs. The increased uptake of BTs@PLGA by DCs might be partially attributable to the enhanced display of PAMPs due to the support of the hybrid membrane by the PLGA core.

Consistent with the enhanced cellular uptake of BTs@PLGA by DCs, BTs@PLGA exhibited superior ability in inducing BMDC maturation compared to BTs and TCMs + OMVs, demonstrated by stronger expression of CD40, CD80, CD86, and MHC-II (Fig. 3D-G). BTs@PLGA also displayed enhanced ability on the induction of BMDC maturation compared to BTs@SiO₂ and BTs@Au, although the differences were not statistically significant.

**Enhanced accumulation of BTs@PLGA in distal lymph nodes**

Lymph nodes are the main sites where DCs present antigens for the priming and expansion of T cells [36,37]. The efficacy of nanovaccines delivered to these sites greatly affects the strength of the induced immune response (Fig. 4A) [38,39]. We next explored the accumulation efficacy of BTs in draining lymph nodes after administration. C57BL/6 mice were subcutaneously (s.c.) injected with DiD-labelled BTs (BTs@DiD) and the draining inguinal lymph nodes were collected at 24 h after injection. It was shown that the DiD signals could be easily detected at the draining inguinal lymph nodes (Fig. 4B), which is partially mediated by OMVs, as evidenced by the lower lymph node enrichment of TCMs (Supplementary Fig. S11). Results of analyzing the main APCs in the lymph nodes showed that BTs were mainly located in DCs and macrophages, the two powerful populations of professional APCs (Fig. 4C and Supplementary Fig. S12) [40]. Immunofluorescence (IF) results also proved the co-localization of BTs with DCs in the draining lymph nodes (Fig. 4D).

The degree of lymph node involvement correlates with the strength of the induced immune response after immunization [41]. Next, we further studied the effect of the cores on the lymph node involvement degree after BT immunization. DiD-labelled BTs and BTs with different cores were s.c. injected into the footpads of mice, respectively, and the draining popliteal lymph
nodes and distal inguinal lymph nodes were harvested at 24 h post injection to analyze the accumulation of the BTs (Fig. 4E). It was shown that the four kinds of BTs exhibited similar efficiency to accumulate in the draining popliteal lymph nodes, but BTs@PLGA manifested a stronger ability to reach distal inguinal lymph nodes compared to bare BTs, BTs@SiO₂, and BTs@Au (Fig. 4F-H), which could be due to the rigidity-induced differences in cell uptake and tissue diffusivity [42]. Collectively, these data indicated that BTs with a PLGA NP core presented stronger ability in the cell uptake, induction of DC maturation, and accumulation in distal lymph nodes. We thus adopted BTs@PLGA for further studies.

**BTs@PLGA promoted DC maturation in vivo**

We next investigated whether BTs@PLGA could induce the maturation of DCs in vivo. C57BL/6 mice were s.c. injected with indicated formulations and the draining inguinal lymph nodes were extracted for flow cytometric analyses at 24 h post-immunization. The results demonstrated that BTs@PLGA, OMVs, and TCMs + OMVs were able to drive significant maturation of DCs, as evidenced by the notable upregulation of CD40, CD80, CD86, and MHC-II on DCs (Fig. 5A-D). The antigen-only TCMs presented apparently reduced activity on inducing the expression of co-stimulatory molecules, further proving the importance of OMVs in inducing the maturation of DCs.

To further verify that the maturation of DCs after treatment with BTs with PLGA is directly caused by the encounter and uptake of BTs (Fig. 5E), BTs@PLGA were labelled with DiD and s.c. injected into mice. After 24 h, leukocytes in the draining lymph nodes were isolated for flow cytometry. According to the gating strategy in Fig. 5F, DCs were divided into two groups based on the DiD intensity, DiD⁺ DCs (that internalized BTs@PLGA) and DiD⁻ DCs (that did not internalize BTs@PLGA). The expression of co-stimulatory molecules on DiD⁺ DCs and DiD⁻ DCs was further analyzed. According to the flow cytometric results, DCs that internalized BTs@PLGA expressed higher co-stimulatory molecules CD40, CD80, CD86, and MHC-II, compared to cells without BTs@PLGA uptake, indicating that the recognition and uptake of BTs@PLGA resulted in the maturation of DCs (Fig. 5G, H).
Antigen cross-presentation is a process in which extracellular antigens are taken up, processed and presented onto MHC-I molecules, which is essential to elicit the proliferation and activation of antigen-specific CD8+ T cells (Fig. 6A). We then studied the effect of BTs on the presentation of tumor antigens by DCs. In this experiment, TCMs of BTs@PLGA were prepared from B16-OVA cells and OVA was used as a model antigen (Fig. 6A). BMDCs were co-cultured with different formulations and the OVA peptide (SIINFEKL)-MHC-I complex on BMDCs was detected by flow cytometry to reflect antigen presentation. As shown in Fig. 6B, C and Supplementary Fig. S13, BTs@PLGA treatment induced stronger antigen presentation compared with the other groups, as evidenced by the higher percentage of DCs expressing H-2Kb-OVAp and the higher geometric mean fluorescence intensity (GMFI) of H-2Kb-OVAp on DCs. The higher antigen presentation of BTs@PLGA was mutually mediated by the BTs and PLGA NP core (Supplementary Fig. S14). Treatment with TCMs did not induce obvious presentation of OVA peptide by DCs, probably due to the insufficient internalization of pristine TCMs by DCs as well as the absence of OMV adjuvant [43,44]. These results further highlight the importance of co-delivery of antigens and adjuvants to DCs. Taken together, these results indicate that BTs@PLGA can induce the enhanced maturation and tumor antigen presentation by DCs, indicating their ability to mount a tumor antigen-specific CD8+ T cell response.

In vivo antigen-specific CD8+ T cell response induced by BTs@PLGA

We next investigated the ability of BTs@PLGA to induce T cell immune response, which is crucial to eliminate tumor cells (Fig. 6A). C57BL/6 mice were s.c. injected with different formulations on three occasions with the dosing interval of 1 week. In this experiment, TCMs were prepared from B16-OVA cells and OVA was used as a model antigen. Draining lymph nodes were dissected and OVA-specific CD8+ T cells were analyzed by flow cytometry 7 days after the last immunization (Fig. 6D). As shown in Fig. 6E and Supplementary Fig. S15, BTs@PLGA mounted a higher percentage of OVA-specific CD8+ T cells, indicating its stronger ability to elicit antitumor immunity. Moreover, immunization with BTs@PLGA generated multiple tumor antigens-specific CD8+ T cells, as evidenced by the increased interferon-γ (IFN-γ) production in response to gp100 and TRP2 peptide stimulation (Supplementary Fig. S16). In addition, BTs@PLGA immunization significantly elicited the proliferation, activation, and immunological memory of T cells, as evidenced by the enhanced expression of Ki-67 and CD69 on T cells and higher percentage of memory CD44+CD62L- T cells (Supplementary Fig. S17 and S18). Based on these above studies, it could be reasonably inferred that BTs@PLGA would generate multiantigenic antitumor immune responses against diverse tumor antigens that were incorporated in the nanovaccines.
Immunization with BTs@PLGA conferred prophylactic effect to inhibit tumor progression

To determine whether the enhanced induction of tumor antigen presentation and antitumor T cell responses generated by BTs@PLGA nanovaccines could translate into effective antitumor activity to reject the encountered tumor cells or to inhibit tumor recurrence after surgery in clinical setting, a prophylactic study was carried out using a B16F10 tumor model with poor immunogenicity, attributing to a lack of effective antigen presentation [45,46]. C57BL/6 mice were s.c. injected with different formulations three times with the dosing interval of 1 week and were challenged with B16F10 tumor cells 7 days after the last immunization (Fig. 7A). The tumor volume was measured starting from the 8th day after tumor inoculation (Fig. 7B-D). It has been reported that systemic injection of 5 μg OMVs could completely eradicate established solid tumors via IFN-γ-dependent immunity [18]. Our recent work showed that systemic injection of about 1.5 μg OMVs only resulted in a transient weight loss, indicating biosafety at a low dose [47]. In the current study, we used an even lower injection dose of 1 μg of OMVs as the adjuvant. Such a dose of OMVs did not cause toxicity to the mice, as evident by that the mouse weight, levels of proinflammatory cytokines and blood biochemical indices remained unchanged after the immunization with BTs (Supplementary Fig. S19). Treatment with OMVs containing no antigenic material showed little protective benefit, as evidenced by the similar tumor growth kinetics to that of the untreated control group. Administration with TCMs without the OMVs adjuvant moderately inhibited the tumor growth. Immunization of a mixture of TCMs + OMVs without membrane integration only showed slight suppression of tumor progression compared to TCMs treatment, highlighting the importance of the co-delivery of tumor antigens and adjuvants. Notably, immunization with BTs@PLGA demonstrated the best antitumor efficacy compared to all the other formulations. Remarkably, two of the seven mice remained tumor-free for over 100 days, further proving the significance of co-delivering antigens and adjuvants to DCs for mounting potent antitumor immunity. These results suggested that immunization with BTs@PLGA conferred sufficient prophylactic effect to inhibit tumor progression.

Immunization with BTs@PLGA generated long-term immunological memory

Vaccines work by triggering a robust immune response and consequent immunological memory that results in a rapid immune response of greater magnitude upon reencountering the same antigens. To test whether immunization with BTs@PLGA generated immunological memory, mice that remained tumor-free for more than 100 days after immunization with BTs@PLGA and subsequent B16F10 inoculation in the prophylactic experiment (tumor-free mice) were challenged with B16F10 cells along with naïve mice (Fig. 7E). All B16F10 tumor-free mice completely rejected the challenged B16F10 cells, while all naïve mice grew tumors, suggesting that immunization with BTs@PLGA triggered long-term immunological memory against B16F10 tumor cells (Fig. 7F), which is consistent with the higher memory T cells elicited by BTs@PLGA immunization (Supplementary Fig. S18).

To further prove the specificity of the immune response elicited by BTs@PLGA immunization, B16F10 tumor-free mice and naïve mice were inoculated with MC38 colon cancer cells. It was shown that MC38 tumors grew in both naïve mice and B16F10 tumor-free mice with similar growth rates, indicating that the immune response induced by BTs@PLGA was tumor type-specific (Fig. 7E, G).

BTs@PLGA immunization delayed tumor progression and potentiated immune checkpoint blockade therapy

The end effector of the immune checkpoint blockade therapy is the tumor antigen-specific T cells, and the deficiency in antigen
Fig. 7. Vaccination with BTs@PLGA conferred prophylactic effect and generated long-term immunological memory. (A) Schedule for the prophylactic experiment process. C57BL/6 mice were s.c. injected with different formulations three times with the dosing interval of 1 week and were challenged with B16F10 tumor cells 7 days after the last immunization. The tumor volume was measured every other day starting from the 8th day after tumor inoculation. The comparisons presentation. (B) Tumor growth curves during the treatment. Reporting of average tumor volumes was halted after the first mouse died in each group. (C, D) Spaghetti plots of tumor volume during the experiment. n = 7 per group. (E) Schematic illustration of the experiment for testing immunological memory and the specificity of immune response. B16F10 tumor-free mice obtained at 100 days after B16F10 tumor cell inoculation from the prophylactic experiment were challenged with B16F10 or MC38 cells. Naive mice were also inoculated with B16F10 or MC38 cells as control groups. The tumor volume was measured every other day. Reporting of average tumor volumes was halted after the first mouse died in each group. (F, G) Tumor growth curves of (F) B16F10 and (G) MC38 after tumor cell inoculation. For F, n = 6 for naive mice group and n = 3 for tumor-free mice group. For G, n = 6 per group. Data are shown as mean ± SEM.

Fig. 8. BTs@PLGA immunization delayed tumor progression and potentiated immune checkpoint blockade therapy. (A) Schematic showing that the tumor antigen-specific CD8+ T cells elicited by immunization with BTs@PLGA potentiated α-PD-1 immunotherapy. (B) Schedule for the combined treatment of BTs@PLGA immunization and α-PD-1 immune checkpoint blockade therapy against B16F10 tumors. After inoculation with B16F10 cells on day 0, mice were s.c. vaccinated with BTs@PLGA (at a TCM protein weight of 100 μg) on days 4, 11, and 18. On days 5, 8, 12, 15, 19, and 22, the mice were i.v. injected with 20 μg α-PD-1. Tumor volume was measured every other day from day 8. (C) Tumor growth curves during the treatment. Reporting of average tumor volumes was halted after the first mouse died in each group. (D) Tumor volumes on day 24. (E, F) Spaghetti plots of tumor volume during the experiment. n = 5 per group. Data are shown as mean ± SEM and analyzed by one-way ANOVA with Tukey's multiple comparisons test. **P < 0.01, n.s., not significant.

presentation would render immunotherapy ineffective [48]. Thus, we studied the potential of the BTs@PLGA nanovaccine system to potentiate programmed cell death protein 1 (PD-1) blockade therapy in treating established B16F10 tumors (Fig. 8A). Mice were first inoculated with B16F10 cells on day 0 and subsequently immunized with BTs@PLGA on days 4, 11, and 18. Starting from the day following the first vaccination, the mice were intravenously (i.v.) administered with 20 μg of anti-PD-1 antibody (α-PD-1) twice a week for 3 weeks (Fig. 8B). The immunization with BTs@PLGA effectively suppressed tumor progression with a ~95% inhibition of tumor growth on day 24 compared to the untreated control group, revealing that the BTs@PLGA nanovaccine elicited a strong antitumor response against established solid tumors (Fig. 8C-E). The α-PD-1 treatment showed little effect on tumor suppression.
on day 24 and rapid tumor growth of the group was observed later on (Fig. 6C-F). Combination of BTs@PLGA with α-PD-1 significantly delayed tumor progression and showed an extra 63.7 % tumor suppression compared with that of the BTs@PLGA group on day 34 (Fig. 8C, F). It is worth noting that one of five mice remained tumor-free for over 50 days. Increasing the dose of TCMs and OMVs could further improve the therapeutic effect of BTs@PLGA against B16F10 tumors (Supplementary Fig. S20). Moreover, the tumor growth suppression effect of BTs@PLGA with similar dosage was further confirmed in MC38 colorectal tumor model and combining BTs@PLGA with α-PD-1 treatment showed a much stronger antitumor effect (Supplementary Fig. S21). These results collectively proved that the BTs@PLGA nanovaccine could generate a much stronger antitumor effect when combined with immune checkpoint blockade therapy, which is commonly used in clinical treatment to reverse immune suppression.

Conclusions

In the present study, we successfully developed a vesicular cancer nanovaccine system by fusing OMVs and TCMs into nanovesicles. In the BTs, OMVs, including various PAMPs from bacteria, enabled DC targeting and promoted full maturation of DCs and presentation by DCs. TCMs, assembling diverse tumor endogenous antigens, were used as training cues to create a diverse T cell repertoire. PLGA NPs were integrated into BTs as the core to support the vesicular structure, improve the display of multivalent epitopes, and facilitate immune recognition. Treatment with BTs@PLGA significantly enhanced the DC targeting, DC maturation, tumor antigen presentation, and amplification of antigen-specific T cell responses. Notably, vaccination with BTs@PLGA containing as low as 1 μg of OMVs showed a stronger prophylactic effect to prevent tumor initiation and progression with ~30 % of treated mice maintaining a tumor-free status for over 100 days. Immunization with BTs@PLGA showed remarkable therapeutic effect in treating established B16F10 tumors and potentiated α-PD-1 immune checkpoint blockade therapy. Cell secreted vesicles, including exosomes, are being assessed as biomarkers and therapeutic delivery vehicles in clinical trials targeting a variety of diseases, such as solid tumors, inflammation, and infection. Among them, the OMV-containing meningitis type B vaccine has been approved by the US FDA and European Medicines Agency [49]. Moreover, given that PLGA has been approved by the FDA for drug delivery, the BTs platform integrating OMVs and endogenous cancer antigens is promising in clinical translation. Overall, our approach holds promise for cancer immunotherapy and might provide a facile and versatile method for developing effective personalized nanovaccines.

Materials and methods

Preparation and characterization of BTs

TCMs were mixed with OMVs at indicated TCMs to OMVs protein ratios. Then the mixture was sonicated at 100 W for 30 min and extruded 21 times through a 200 nm polycarbonate membrane (Whatman, Chicago, IL, USA). BTs-coated NPs were prepared by mixing 101 μg BTs with 1 mg PLGA NPs, 30 μg SiO₂ NPs, or 100 μg Au NPs, respectively, and sonicating for 30 min. The BTs were prepared in biosafety cabinets, which could filter 99.99 % of airborne particles.

Size and zeta potential measurements were performed using a Malvern Zetasizer Nano ZS90 DLS instrument (Malvern Instruments Ltd., Worcs, UK) at 25 °C. The morphology was examined using TEM. For the morphology of SiO₂ NPs, Au NPs, PLGA NPs and BTs@Au, a drop of the nanoparticle solution at a concentration of 5 mg/mL was deposited onto a glow-discharged carbon-coated grid. The grid was subsequently dried and visualized by Tecnai Cryo-TEM (FEI, Hillsboro, OR) at an accelerating voltage of 120 kV. For the morphology of BTs@SiO₂ and BTs@PLGA, a drop of the nanoparticle solution at a concentration of 5 mg/mL was deposited onto a glow-discharged carbon-coated grid. A drop of phosphotungstic acid (1%) stain was added to the grid. After 10 min, the grid was further rinsed with 5 drops of MilliQ water. The grid was subsequently dried and observed by TEM.

To analyze whether the BTs contain proteins derived from each kind of membrane, SDS-PAGE and Coomassie brilliant blue staining were performed. All samples were mixed with SDS loading buffer, heated at 99 °C for 15 min to denature protein, and added into wells within a gel. After electrophoresis, proteins were stained with Coomassie brilliant blue and imaged after staining. For western blotting assay, after SDS-PAGE, the proteins were transferred to 0.45 μm PVDF membrane (Merck Millipore, Billerica, MA, USA) at 300 mA for 75 min. After blocking with 5% milk, the membrane was stained with rabbit anti-mouse gp100 (Abcam, Cambridge, UK), rabbit anti-mouse TRP2 (Abcam), or rabbit anti-LPS (Anyan Biotech., Nanjing, China). After thorough washing, the membranes were further stained with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (Bioznharp, Hefei, China) and developed with ECL substrate (Advantec, Menlo Park, CA, USA).

To confirm the successful coating of BTs on PLGA NPs, BTs and PLGA NPs were labeled with DiO and DiD, respectively. The resulted BTs@PLGA were observed under confocal microscope.

To determine whether two kinds of membranes could fuse, TCMs and OMVs were labelled with DiD and DiO, respectively, by incubation at 37 °C for 10 min and then removed free dyes by centrifugation (845 g, 4 °C, 5 min) for 3 times. The mixture of TCMs@DiD and OMVs@DiO was sonicated and extruded to obtain BTs for analysis by a confocal microscope (LSM 880 with Airyscan, Carl Zeiss, Jena, Germany).

Analyses of BT accumulation and uptake by immune cells in lymph nodes

To assess the accumulation of TCMs and BTs in lymph nodes, DiD-labelled TCMs and BTs (at TCM and OMV protein weight of 100 and 1 μg respectively) were s.c. injected into the right flank of female C57BL/6 mice. After 24 h, the right inguinal lymph nodes were extracted and the DiD fluorescent signals were detected with IVIS Spectrum (Perkin Elmer, Waltham, MA, USA). To analyze the uptake of BTs by multiple kinds of immune cells, the extracted lymph nodes were mechanically disrupted, and digested with 1% collagenase IV in complete DMEM medium for 30 min. After centrifugation, the pelleted cells were suspended in PBS for flow cytometry. To analyze the lymph node involvement degree after BTs immunization, DiD-labelled indicated formulations (at TCM and OMV protein weight of 100 and 1 μg respectively) were s.c. injected into the footpad of female C57BL/6 mice. After 24 h, the popliteal and inguinal lymph nodes were extracted and the DiD fluorescent signals were detected with IVIS.

In vivo DC maturation

To assess in vivo DC maturation, indicated formulations (at TCM and OMV protein weight of 100 and 1 μg respectively) were s.c. injected into the right flank of female C57BL/6 mice. After 24 h, the right inguinal lymph nodes were harvested, mechanically disrupted, and digested with 1% collagenase IV in complete DMEM medium for 30 min. After centrifugation at 652 g for 5 min, the pel-
leted cells were suspended in PBS for flow cytometric analysis of the expression of CD40, CD80, CD86, and MHC-II on DCs.

**Antigen-specific T cell generation analysis**

To determine the induction of antigen-specific T cell responses, female C57BL/6 mice were s.c. injected with 100 μL different formulations (at TCM and OMV protein weight of 100 and 1 μg, respectively) at the right flank on days 0, 7, and 14. TCMs were extracted from B16-OVA cells. On day 21, the right inguinal lymph nodes were harvested and mechanically disrupted. After centrifugation, the pelletted cells were stained with APC-anti-H-2K^d/OVA peptide (SIINFEKL) tetramer (NIH, USA), FITC-anti-CD8, and APC-Cy7-anti-CD3 for flow cytometric analysis.

**Prophylactic efficacy of BTS on B16F10 tumor**

To determine the protection efficacy conferred by BT immunization, female C57BL/6 mice were s.c. injected with 100 μL different formulations (at TCM and OMV protein weight of 100 and 1 μg, respectively) on days 0, 7, and 14. On day 21, these mice were s.c. inoculated with 1×10^6 B16F10 cells. Tumors were measured every other day and tumor volumes were calculated according to the formula: tumor volume = 0.5 × length × width^2. Either death of mouse or tumor volume greater than 2000 mm^3 were defined as the experimental endpoint.

**Immunological memory evaluation**

To test whether immunization with BTS@PLGA generated immunological memory, mice that remained tumor-free for more than 100 days after immunization with BTS@PLGA and subsequent B16F10 inoculation in the prophylactic experiment (tumor-free mice) were challenged with 1×10^6 B16F10 cells along with naive mice. Tumors were measured every other day.

To verify the specificity of the immune response elicited by BTS@PLGA immunization, B16F10 tumor-free mice and naive mice were inoculated with 5×10^5 MC38 colon cancer cells. Tumors were measured every other day.

**Therapeutic efficacy of BTS on B16F10 and MC38 tumors**

To study the therapeutic effect of BTS, female C57BL/6 mice were s.c. inoculated with 1×10^6 B16F10 cells or 2×10^5 MC38 cells on the right flank on day 0. These tumor-bearing mice were randomly divided into four groups: blank, BTS@PLGA, α-PD-1, and BTS@PLGA + α-PD-1. For the B16F10 tumor-bearing mice, on days 4, 8, 11, and 18, mice were s.c. injected with 100 μL BTS@PLGA (at TCM and OMV protein weight of 100 μg and 1 μg respectively) at the right abdomen. On days 5, 8, 12, 15, 19, and 22, mice were i.v. injected with 20 μg α-PD-1 (clone RMP1-14; BioXCell, West Lebanon, NH, USA). For the MC38 tumor-bearing mice, on days 4, 8, 15, and 22, mice were s.c. injected with 75 μL BTS@PLGA (at TCM and OMV protein weight of 150 μg and 1.5 μg respectively) at the right abdomen. On days 5, 9, 12, 16, 19, and 23, mice were i.v. injected with 100 μg α-PD-1 (clone RMP1-14, BioXCell). Tumors were measured every other day. Either death of mouse or tumor volume greater than 2000 mm^3 were defined as the experimental endpoint.

**Statistical analysis**

Data are expressed as the mean ± SEM or mean ± SD and analyzed using GraphPad Prism 8. Unpaired two-tailed Student’s t test and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to analyze the statistically significant differences and data were considered statistically significant when the values of *P < 0.05, *P < 0.01, **P < 0.001, and ****P < 0.0001. n.s., not significant. N.D., not detected.

**Credit authorship contribution statement**

**Min Li:** Conceptualization, Investigation, Visualization, Formal analysis, Writing - original draft. **Han Zhou:** Investigation, Visualization. **Wei Jiang:** Investigation, Visualization, Writing - original draft. **Chen Yang:** Visualization. **Hui Miao:** Visualization. **Yucai Wang:** Supervision, Funding acquisition, Writing - review & editing.

**Declaration of Competing Interest**

The authors reported no declarations of interest.

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**Supplementary material**

Supplemental material related to this article can be found, in the online version, at doi: [https://doi.org/10.1016/j.nantod.2020.101007](https://doi.org/10.1016/j.nantod.2020.101007).

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