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Nanocarriers based on bacterial membrane materials for cancer vaccine delivery

Xiao Zhao[1,2,3,5], Ruifang Zhao[1,2,4,5] and Guangjun Nie[1,2,4]✉

Here we present a protocol for the construction and use of two types of nanocarrier based on bacterial membrane materials for cancer vaccine delivery. Cancer vaccines induce tumor regression through triggering the specific T-cell responses against tumor neoantigens, a process that can be enhanced by nanocarrier delivery. Inspired by the body's natural immune defenses against bacterial invasion, we have developed two different types of nanocarrier based on bacterial membrane materials, which employ genetically engineered outer-membrane vesicles (OMVs), or hybrid membrane vesicles containing bacterial cytoplasmic membrane, respectively. The OMV-based nanocarriers can rapidly display different tumor antigens through the surface modified Plug-and-Display system, suitable for customized cancer vaccines when the tumor neoantigens can be identified. The hybrid membrane-based nanocarriers are prepared through fusion of the bacterial cytoplasmic membrane and the primary tumor cell membrane from surgically removed tumor tissues, possessing unique advantages as personalized cancer vaccines when the neoantigens are not readily available. Compared with chemically synthesized nanocarriers such as liposomes and polymer without intrinsic adjuvant properties, owing to the large amounts of pathogen-associated molecular patterns, the two nanocarriers can activate the antigen-presenting cells while delivering multiple antigens, thus inducing effective antigen presentation and robust adaptive immune activation. Excluding bacterial culture and tumor tissue collection, the preparation of OMV- and hybrid membrane-based nanocarriers takes ~8 h and 10 h for tumor vaccine construction, respectively. We also detail how to use these nanocarriers to create cancer nanovaccines and evaluate their immunostimulatory and antitumor effects.

Introduction

As an emerging class of immune-oncology therapy, therapeutic cancer vaccines have demonstrated robust tumor-specific immunogenicity and antitumor activity in patients with melanoma, glioblastoma and other cancers in the past decade[1–5]. Different from the previously developed cancer vaccines targeting tumor-associated antigens that overexpress in tumor cells, the recent cancer vaccines trigger specific immune responses against neoantigens that are produced by genetic mutations in tumor cells, thus potentially avoiding off-target effects[6,7]. However, the immunogenicity of tumor neoantigens alone is often disappointing, and the use of immune adjuvants and/or delivery vehicles is an effective approach to improve the immunogenicity[8,9]. Immune adjuvants can stimulate the antigen-presenting cells (APCs) to provide the necessary costimulatory signals for successful antigen presentation, while delivery vehicles can enhance the uptake and processing efficiency of neoantigens by APCs[10,11].

Currently, some promising nanocarriers with intrinsic immune adjuvant properties, such as polymeric and lipid nanoparticles with stimulator of interferon genes (STING) pathway activation ability, have been developed[12,13]. These nanocarriers can ensure that immune activation and antigen delivery occur in the same APCs, which is necessary for effective antigen presentation. Inspired by the body's natural immune defenses against bacterial invasion, our group has developed two different types of nanocarrier based on bacterial membrane materials for cancer vaccine delivery[14,15]. Owing to the large amounts of pathogen-associated molecular patterns (PAMPs), the bacterial membrane materials can act as excellent nanocarriers with intrinsic immune adjuvant properties[16–18]. To make these nanocarriers more suitable for use in cancer vaccines, two different loading methods for known

[1]CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, China, Beijing, China. [2]Center of Materials Science and Optoelectronics Engineering, University of Chinese Academy of Sciences, Beijing, China. [3]IGDB-NCNST Joint Research Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. [4]GBA Research Innovation Institute for Nanotechnology, Guangzhou, China. [5]These authors contributed equally: Xiao Zhao, Ruifang Zhao. ✉e-mail: niegj@nanoctr.cn

Table 1 | Properties and applications of two types of nanocarrier based on bacterial membrane materials for cancer vaccines

	OMV-based nanocarriers	HM-NPs
Bacterial components	OMVs (mainly outer membrane)	Cytoplasmic membrane
TLR activation	TLRs 2, 4 and 5	TLRs 1, 2 and 6
Preparation method	Rapid antigen display via the Plug-and-Display system	Utilization of autologous tumor antigens via membrane fusion technology
Application	Customized cancer vaccines for metastatic and primary tumor treatment	Personalized cancer vaccines for the prevention of postoperative recurrence
Acquisition of tumor antigen	Synthesis of known tumor antigen	Unknown whole-tumor antigen on cell membrane

and unknown tumor neoantigens have been applied for different application purposes, respectively (Table 1).

In the first study, the bacteria-derived outer-membrane vesicles (OMVs) were extracted through ultracentrifugation, which are the natural nanosized vesicles secreted by Gram-negative bacteria (Fig. 1a)[19,20]. To simplify the display of tumor neoantigens on OMVs, we employed a Plug-and-Display system to decorate OMVs through genetic engineering (Fig. 1a)[14]. The Plug-and-Display system comprises the tag/catcher protein pairs, and the protein catchers were fused with the surface protein ClyA on OMVs[21,22]. Then, the OMVs decorated with different protein catchers can simultaneously display multiple tumor neoantigens labeled with the corresponding tags, thereby rapidly constructing the tumor nanovaccines (Fig. 1a). These OMV-based nanovaccines have been used to inhibit tumor growth and metastasis, which are more suitable for customized design when the tumor neoantigens have been identified and/or can be identified. According to the identified antigen information, the individualized matched neoantigens were synthesized and displayed on the OMV-based nanocarriers to create antigen-suited nanovaccines for patients with cancer.

In the second study, we developed hybrid membrane-based nanocarriers (HM-NPs), containing the bacterial cytoplasmic membrane from *Escherichia coli* (EM) and tumor cell membrane (TM) from the resected autologous tumor tissues (Fig. 1b)[15]. Briefly, the poly(lactic-co-glycolic acid) (PLGA) nanoparticles were constructed as a core, and the EM and TM were simultaneously coated onto the PLGA nanoparticles to form the final HM-NPs (Fig. 1b). The EM acts as the immune adjuvant to activate the innate immune response, and the autologous tumor neoantigens in the TM are the key messengers for transmitting the tumor-specific identification to the adaptive immune system[23–26]. These HM-NPs possess unique advantages as personalized cancer vaccines when the tumor neoantigens are not readily available and are more suitable for prevention of postoperative recurrence.

Here we provide protocols for the construction of these two types of vaccine based on bacterial membrane materials and the matched tumor antigens from different sources, and the potential applications and limitations of the nanovaccines are discussed. The characterization methods are validated and presented in detail, and the antigen delivery effect and immune stimulation mechanisms were validated both in vitro and in vivo.

Development of the protocol

Development of OMV-based nanocarriers and vaccines

Owing to their excellent immunostimulatory ability, OMVs are attractive candidates as vaccine carriers in the field of prophylactic vaccines against pathogenic microorganisms[20]. For example, the OMV-based vaccines against Group B meningococcus, namely MeNZB, have effectively decreased the incidence and mortality of meningitis in New Zealand[27]. In addition to preventing the invasion of their parental microorganisms, OMVs can be employed as a display platform of heterologous antigens to form vaccines against other pathogens through genetic engineering, chemical bonding and physical mixing[28]. To exploit OMVs as nanocarriers for cancer vaccines, the loading method of tumor neoantigens is the key issue. First, we adopted fusion expression to display a pattern antigenic

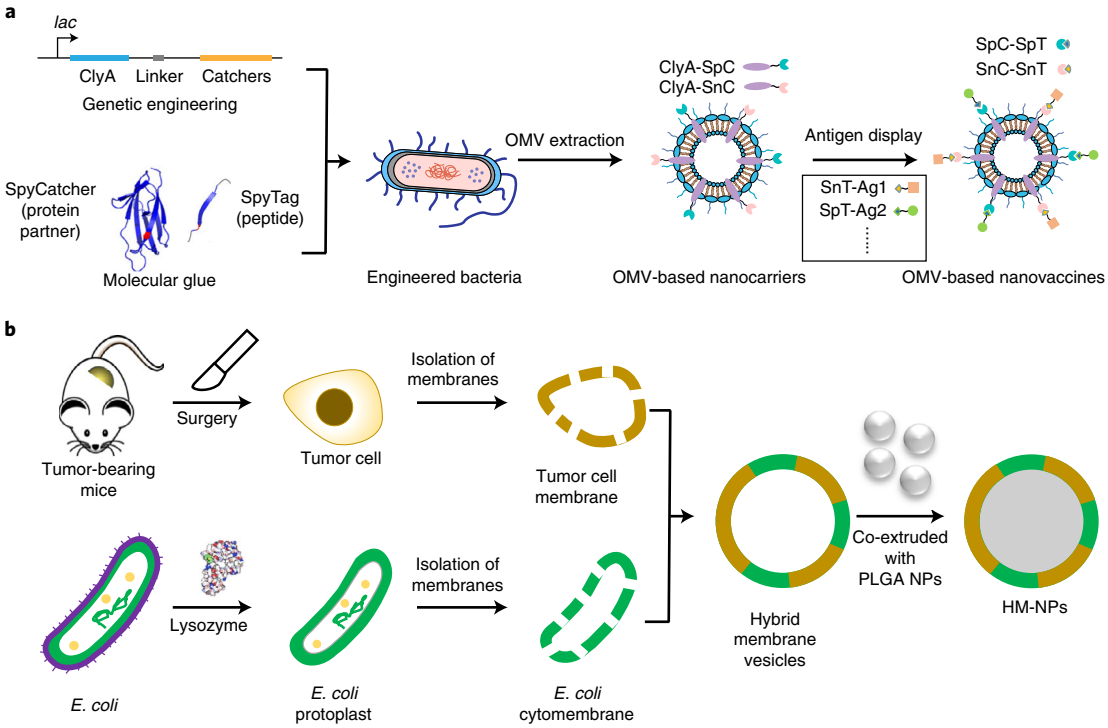


Fig. 1 | Preparation of OMV- and HM-NPs for cancer vaccines. a, Preparation of OMV-based nanocarriers. The catchers (SpC and SnC) in the Plug-and-Display system are fused with the ClyA protein (ClyA-catchers) on OMVs' surface, through *E. coli* genetic engineering. Then, the engineered bacteria-derived OMVs are extracted through ultracentrifugation. The OMVs decorated with two different catchers can simultaneously display multiple tumor neoantigens (Ag) labeled with corresponding tags (SpT and/or SnT), thereby rapidly constructing the antitumor nanovaccines. **b**, Preparation of HM-NPs. Tumors are surgically removed from tumor-bearing mice to obtain TM. *E. coli* are treated with lysozyme to remove the cell wall to generate protoplasts, and the cytomembrane is extracted with an extraction buffer to prepare *E. coli* cytomembrane (EM). HM vesicles are generated by mixing EM and TM, followed by extruding through an extruder. PLGA nanoparticles (NPs) as polymeric cores are then added to generate HM-NPs. Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0 (**a**) and ref. [15], AAAS (**b**).

epitope of ovalbumin (OVA), $OVA_{257–264}$ (SIINFEKL) on the OMVs' surface, in which $OVA_{257–264}$ was fused with the C terminal of the surface protein ClyA (ClyA-OVA) on OMVs[29]. The strong antigen-specific immune response and antitumor effect induced by ClyA-OVA OMVs indicated that OMVs are outstanding vaccine nanocarriers for displaying tumor neoantigens to elicit antitumor immunity.

However, the tumor neoantigens generated from the gene mutations have high heterogeneity and variability[30], making it impractical and time-consuming to produce OMV-based nanovaccines for every patient via fusion expression with neoantigens. The antigen display of tumor vaccine carriers should be rapid and flexible to meet the clinical requirement. Therefore, we employed the Plug-and-Display system to decorate OMVs, including a SpyTag (SpT)/SpyCatcher (SpC) pair and a SnoopTag (SnT)/SnoopCatcher (SnC) pair[21,22]. The SpC and SnC catchers were fused with the ClyA protein (ClyA-catchers, CC) on OMVs' surface and can spontaneously bind to the neoantigens labeled with SpT and SnT tags through isopeptide bond formation, respectively. These bioengineered CC OMVs were able to simultaneously and rapidly display multiple tumor neoantigens, thereby being competent for rapid preparation of the customized cancer vaccines when tumor antigens can be predicted and identified.

Development of HM-NPs and vaccines

Surgical resection is the primary treatment option for most solid tumors, and the recurrence and metastasis after surgery are still unmet clinical challenges[31,32]. Vaccination after surgery to induce the antigen-specific immune responses to eliminate residual tumor cells may have wide application[33]. TMs contain a high proportion of antigenic motifs and have similar antigen patterns as are displayed on the surface of tumor cells in vivo[34,35]. However, autologous tumor antigens can be recognized as 'self', and they are more likely to induce antigen-specific tolerance rather than antitumor immunity[36]. The body's immune defenses against bacterial invasion are rather sensitive; utilizing bacterial

constituents that act as adjuvants to enhance immunogenicity is a promising strategy to overcome this limitation.

However, lipopolysaccharide (LPS) and other cell wall components of bacteria as the first line of contact with immune cells could cause an undesirable immunopathological state[23]. The bacterial cytoplasmic membrane, which is spatially separated from the organism's cell wall, may be used as a potential adjuvant to reduce the magnitude of 'danger signals'[17,18]. Membrane fusion is a technology that can confer a hybrid membrane (HM) with properties inherited from two source cell membranes[24–26]. Thus, we developed the immunotherapy strategy using bacterial cytoplasmic membranes from *E. coli* and autologous tumor membranes from resected tumor tissue to confer a HM with the properties inherited from two source cell membranes[15]. These HM-NPs were able to codeliver antigen and adjuvant to APCs.

Overview of the procedures

First, we describe the preparation of the two types of nanocarrier based on bacterial membrane materials and the related nanovaccine constructions (Procedure 1, Steps 1–18 for OMV-based nanocarriers; Procedure 2, Steps 1–38 for HM-NPs). Next, the methods and processes for characterization of physicochemical properties and biological components are described in detail (Procedure 1, Steps 19–30 for OMV-based nanocarriers; Procedure 2, Step 39 for HM-NPs). Then, the antigen delivery efficiency and the immune response analysis are performed (Procedure 1, Steps 31–48 for OMV-based nanocarriers; Procedure 2, Step 40 for HM-NPs). Finally, we discuss the strategies to evaluate the in vivo antitumor effect in different mouse models using multiple antigens (Procedure 1, Step 49 for OMV-based nanocarriers; Procedure 2, Step 41 for HM-NPs). More detailed information on the two procedures is provided below.

Procedure 1: OMV-based nanocarriers and vaccines

Through genetic engineering, we splice the gene fragments fusing SpC or SnC and ClyA (ClyA-SpC and ClyA-SnC) respectively, and insert them into the co-expression plasmid pETDuet-1. Then, the co-expression plasmid is transformed into the engineered bacteria *E. coli* Rosetta (DE3). Finally, CC OMVs are extracted from the culture medium in massive quantities by bacterial fermentation and ultracentrifugation, followed by antigen display, vaccine preparation and functional characterization. In this protocol, the size and morphology of CC OMVs are characterized using dynamic light scattering (DLS) and transmission electron microscopy (TEM), respectively. The antigen display ability is evaluated by western blot. Finally, CC OMVs are used to display multiple tumor antigens including $OVA_{257–264}$ (termed 'OTI', an epitope that can stimulate the production of MHC class I-restricted OVA-specific $CD8^+$ T cells in mice), $OVA_{223–339}$ (ISQAVHAAHAEINEAGR; 'OTII', an epitope that can stimulate the production of MHC class II-restricted OVA-specific $CD4^+$ T cells in mice), an antigenic epitope of tyrosinase-related protein 2 (TRP2), $TRP2_{180–188}$ (SVYDFFVWL) and an antigenic epitope of Adpgk (CGIPVHLELASMTNMELMSSIVHQQVFPT)[37,38]. The in vitro and in vivo immune response and antitumor effect are determined in the pulmonary metastatic melanoma model and subcutaneous colon cancer model.

Procedure 2: HM-NPs and vaccines

HM-NPs are synthesized by preparation of the HM of EM and TM and coating the HM onto the synthesized PLGA nanoparticles. In this protocol, the size and morphology of HM-NPs are characterized using DLS and TEM, respectively. Immunogold staining for specific markers to identify EM and TM on HM-NPs is also observed by TEM. The enhanced tumor antigen uptake and maturation of bone marrow-derived dendritic cells (BMDCs) by HM-NPs are evaluated by flow cytometry and enzyme-linked immunosorbent assay (ELISA). The mouse tumor models of 4T1, CT26 and B16-F10 cells are then used to investigate the antitumor immune effects of HM-NPs.

Applications of the method

Potential applications of OMV-based strategy for vaccine development

In this protocol, we used the OMV-based nanocarriers to rapidly display multiple antigens, including OTI, OTII, $TRP2_{180–188}$ and Adpgk. Of course, this platform can be also employed to deliver any other antigenic peptide, including human and murine antigens. Because of the high compatibility, this platform is suitable for the preparation of customized cancer vaccines for each patient with cancer. In the future, if a tumor antigen library is established, we can quickly identify the antigen

information of patients with tumors by gene sequencing, so as to make use of the rapid antigen display capability of this platform for the preparation of customized cancer vaccines. In addition, OMV-based nanocarriers can also be used for rapid screening and identification of tumor antigens owing to their excellent immune stimulation, antigen transport and rapid antigen display capabilities.

As mentioned earlier, previous studies have focused on using OMVs to build vaccines against microorganisms. Therefore, in addition to cancer vaccines, prophylactic vaccines against infectious diseases can also use our OMV-based nanocarriers, especially for pathogenic microorganisms with highly variable antigens, such as influenza virus.

Potential applications of HM-based strategy for vaccine development

The HM-NPs are integrated into a vaccine delivery nanoplatform with both bacterial cytoplasmic membranes and surgically derived TM to enhance both innate and adaptive immune responses. This HM-derived vaccine strategy is beneficial to multiple tumor models by codelivery of individualized tumor antigens and adjuvants into dendritic cells (DCs). In addition, these HM-NPs can be genetically or chemically modified to further expand their multifunctionality. The core of the nanocarriers can also load various cargos for integration of other treatment modalities to enhance the effects of immunotherapy.

Comparison with other methods

Several materials have been used to develop nanocarriers for tumor vaccine delivery, such as lipid, polymer, synthetic high-density lipoprotein and DNA origami[39–44]. Compared with these materials, the bacterial membrane material-based nanocarriers have a unique immune stimulation function and play the dual roles of immune adjuvants and antigen carriers. In addition, compared with the complexity of chemical synthesis, bacterial membrane material-based nanocarriers can be obtained in relatively large quantities through bacterial fermentation. This feature may ensure compatibility with industrial production in the future.

In the first Procedure, the Plug-and-Display system is employed to achieve the rapid display of peptide antigens on OMVs. The current clinical trials of tumor vaccines based on peptide antigens are mainly conducted by using a mixture of peptides and adjuvants[1,3–5]. Nanocarriers can improve stability and immune system targeting of the antigens and realize the codelivery of antigens and adjuvants[9,10]. Therefore, the tumor vaccines based on nanocarriers have received more and more attention[9,10]. As mentioned above, the bacteria-derived OMVs have the dual functions of both carriers and adjuvants, while the chemical synthetic nanocarriers are mainly delivery vehicles. Therefore, the nanovaccines based on OMVs do not require additional adjuvants. In addition, our study demonstrated that the OMV-based nanovaccines can activate an antigen-specific T-cell response, as distinct from the previously reported B-cell-mediated antibody responses in the OMV-based prophylactic vaccines against pathogenic microorganisms[20,27].

More importantly, we employed the Plug-and-Display system to achieve the rapid display of multiple peptide antigens. Compared with the fusion expression, physical encapsulation or chemical conjugation, this flexible approach of antigen display makes our OMV-based nanocarriers more suitable for rapid preparation of customized cancer vaccines. The Plug-and-Display system has been used in the construction of vaccines against microorganisms based on virus-like particle[45–47]. We firstly demonstrate the feasibility of this system for constructions of tumor vaccines. Compared with virus-like particle, OMVs have the advantage of immune adjuvant function, despite the disadvantage of complex composition. Furthermore, the current practice for antigen encapsulation into the nanoparticles during the production process lacks flexibility to load multiple antigens under different scenarios[41,43]. The vehicle and antigen(s) in our nanovaccine can be separately synthesized and used to rapidly prepare the vaccine through a simple combination procedure before immunization. This modular design allows one to establish a neoantigen library in advance and rapidly select appropriate antigens for individual patients, which may reduce the production time and realize the bedside preparation of tumor vaccines for individual patients in the future[14].

In the second Procedure, the cancer vaccines are constructed through hybrid fusion of the surgically removed TM and the bacterial cytoplasmic membranes. Traditional methods of autologous tumor cell-based vaccines use whole-cell tumor vaccines or tumor lysate vaccines to elicit immunity against the entire collection of antigens expressed by the tumor[33,48,49]. Although whole-cell tumor or tumor lysate vaccines open a promising area for cancer immunotherapy, therapeutic efficacy may be severely limited as the immunogenicity of antigens in these vaccine formulations may be diluted or

inhibited by the unrelated proteins and nucleic acids. The current HM tumor vaccines are constructed through the fusion of the surgically removed TM and the bacterial cytoplasmic membranes, possessing unique advantages as personalized cancer vaccines with highly enriched antigens when the identified ones are not sufficient to trigger antitumor immunity or the neoantigens are not readily available.

Although TM are naturally enriched with a specific tumor antigen pool, these membrane antigens usually have low immunogenicity since tumorigenesis implies adaptation of tumor cells to the host immune system. Compared with the other tumor membrane-based nanocarriers, the introduction of the bacterial cytoplasmic membranes enhances the recognition of tumor membrane antigens by immune system. Meanwhile, bacterial cytoplasmic membranes also take advantage of the antibody-dependent, cell-mediated cytotoxicity of NK cells, which lyse malignant cells even before the stimulation of specific T cells in an antigen-independent manner. Bacteria-derived substances (inactivated or attenuated bacteria, lipids, proteins and/or nucleic acids) provide 'danger signals' that alert the immune system to the potential infection and invasion[17]. However, bacteria-derived formulations, especially LPS, can lead to severe side effects, such as cytokine storm and sepsis[18]. Using bacterial cytoplasmic membranes can reduce the magnitude of 'danger signals' since most of the bacterial LPS is removed. Meanwhile, the bacterial cytoplasmic membranes can be rapidly and manageably prepared from inexpensive cultures of bacteria. This vaccine formulation protocol provides an appropriate balance among accessibility, safety and effectiveness.

Limitations

Some limitations of OMV-based nanocarriers for cancer vaccines should be considered. First, the OMVs contain large amounts of proteins, polysaccharides, lipids and trace amounts of nucleic acids from bacteria, and the complicated components may cause difficulty for the quality control of vaccine production. In this Procedure, we use DLS to detect the particle size and TEM to observe the morphology for quality control of OMVs. The ideal OMVs should have a low polydispersity index (PDI, <0.2). Another quality control from the production aspect of OMV-based products may be the presence of some characteristic proteins in the OMVs, such as outer-membrane protein A/C/F, which can be used as the fingerprint for OMV identification[20]. Second, the amount of LPS in OMVs is the main component of endotoxin, and the elimination of LPS in OMVs by genetic engineering is a feasible option for further improvement[50]. Third, the antigens used in this protocol include model antigens and murine tumor antigens, and the adaptability of this platform to human tumor antigens needs further study. Fourth, ultracentrifugation is used to isolate and purify OMVs in this protocol, but this method may not be suitable for mass production. Appropriate purification methods for industrial practices are in urgent need, and chromatographic separation according to the size of OMVs is an optional solution. Finally, the Plug-and-Display system used to create CC OMVs may be further optimized. In addition to ClyA protein, there are some other surface proteins on OMVs that can be employed as scaffold for the Plug-and-Display system, such as hemoglobin protease and outer-membrane protein A/C/F[20]. In addition, updated versions of the Plug-and-Display system have been described, such as spycatcher003, with a stronger integration efficiency[51]. These novel scaffolds and Plug-and-Display systems should be further evaluated to determine whether they will be able to enhance the antigen display capability of the OMV-based nanocarriers.

One limitation of HM nanovaccines is that the immunosuppressive proteins of the TM have not been investigated. As tumor cells interact with the infiltration of immune cells and form the immunosuppressive environment, there is a high portion of immunosuppressive proteins. For example, the well-known immune checkpoint inhibitor, programmed cell death 1 ligand (PD-L1), is located in the TM[52]. Enhanced antitumor efficacy may be achieved by regulating these immunosuppressive factors. In addition, the contribution of other cell membranes to the antitumor immunity should be considered, since immune cells and stromal cells in the resected tumor tissue are not eliminated during HM nanovaccine preparation. Tumor cells could firstly be isolated and purified by the negative selection-based tumor cell isolation kit or culture plate adherent passaging, then the effectiveness of HM vaccines prepared from purified TM could be assessed in comparison. The fabricating methods could also be improved by the use of microfluidic methods instead of manual preparation and procedures. Last but not least, specific antigens on the TM recognized by the T cells are not yet identified. All these factors could either limit or benefit translation to the clinic.

In addition, the sterile production of the OMV-based nanocarriers and HM-NPs requires special attention. In the Procedure for the OMV-based nanocarriers, almost all the bacteria were removed by

centrifugation at 5,000*g* for 10 min. In the following steps to separate OMVs from the supernatant, two filtration processes were carried out through 0.45 μm and 0.22 μm filters to ensure that the final OMVs did not contain bacteria. In the Procedure for the HM-NPs, the lysozyme and extraction buffer can lyse almost all the bacteria to obtain the cytoplasmic membranes of *E. coli*. In addition, in the following steps to prepare the HM-coated nanoparticles, the HM were extruded at least 13 times through the 400 and 200 nm cutoff sterile extruders, respectively. Therefore, the final HM-NPs do not contain bacteria. For future industrial-scale preparation, radiation sterilization may be suitable for large-scale production of the OMV-based nanocarriers and HM-NPs.

Experimental design

Selecting tumor antigens

In our first Procedure, we adopt several model antigens (OTI and OTII) and murine tumor antigens ($TRP2_{180–188}$ and Adpgk) to make tumor vaccines against different cancer cells. Other tumor antigens can be selected according to the cancer cell type. For example, the gp100 antigen can be used to establish a cancer vaccine against B16-F10 melanoma cells, and the gp70 antigen is suitable for the treatment of CT26 colon cancer cells[53,54]. In addition, the combination of mass spectrometry and exome sequencing can effectively predict and identify neoantigens in tumor cells, which lays the foundation for customized cancer vaccines[38]. These identified tumor antigens can also be used in the experiments according to our protocol. Once the tumor antigens are selected, the SpT or SnT tag needs to be added at the N terminal, and the tag-labeled antigen peptides are synthesized via the Fmoc solid-phase peptide synthesis method. When there is only one type of tumor antigen, either SpC–SpT or SnC–SnT pair can be used for rapid display, and there is no notable difference between them. If there are two types of tumor antigen, it is recommended to use different pairs to display, so as to increase the efficiency of display. If there are more than two types of tumor antigen, we recommend that each antigen can be displayed separately using either SpC–SpT or SnC–SnT pair to construct different nanovaccines, and eventually mix together for immunization.

Selecting murine cancer models and mouse strains

In our two Procedures, several murine cancer models are used, including B16-F10 and OVA-expressing B16-F10 (B16-OVA) melanoma, MC38 and CT26 colon cancer and 4T1 breast cancer. According to the origin of the tumor cells, different mice were used to make tumor models, including BALB/c mice for CT26 and 4T1 and C57BL/6 mice for B16-F10, B16-OVA and MC38. This protocol is also suitable for use with other murine tumor models. If the tumor vaccine is to be built against human tumor cells, mice with a humanized immune system should be used.

Design of controls

In the first Procedure, for OMV-based nanocarriers, the critical control group is a mixture of CN OMVs (from the bacteria expressing ClyA without the fused Catchers, ClyA-none) and tumor antigens, thus proving the importance of antigen display on CC OMVs for efficient immune stimulation. The control groups of separate antigens or separate empty CC OMVs are also necessary to illustrate the advantages of the system. If multiple antigens are displayed on the surface of CC OMVs to prepare tumor vaccines, control vaccines containing individual antigens should also be evaluated to determine synergistic effect between different antigens.

In the second Procedure, for HM-NPs, the critical control groups include the EM-coated nanoparticles (EM-NPs) and TM-coated nanoparticles (TM-NPs), and the physical mixture of two types of NPs (Mix NPs). The codelivery ones (HM-NPs) should result in a synergistic stimulation of an immune response, compared with the individual antigen and adjuvant nanoparticles or mixed ones.

Materials

Biological materials: Procedure 1

- Bacterial strain: *E. coli* Rosetta (DE3; Tiangen Biotech Beijing, cat. no. CB108-02). In this bacterial strain, the T7-lac promoter can be activated by adding the expression inducer isopropyl-β-D-thiogalactoside (IPTG). The competent cells of *E. coli* Rosetta (DE3) are purchased from Tiangen Biotech and stored at −80 °C until use **! CAUTION** If you are concerned about LPS in OMVs, you can

Table 2 | The DNA sequences encoding ClyA-SpC and the ClyA-SnC

ClyA-Catchers	Sequence
ClyA-SpC	CC**ATG**GGCATGACTGAAATCGTCGCGGATAAAACCGTTGAGGTGGTGAAAAACGCAATCGAAACCGCTGATGGCGC TCTGGATCTGTATAACAAATACCTGGACCAGGTTATTCCGTGGCAGACTTTTGATGAAACCATTAAAGAACTGTCC CGCTTCAAACAGGAATACTCTCAGGCGGCGTCCGTACTGGTAGGCGACATCAAAACTCTGCTGATGGACTCCCAGGA CAAATACTTCGAAGCCACTCAGACCGTTTACGAGTGGTGCGGTGTTGCTACCCAGCTGCTGGCTGCATACATTCTGCT GTTCGACGAATATAATGAGAAAAAAGCGTCTGCTCAAAAAGACATCCTGATCAAAGTTCTGGATGATGGTATCACT AAGCTGAACGAAGCGCAGAAAAGCCTGCTGGTTAGCAGCCAGAGCTTCAACAACGCGTCTGGTAAGCTGCTGGCTC TGGATTCCCAGCTGACCAACGATTTCTCCGAAAAATCTTCCTACTTCCAGTCCCAGGTAGACAAAATCCGCAAAGAA GCTTACGCTGGCGCAGCTGCTGGTGTTGTAGCAGGTCCGTTCGGTCTGATCATTAGCTATTCTATTGCTGCAGGTGTGGT CGAGGGCAAACTGATTCCGGAGCTGAAAAACAAACTGAAATCTGTACAAAACTTCTTTACCACCCTGTCTAACACT GTTAAACAGGCGAACAAAGACATCGATGCGGCAAAACTGAAACTGACCACCGAGATCGCGGCCATCGGTGAAATC AAAACTGAAACCGAAACCACCCGTTTTTACGTTGATTACGACGACCTGATGCTGAGCCTGCTGAAAGAGGCAGCGAA AAAAATGATCAACACTTGTAACGAATACCAGAAACGTCACGGTAAAAAAACCCTGTTTGAAGTGCCGGAAGTAGGCT CCTCTATGGTTACCACCCTGTCTGGTCTGTCTGGTGAACAGGGTCCATCTGGCGATATGACCACCGAAGAGGACTC CGCCACTCATATTAAGTTCTCCAAGCGTGACGAAGATGGTCGTGAGCTGGCTGGCGCAACTATGGAACTGCGCGA CAGCTCTGGTAAGACCATTTCTACCTGGATCAGCGATGGTCACGTCAAAGACTTCTACCTGTACCCGGGCAAATAC ACCTTCGTAGAGACTGCAGCGCCGGATGGCTACGAAGTGGCAACCGCTATTACGTTCACCGTCAACGAACAGGGTC AGGTGACTGTTAACGGCGAAGCGACCAAAGGTGACGCTCACACCGGTTCTTCTGGTAGCTAAGTCGAC
ClyA-SnC	CAT**ATG**ACGGAGATCGTAGCAGACAAGACGGTAGAAGTTGTAAAAAACGCAATCGAAACCGCTGATGGCGCGCTGG ACCTGTATAACAAATACCTGGACCAGGTCATTCCGTGGCAAACTTTCGACGAAACCATCAAAGAACTGTCTCGCTTC AAACAGGAATACTCCCAGGCTGCATCTGTCCTGGTGGGCGATATCAAGACCCTGCTGATGGACTCTCAGGACAAAT ATTTCGAAGCGACCCAGACCGTTTACGAATGGTGCGGTGTAGCAACCCAGCTGCTGGCCGCTTACATCCTGCTGTT CGACGAATATAACGAAAAAAAGGCATCCGCGCAGAAAGACATTCTGATCAAGGTTCTGGACGATGGCATCACCAAA CTGAACGAAGCGCAGAAGTCCCTGCTGGTTAGCTCCCAGTCCTTCAATAACGCATCTGGTAAACTGCTGGCTCTGGAC TCCCAACTGACCAACGACTTTAGCGAGAAATCCTCTTACTTCCAATCTCAGGTTGATAAAATCCGTAAAGAAGCTTAC GCTGGTGCAGCAGCTGGTGTTGTAGCGGGTCCATTCGGTCTGATCATCAGCTACAGCATCGCAGCTGGCGTTGTAGA AGGCAAACTGATCCCGGAACTGAAAAACAAACTGAAGTCTGTTCAGAACTTCTTCACTACCCTGTCTAACACCGTTA AACAGGCCAACAAAGACATCGACGCGGCTAAACTGAAACTGACCACCGAAATCGCCGCCATCGGTGAAATTAAAACTGA GACCGAAACTACTCGCTTCTACGTTGACTACGACGACCTGATGCTGTCTCTGCTGAAAGAAGCGGCTAAAAAAATGATT AACACTTGTAACGAGTACCAGAAACGTCACGGCAAAAAAACCCTGTTTGAGGTTCCGGAAGTTGGCTCCTCCAAACC GCTGCGTGGTGCAGTGTTCAGCCTGCAGAAACAGCACCCGGATTATCCGGACATCTATGGTGCGATCGACCAGAAC GGTACTTATCAGAACGTTCGCACCGGTGAAGACGGCAAACTGACCTTCAAAAATCTGTCTGATGGCAAATATCGCCTG TTTGAGAACTCTGAACCTGCGGGTTACAAACCGGTTCAGAACAAGCCGATCGTTGCGTTTCAGATCGTTAACGGTG AAGTGCGCGATGTTACCTCCATTGTGCCACAGGATATCCCGGCCACTTACGAGTTCACTAACGGCAAGCACTATATT ACGAACGAACCAATTCCGCCAAAATAAGGTACC

The initiation codons are highlighted in bold, and the restriction enzyme cutting sites of NcoI, SalI, NdeI and KpnI enzymes are underlined.

use the ClearColi BL21 (DE3) (Lucigen, cat. no. 60810-1), in which LPS is genetically modified to not trigger the endotoxic response in human cells.

Plasmid

- pETDuet-ClyA-Catchers (synthesized by Suzhou Genewiz). The DNA fragments encoding the ClyA-SpC and ClyA-SnC (Table 2) are inserted into the backbone plasmid pETDuet-1 simultaneously to construct the co-expression plasmid pETDuet-ClyA-Catchers. The restriction enzymes NcoI and SalI are used for the ClyA-SpC insertion, and the restriction enzymes NdeI and KpnI are used for the ClyA-SnC insertion

Mice

- C57BL/6 mice (6–8 weeks old; Vital River Laboratory Animal Technology). Mice are housed in a room at 20–22 °C with a 12-h light/dark cycle and a humidity of 30–70%, and provided food and water ad libitum ▲ **CRITICAL** All animal protocols were approved by the Institutional Animal Care and Use Committee of the National Center for Nanoscience and Technology. ! **CAUTION** The mouse strain should be determined according to the source of tumor antigens and tumor cells in your experiments, such as using the C57BL/6 and BALB/c mice for the B16-F10 and CT26 cells, respectively. The sex and sex chromosomes do not affect the tumor growth or immune activation of vaccines. However, some sex-hormone-dependent tumors should mainly be used in mice of the corresponding sex, such as female mice for breast cancer and male mice for prostate cancer. In addition, we recommend the use of female mice because their body weight is relatively stable, which make it easy to control the dose–weight ratio.

Cell lines

- B16-F10 murine melanoma cell line (ATCC, cat. no. CRL−6475, RRID: CVCL_0159, sex chromosomes: XY)
- MC38 murine colon cancer cell line (CRC cat. no. 1101MOU-PUMC000523, RRID: CVCL_B288, sex chromosomes: XX), Cell Resource Center, IBMS, CAMS/PUMC
- B16-OVA cell (provided by Wang Hao at the National Center for Nanoscience and Technology)

! CAUTION All cell lines are validated with cell line authentication by the short tandem-repeat DNA profiling and mycoplasma contamination detection.

Procedure 2

Mice

- C57BL/6 and BALB/c Mice (6–8 weeks old; Vital River Laboratory Animal Technology) and maintained under pathogen-free conditions **! CAUTION** Sex and strain choice of mice is recommended as described above for Procedure 1.

Bacteria

- *E. coli* DH5α (Invitrogen, cat. no. 18265017)

Cell lines

- Murine 4T1 breast tumor cell (ATCC, cat. no. CRL−2539, RRID: CVCL_0125, sex chromosomes: XX)
- Murine B16-F10 melanoma cell (ATCC cat. no. CRL-6475, RRID: CVCL_0159, sex chromosomes: XY)
- Murine CT26 colon carcinoma cell (ATCC cat. no. CRL-2638, RRID: CVCL_7256, sex chromosomes: unknown)

Reagents

▲ CRITICAL All reagents mentioned in this protocol are stored and prepared according to the manufacturer's recommendations.

Procedure 1

- Tryptone (Solarbio, cat. no. T8490)
- Yeast extract (Solarbio, cat. no. Y8020)
- NaCl (Solarbio, cat. no. S8210)
- Agar (Solarbio, cat. no. A8190)
- Ampicillin (Amresco, cat. no. 0339)
- IPTG (Biosharp, cat. no. BS119)
- Phosphate-buffered saline (PBS; Wisent, cat. no. 311-010-CL)
- Fetal bovine serum (FBS; Wisent, cat. no. 085-150)
- RMPI 1640 medium (Wisent, cat. no. 350-000-CL)
- Penicillin G and streptomycin (Wisent, cat. no. 450-201-EL)
- HEPES, 1 M, pH 7.4 (Sigma-Aldrich, cat. no. 83264)
- β-Mercaptoethanol (β-ME; Gibco, cat. no. 21985023)
- IL-4 (Peprotech, cat. no. 214−14)
- Granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, cat. no. 315-03)
- Matrigel (BD, cat. no. 354234)
- Ammonium chloride potassium (ACK) lysing buffer (Solarbio, cat. no. R1010)
- Monensin (BioGems, cat. no. 2237803)
- Phorbol 12-myristate 13-acetate (PMA; BioGems, cat. no. 1652981)
- Ionomycin (BioGems, cat. no. 5608212)
- RIPA buffer (Solarbio, cat. no. R0010)
- Pierce BCA Protein Assay Kit (Thermo, cat. no. 23227)
- Anti-HA tag antibody (Abcam, cat. no. ab236632, Clone: EPR22819-101, RRID: AB_2864361)
- HRP-conjugated secondary antibody, goat anti-rabbit IgG (Invitrogen, cat. no. A16096, Clone: polyclone, RRID: AB_2534770)
- SnT-TRP2 peptide (GKLGDIEFIKVNKGYGGSVYDFFVWL, synthesized by Top Peptide)
- SpT-OTI peptide (VPTIVMVDAYKRYKGGSIINFEKL, synthesized by Top Peptide)
- SnT-OTII peptide (GKLGDIEFIKVNKGYGGISQAVHAAHAEINEAGR, synthesized by Top Peptide)
- SpT-Adpgk peptide (VPTIVMVDAYKRYKGGCGIPVHLELASMTNMELMSSIVHQQVFPT, synthesized by Top Peptide)

- TRP2 peptide (SVYDFFVWL, synthesized by Top Peptide)
- OTI peptide (SIINFEKL, synthesized by Top Peptide)
- OTII peptide (ISQAVHAAHAEINEAGR, synthesized by Top Peptide)
- Adpgk peptide (CGIPVHLELASMTNMELMSSIVHQQVFPT, synthesized by Top Peptide)
- Uranyl acetate staining solution (3%, Beijing Zhongjingkeyi Technology, cat. no. GZ02625)
- FITC-anti-mouse CD11c (BioLegend, cat. no. 117306, Clone: N418, RRID: AB_313775)
- PE/Cy7-anti-mouse CD80 (BioLegend, cat. no. 104734, Clone: 16-10A1, RRID: AB_2563112)
- APC-anti-mouse CD86 (BioLegend, cat. no. 105012, Clone: GL-1, RRID: AB_493342)
- PE/Cy7-anti-mouse H-2Kb bound to SIINFEKL (BioLegend, cat. no. 141608, Clone: 25-D1.16, RRID: AB_11218593)
- FITC-anti-mouse CD3 (BioLegend, cat. no. 100204, Clone: 17A2, RRID: AB_312661)
- APC-anti-mouse CD8 (BioLegend, cat. no. 100712, Clone: 53-6.7, RRID: AB_312751)
- PE/Cy7-anti-mouse IFNγ (BioLegend, cat. no. 505826, Clone: XMG1.2, RRID: AB_2295770)
- PE-anti-mouse CD4 (Invitrogen, cat. no. 2013481, Clone: RM4-5)
- APC-anti-mouse CD3 (BioLegend, cat. no. 100236, Clone: 17A2, RRID: AB_2561456)
- FITC-anti-mouse CD8 (Invitrogen, cat. no. 2002714, Clone: 53-6.7)
- FITC-anti-mouse CD4 (BioLegend, cat. no. 100406, Clone: GK1.5, RRID: AB_312691)
- PE-anti-mouse Foxp3 (BioLegend, cat. no. 126404, Clone: MF-14, RRID: AB_1089117)
- FITC-anti-mouse Ly6G (BioLegend, cat. no. 127606, Clone: 1A8, RRID: AB_1236494)
- APC-anti-mouse CD11b (BioLegend, cat. no. 101212, Clone: M1/70, RRID: AB_312795)
- PE/Cy7-anti-mouse F4/80 (BioLegend, cat. no. 123114, Clone: BM8, RRID: AB_893478)
- PE-anti-mouse CD11b (BioLegend, cat. no. 101208, Clone: M1/70, RRID: AB_312791)
- APC-anti-mouse Gr1 (BioLegend, cat. no. 108412, Clone: RB6-8C5, RRID: AB_313377)
- PE-anti-mouse CD44 (BioLegend, cat. no. 103008, Clone: IM7, RRID: AB_312959)
- PE/Cy7-anti-mouse CD62L (BioLegend, cat. no. 104418, Clone: MEL-14, RRID: AB_313103)
- Fixation buffer (BioLegend, cat. no. 420801)
- Intracellular staining perm wash buffer (BioLegend, cat. no. 421002)
- CCK8 assay (Dojindo, cat. no. CK04)
- Mouse IFNγ precoated enzyme-linked immunospot assay (ELISPOT) kit (Dakewe Biotech, cat. no. 2210005)
- Trypsin (Wisent, cat. no. 325-043-EL)
- Collagenase IV (Invitrogen, cat. no. 17104019)
- DNase-I (Solarbio, cat. no. D8071)

Procedure 2

- PBS (Wisent, cat. no. 311-010-CL)
- Tryptone (Oxoid, cat. no. 2858574)
- Yeast extract (Oxoid, cat. no. 2194133)
- $MgCl_2$ (Aladdin, cat. no. M140788)
- Glycerol (Aladdin, cat. no. G116205)
- Triton-X-100 (Sigma-Aldrich, cat. no.9002-93-1)
- Lysozyme (Sigma-Aldrich, cat. no.L6876)
- DNAse-I (Solarbio, cat. no. D8071)
- GBSS buffer (Macgene, cat. no. CC048)
- Tris–HCl, 1 M, pH 7.4 (Solarbio, cat. no. T1090)
- Tris–HCl, 1 M, pH 8.0 (Solarbio, cat. no. T1150)
- Collagenase IV (Gibco, cat. no. 17104-019)
- Hyaluronidase (Macklin, cat. no. C10717617)
- Sucrose (Macklin, cat. no. S818046)
- Rhodamine B (Sigma-Aldrich, cat. no.83689)
- PLGA, 75:25, relative molecular mass 20,000 Da (Jinandaigang Biotechnology)
- Dichloromethane (DCM; Innochem, cat. no. A46703)
- KOH (Macklin, cat. no. P816399)
- NaCl (Sangon, cat. no. A610476-0005)
- Hydrochloric acid (HCl), 37% (vol/vol) (Innochem, cat. no. 124620010)
- Pentobarbital sodium (Solarbio, cat. no. P8410-5)
- Bull serum albumin (BSA; Sigma-Aldrich, cat. no. A1933)

- Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Aladdin, cat. no. E104432-25g)
- Sodium cholate (Macklin, cat. no. S821473)
- D-Mannitol (Solarbio, cat. no. 69-65-8)
- Protease inhibitor and phosphatase inhibitor cocktail (Solarbio, cat. no. P1260)
- Phosphotungstic acid (Abcam, cat. no. ab146206)
- Anti-mouse IgG-gold conjugate, 10 nm (Fitzgerald, cat. no. 43R-IG084GD, RRID: AB_1286630)
- Anti-rabbit IgG-gold conjugate, 5 nm (Fitzgerald, cat. no. 43R-IG100GD, RRID: AB_1286646).
- Anti-mouse Na^+/K^+ ATPse (Abcam, cat. no. ab76020, Clone: polyclone, RRID: AB_1310695).
- Anti-Ftsz (Agrisera, cat. no. AS10-715, Clone: polyclone, RRID: AB_10754647).
- Anti-mouse TLR4 (Abcam, cat. no. ab13867, Clone: polyclone, RRID: AB_300696)
- Anti-mouse TLR1 (Abcam, cat. no. ab180798, Clone: polyclone)
- Anti-mouse TLR2 (Abcam, cat. no. ab209216, Clone: EPR20302-119)
- Anti-mouse TLR6 (CST, cat. no. 12717, Clone: D1Z8B, RRID: AB_2798005)
- Anti-mouse NF-κB (Abcam, cat. no. ab16502, Clone: polyclone, RRID: AB_443394)
- Anti β-actin (Abcam, cat. no. ab8226, Clone: Abcam 8226, RRID: AB_306371)
- FITC-anti-mouse CD11c (Biolegend, cat. no. 117305, Clone: N418, RRID: AB_313774)
- PE-anti-mouse CD80 (Biolegend, cat. no. 104707, Clone: 16-10A1, RRID: AB_313128)
- PE-anti-mouse CD86 (Biolegend, cat. no. 105007, Clone: GL-1, RRID: AB_313150)
- Mouse IFN gamma ELISA Ready-SET-Go! (eBioscience, cat. no. 88-7314-76, Clone: unknown, RRID: AB_2575067)
- Mouse TNF alpha ELISA Ready-SET-Go! (eBioscience, cat. no. 88-7324-88, Clone: unknown, RRID: AB_2575080)
- Mouse IL-1 beta ELISA Ready-SET-Go! (eBioscience, cat. no. 88-7013-88, Clone: unknown, RRID: AB_2574946)
- Glutaraldehyde (Macklin, cat. no. G849973)

Equipment

▲ **CRITICAL** All equipment is operated according to the operation instructions.

Procedure 1

- Culture plate, 10 cm (Corning, cat. no. 430167)
- Centrifuge tube, 50 ml (Corning, cat. no. 430829)
- Centrifuge tube, 1.5 ml (Kirgen, cat. no. KG2211)
- Six-well plate (Corning, cat. no. 3516)
- 48-Well plate (Corning, cat. no. 3513)
- 96-Well plate (Corning, cat. no. 3599)
- Sealing film (Paraflim, cat. no. PM-996)
- Filter, 0.45 μm (Millipore, cat. no. SLHPR033RB)
- Filter, 0.22 μm (Millipore, cat. no. SLGPR033RB)
- Cell strainer, 70 μm (BD Falcon, cat. no. 352350)
- Ultrafiltration tube, 50 kDa (Millipore, cat. no. UFC9050964)
- Ultracentrifugation tube (Beckman, cat. no. 355618)
- Sterile breathable sealing film (BKMAN, cat. no. B-FK14-50E)
- DLS cuvette (Malvern, cat. no. DTS0012)
- Carbon-coated copper 230-mesh grid (Beijing Zhongjingkeyi Technology, cat. no. BZ110323a)
- Refrigerator (−80 °C) (Haier, HYCD-290)
- Refrigerator (4 and −20 °C) (Haier, DE-25W262)
- Autoclave (STIK Instrument Equipment, IMJ-54A)
- Water bath (BluePard, HWS-24)
- Bacteriological incubator (BluePard, THZ-98C)
- Clean bench (for bacteria) (Beijing Dinglian Har Instrument Manufacture, DL-CJ-1NDII)
- High-speed centrifuge (Thermo Fisher Scientific, Sorvall ST 8R)
- Ultra-speed centrifuge (Beckman, OPTIMA XPN-100)
- DLS instrument (Malvern, Zetasizer Nano ZS90)
- TEM instrument (FEI, Tecnai G2 F20 U-TWIN)
- Flow cytometer (BD Biosciences, BD Accuri C6)

- Spectrophotometer (Asone, ASV-S3)
- Automated Cell Counter (Invitrogen, Countess 3)

Procedure 2
- Carbon-coated copper 200-mesh grid (Beijing Zhongjingkeyi Technology, cat. no. BZ1102XX)
- Round-bottom flasks (50 ml and 250 ml; Synthware)
- Microcentrifuge tubes, 1.5 ml (Eppendorf, cat. no. 0030120086)
- 200 nm and 400 nm cutoff extruder (Hamilton Company, Reno, Nevada, USA)
- Six-well plate (Corning, cat. no. 3599)
- 70 μm cell strainer (Corning, cat. no. 431751)
- Autoclave (STIK Instrument Equipment, IMJ-54A)
- Incubator at 37 °C for *E. coli* growth (Jiangetech, 2102C)
- CO_2 incubator for tissue culture (Thermo Fisher Scientific, Forma 310)
- Magnetic stirrer (IKA, RCT basic)
- High-speed centrifuge (Thermo Fisher Scientific, cat. no. 75004250)
- Ultra-speed centrifuge (Beckman, OPTIMA XPN-100)
- TEM instrument (Hitachi, HT7700)
- pH meter (Mettler Toledo, S210FE20K)
- Rotary evaporator (Tokyo Rikakikai, cat. no. N-1100V-W(WD))
- DLS instrument (Malvern, Zetasizer Nano ZS90)
- Ultrasonicator (NingBo Scientz Biotechnology, Scientz-IID)
- Liposome extruders (Avestin, LiposoFast-Basic & Stabilizer)

Reagent setup

Procedure 1

Solid LB medium. Prepare solid LB medium by dissolving 1 g NaCl, 0.5 g yeast extract, 1 g tryptone and 1.5 g agar in 100 ml deionized water. After sterilization under high temperature and high pressure, antibiotics (such as ampicillin sodium, kanamycin, etc.) were added when the temperature reduced to 60 °C. After fully mixing, 10 ml sterilized solution was added to 10 cm dish and sealed with sealing film after solidification, which could be stored at 4 °C for 1 month. **! CAUTION** Temperature will seriously affect the activity of antibiotics, so antibiotics should be added when the liquid temperature drops to ~50–60 °C.

Liquid LB medium. Prepare liquid LB medium by dissolving 10 g NaCl, 5 g yeast extract and 10 g tryptone in 1 L deionized water. After sterilization under high temperature and high pressure, it can be stored at 4 °C for 1 month.

Fekete's buffer. Prepare Fekete's buffer by mixing 70 ml ethanol, 10 ml formalin and 5 ml glacial acetic acid. The solution can be stored at 4 °C for 1 week. **! CAUTION** Ethanol, formalin and glacial acetic acid are highly volatile. Wear acid-resistant gloves, lab coat and goggles, and handle with caution in a fume hood.

PMA and ionomycin (PI) solution. Prepare PI solution by dissolving 5 μg PMA and 100 μg ionomycin in 1 ml sterilized PBS. The solution can be stored at 4 °C for 1 week.

Tissue digestion solution. Prepare tissue digestion solution by dissolving 5 mg trypsin, 500 mg collagenase IV and 1 mg DNase-I in 100 ml RPMI 1640. The solution can be stored at 4 °C for 3 d.

Procedure 2 **▲ CRITICAL** For preparation of plasma membranes from tumor tissue, all glassware should be washed with deionized water to avoid Ca^{2+} contamination. Calcium contamination can cause swelling of mitochondria and rupture of the outer mitochondrial membrane. Special attention should be paid to use deionized water instead of ice and tap water in all preparations.

LB medium

Dissolve 10 g tryptone, 5 g yeast extract and 10 g sodium chloride in 1,000 ml deionized water, and the mixture sterilized at 121 °C for 30 min. The solution can be stored at 4 °C for 1 month.

Extraction buffer
Dissolve 1 mg DNAse-I and 95 mg $MgCl_2$ in 100 ml deionized water, and add 5 ml Tris–HCl (1 M, pH 8.0). The solution can be stored at 4 °C for 1 week.

Tissue dissociation solution
Add 5 mg collagenase IV, 0.5 mg DNAse-I and 5 mg hyaluronidase to 5 ml GBSS buffer. The solution can be stored at 4 °C for 3 d.

100 mM EGTA, pH 7.4
Dissolve 38.04 mg EGTA in 1 ml deionized water, and adjust the pH to 7.4 with KOH. The solution can be stored at 4 °C for 1 week.

Isolation buffer (IB)
Dissolve 4.1 g mannitol and 2.6 g sucrose in 100 ml deionized water, and add 3 ml Tris–HCl (1 M, pH 7.4). Place the buffer at 4 °C for ~30 min to allow it to cool. Check the pH of the buffer, and adjust it (if necessary) with KOH (1 M, if too low) or HCl (1 M, if too high). Dissolve 0.5 g of albumin in 100 ml solution above mentioned, add 500 µl 100 mM EGTA (pH 7.4). Protease inhibitor cocktail (20 µl/4 ml) and 1% (vol/vol) phosphatase inhibitor cocktail (final concentration) should be supplemented in IB buffer to avoid sample degradation. The solution can be stored at 4 °C for 3 d. **! CAUTION** EGTA is recommended for removing traces of Ca^{2+}. It can be replaced by EDTA; however, a lower concentration (not greater than 0.1 mM) should be used. **! CAUTION** IB buffer must be made fresh, not more than 1 d before the experiment.

ACK lysing buffer
Dissolve 8.02 g NH_4Cl, 1 g $KHCO_3$ and 37.2 mg Na_2EDTA in 850 ml deionized water. Adjust the pH to 7.2–7.4, and add deionized water to 1,000 ml. The solution can be stored at 4 °C for 1 week.

1% (wt/vol) sodium cholate
Dissolve 0.2 g sodium cholate into 20 ml deionized water. The solution can be stored at 4 °C for 1 week.

1% (wt/wt) phosphotungstic acid stain solution (pH 6.0)
Dissolve 0.1 g phosphotungstic acid into 0.1 ml deionized water. Adjust pH to 6.0. The solution can be stored at 4 °C for 1 week.

1% (wt/vol) BSA solution
Dissolve 0.25 g BSA into 25 ml deionized water. The solution can be stored at 4 °C for 1 week.

1% (wt/vol) glutaraldehyde
Dissolve 0.025g glutaraldehyde into 2.5 ml deionized water. The solution can be stored at 4 °C for 1 week.

Procedure

▲ CRITICAL The methods for the preparation of OMV-based nanocarriers and HM-NPs for cancer vaccines, are described in Procedures 1 and 2, respectively.

Procedure 1: plasmid transformation and bacterial culture ● Timing ~2 d

1 Place the centrifuge tube containing 100 µl competent cells (*E. coli* Rosetta (DE3)) in an ice bath until completely thawed.
▲ CRITICAL STEP The competent cells should be stored at −80 °C, and repeated freezing and thawing should be avoided to ensure the transformation efficiency.
2 Add 1 ng co-expression plasmid pETDuet-ClyA-Catchers into the competent cells. Mix gently using a 10 µl pipette ten times, and let stand in an ice bath for 30 min.
3 Place the centrifuge tube in a 42 °C water bath for 60–90 s, and then transfer the tube quickly to an ice bath to cool the cells for 2–3 min without shaking during this process.
4 Add 900 µl sterile LB medium to the centrifuge tube, and incubate in a 150 rpm shaker at 37 °C for 45 min.

▲ **CRITICAL STEP** The LB medium in this step must be antibiotic-free because the resistance gene has not yet been expressed.

5 Add 100 µl transformed cells to a 10 cm culture plate of solid LB medium containing 50 µg/ml ampicillin, and gently spread the cells evenly with a sterile glass stick.
▲ **CRITICAL STEP** The 10 cm culture plate of solid LB medium should be prepared in advance and stored in a sterile environment of 4 °C (see 'Reagent setup').

6 Place the culture plate at room temperature (20–25 °C) until the liquid is absorbed, and culture at 37 °C for 12–16 h.
▲ **CRITICAL STEP** The amount of bacteria in the plate can be adjusted, ideally to obtain several dozens of colonies in a 10 cm plate.
■ **PAUSE POINT** The bacteria in the plate can be stored at 4 °C for 1 month.
? TROUBLESHOOTING

7 Pick one bacteria colony on the plate carefully, and add it into 200 ml LB medium containing 50 µg/ml ampicillin in a conical flask.
▲ **CRITICAL STEP** The conical flask is sealed using the sterile breathable sealing film to ensure oxygen supply for bacterial growth.

8 Incubate at 37 °C with shaking at 180 rpm. Monitor the optical density at 600 nm (OD_{600}) of bacterial culture medium using a spectrophotometer. When the OD_{600} reaches 0.6, add 0.1 mM IPTG into the medium and incubate at 16 °C with shaking at 160 rpm for another 14 h.
▲ **CRITICAL STEP** The IPTG-induced expression can be performed at 37 °C for 2 h, but the expression efficiency is not as good as induction at 16 °C.

OMV extraction ● Timing ~7 h

9 Divide the 200 ml bacteria medium into four 50 ml centrifuge tubes, and collect the supernatant after centrifugation at 5,000*g* and 4 °C for 10 min.
▲ **CRITICAL STEP** The weight of the centrifugal tubes must be balanced.

10 Filter the resulting 200 ml supernatant through a 0.45 µm filter, and then concentrate to 50–60 ml using a 50 kDa ultrafiltration tube through centrifugation at 3,000*g* and 4 °C for ~5–10 min.

11 Filter the concentrated solution through a 0.22 µm filter.

12 Put the concentrated solution into two ultracentrifugation tubes.
▲ **CRITICAL STEP** The ultracentrifugation tubes should be cleaned and dried in advance.

13 Separate the CC OMVs through an ultracentrifugation at 150,000*g* and 4 °C for 3 h.
▲ **CRITICAL STEP** Bubbles are strictly prohibited in the liquid in the ultracentrifugation tubes. The weight of ultracentrifugation tubes must be balanced! All ultracentrifugation tubes have a weight error of less than 50 mg. The tube cover must be tightened.

14 Discard the supernatant, and resuspend the deposited CC OMVs at the bottom of the tubes using PBS to fill up the ultracentrifugation tubes. Repeat the ultracentrifugation at 150,000*g* and 4 °C for 2 h.

15 Pour the supernatant out after ultracentrifugation as soon as possible, and resuspend the deposited CC OMVs in each tube with 200 µl PBS.
■ **PAUSE POINT** The OMVs can be stored at −20 °C or −80 °C for 1 month.
? TROUBLESHOOTING

16 Add 20 µl CC OMVs into 20 µl RIPA buffer, and incubate at 4 °C for 30 min to extract the total protein, and evaluate the total protein concentration using the bicinchoninic acid assay (BCA) according to the manufacturer's protocols.
▲ **CRITICAL STEP** In this protocol, the dose of OMVs is measured in terms of total protein mass. Normally, ~1–1.2 mg OMVs can be extracted from 200 ml bacterial medium, and the concentration of the final CC OMVs is ~2.5–3 mg/ml.

Antigen display ● Timing ~1 h

17 Mix 50 µg CC OMVs from Procedure 1, Step 15 and 50 µg SpT- and/or SnT-labeled antigens in 50 µl PBS, and put the mixture at room temperature for 30 min.
▲ **CRITICAL STEP** The antigens can be customized. When there is only one tumor antigen, either SpT or SnT labeling can be used for rapid display, and there is no notable difference between them. If there are two or more tumor antigens, it is recommended to use different pairs to display, so as to increase the efficiency of display.

Table 3 | The sequence of the SnT- or SpT-labeled antigens used in this protocol

Antigen	Sequence	Vaccine name
SnT-TRP2	GKLGDIEFIKVNKGYGGSVYDFFVWL	CC-SnT-TRP2 OMVs
SpT-OTI	VPTIVMVDAYKRYKGGSIINFEKL	CC-SpT-OTI OMVs
SnT-OTII	GKLGDIEFIKVNKGYGGISQAVHAAHAEINEAGR	CC-SnT-OTII OMVs
SpT-OTI/ SnT-OTII	VPTIVMVDAYKRYKGGSIINFEKL/ GKLGDIEFIKVNKGYGGISQAVHAAHAEINEAGR	CC-SpT-OTI/SnT-OTII OMVs
SpT-Adpgk	VPTIVMVDAYKRYKGGCGIPVHLELASMTNMELMSSIVHQQVFPT	CC-SpT-Adpgk OMVs

The final OMV-based vaccines are named according to the display antigens.

18 Name the final OMV-based nanovaccines according to the display antigens, e.g., CC-SnT-TRP2 OMVs, CC-SpT-OTI OMVs, CC-SnT-OTII OMVs, CC-SpT-OTI/SnT-OTII OMVs and CC-SpT-Adpgk OMVs (Table 3).

▲ **CRITICAL STEP** To accurately control the amount of antigens in the nanovaccines and ensure the same amount of antigens in the different groups, the SpT- or SnT-labeled antigens unbound to CC-OMVs are intended not to be removed. The 50 μg CC-SpT (or SnT)-antigen OMVs mean that there are 50 μg SpT (or SnT)-antigen and 50 μg CC-OMVs in the final nanovaccine. The 50 μg CC-SpT-OTI/SnT-OTII OMVs mean that there are 25 μg SpT-OTI, 25 μg SnT-OTII and 50 μg CC-OMVs in the final nanovaccine.

▲ **CRITICAL STEP** To evaluate the loading rate of SpT- or SnT-labeled antigens, the total proteins of the final OMV-based nanovaccines (containing 3 μg antigens and 3 μg CC OMVs) are extracted according to Procedure 1, Step 16. The proteins are analyzed by electrophoresis and Coomassie blue staining[21,22]. According to the quantitative analysis of the bands in the gel using ImageJ software, the mass of the unbound antigens can be calculated, thereby evaluating the loading rate. The loading rates of SpT-labeled antigens and SnT-labeled antigens are ~8.5% and 19.5%, respectively (Supplementary Fig. 1a,b).

■ **PAUSE POINT** The final OMV-based nanovaccines can be stored at −20 °C or −80 °C for 1 month.

Characterization of OMV-based nanovaccines ● Timing ~2 d

19 *DLS analysis of size* (~0.5 h). Dilute 100 μl of the final OMV-based nanovaccines from Procedure 1, Step 18 into 1 ml ultrapure water in a centrifuge tube.

20 Mix the solution by vortex, and transfer it to a DLS cuvette.

21 Test the size distribution using a Malvern Zetasizer[14].

▲ **CRITICAL STEP** The salt in PBS can seriously affect the DLS measurement, so OMVs must be diluted in pure water. To reduce the effect of osmotic pressure, measurements should be taken within 2 min after dilution in Step 19.

22 *TEM observation of morphology* (~1 h). Drop 10 μl of the final OMV-based nanovaccines from Procedure 1, Step 18 onto the carbon-coated copper 200-mesh grids and incubate for 10 min at room temperature.

23 Drain the solvent with a filter paper.

24 Stain the sample using 1% (wt/wt) uranyl acetate staining solution (pH 6.0) for 5 min.

25 Carry out TEM imaging[14].

▲ **CRITICAL STEP** The staining time can be adjusted to achieve the best contrast in TEM imaging.

26 *Evaluation of the stability of CC OMVs* (~1 d). To evaluate the stability of CC OMVs in the cryopreservation, put 100 μl CC OMVs from Procedure 1, Step 15 into a 1.5 ml centrifuge tube, and repeat five freeze–thaw cycles at −80 °C. Then, evaluate the stability of CC OMVs through DLS analysis and TEM observation according to Procedure 1, Steps 19–25.

27 To evaluate the stability of CC OMVs in the physiological environment, add 100 μl CC OMVs from Procedure 1, Step 15 into 100 μl PBS containing 10% FBS, and incubate at 37 °C for 24 h. Then, evaluate the stability of CC OMVs through DLS analysis and TEM observation according to Procedure 1, Steps 19–25.

28 *Validation of antigen display* (~1 d). To confirm the successful antigen display on CC OMVs, add a peptide tag into the antigens during the synthesis process, such as HA tag, Flag tag or c-myc tag.
▲ **CRITICAL STEP** The peptide tag should be placed between the antigen and SpT or SnT to avoid affecting the connection of tag/catcher protein pairs.

29 Then, prepare the OMV-based nanovaccines according to Procedure 1, Steps 17–18 and extract the total protein according to Procedure 1, Step 16.

30 Use western blot analysis to detect the displayed antigens[14]. Incubate the membranes first with primary antibodies against these peptide tags (1:1,000) and then HRP-conjugated secondary antibodies (1:10,000) before visualization. The expected band is at ~45 kDa, the molecular weight of ClyA-SpC and ClyA-SnC.
▲ **CRITICAL STEP** It must be noted that the addition of these tags has no effect on the immune stimulation of the final OMV-based nanovaccines.

Immune stimulation evaluation of OMV-based nanovaccines in vitro ● Timing ~8 d

31 Keep the C57BL/6 mice (6–8 weeks old) in a room at 20–22 °C with a 12-h light/dark cycle and a humidity of 30–70%. Provide food and water ad libitum.

32 Kill the C57BL/6 mice by cervical dislocation. Dissect the mice, and obtain the femurs and tibias from the killed C57BL/6 mice. Flush the bone marrow cells using an injection syringe and RMPI 1640 medium containing 2% FBS, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin. 40 ml medium is enough for femurs and tibias from one mouse.
! **CAUTION** The animal study should comply with the relevant ethical regulations for animal testing and research. Our animal protocols are approved by the Institutional Animal Care and Use Committee of the National Center for Nanoscience and Technology.

33 Collect the bone marrow cells through centrifugation at 800*g* and room temperature for 5 min.

34 Discard the supernatant, and resuspend the precipitated cells with 1 ml ACK lysis buffer to lysis the red blood cells. Incubate at room temperature for 90 s.

35 Add 3 ml PBS to stop the lysis, and filter the cells through a 70 μm cell strainer.

36 Collect the cells through centrifugation at 800*g* and room temperature for 5 min.

37 Discard the supernatant and resuspend the precipitated cells with 12 ml RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin, 1% HEPES, 0.05 mM β-ME, 20 ng/ml IL-4 and 20 ng/ml GM-CSF, and divide into six wells in a six-well plate.
▲ **CRITICAL STEP** The medium should be prepared in advance.

38 Replace half of the medium every 2–3 d.

39 Collect non-adherent cells on day 6 for further investigation, which are known as BMDCs, through centrifugation at 800*g* and room temperature for 5 min.

40 Discard the supernatant, and resuspend the precipitated BMDCs with 10 ml RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin. Quantify the cell density using the Automated Cell Counter.

41 Place 100,000 BMDCs into a 1.5 ml centrifuge tube with 500 μl RPMI-1640 medium supplemented with 100 U/ml penicillin G sodium and 100 μg/ml streptomycin.

42 Add 50 μg OMV-based nanovaccine from Procedure 1 Step 18 into the BMDCs, and incubate in the cell incubator for 24 h.
▲ **CRITICAL STEP** Herein, we use the nanovaccine containing the OTI antigen (SIINFEKL), CC-SpT-OTI OMVs as an example. Meanwhile, two necessary control groups should be evaluated, including the only antigen group (SpT-OTI, 50 μg) and the mixture group (SpT-OTI + CN OMVs, 50 μg + 50 μg).
▲ **CRITICAL STEP** The centrifuge tube should be sealed with sealing film, but the air hole should be preserved to ensure oxygen supply.

43 Collect the stimulated BMDCs through centrifugation at 800*g* and room temperature for 5 min.

44 Discard the supernatant, and resuspend the precipitated BMDCs with 200 μl RPMI-1640 medium containing 2% FBS.

45 Add the proper antibodies to analyze the maturation and cross-presentation of BMDCs. For the maturation assay, stain the BMDCs with FITC-anti-mouse CD11c (1:200, 1 μl), PE/Cy7-anti-mouse CD80 (1:40, 5 μl) and APC-anti-mouse CD86 (1:80, 2.5 μl). For the cross-presentation assay, stain the BMDCs with PE/Cy7-anti-mouse H-2Kb bound to SIINFEKL (MHCI-OVA) (1:80, 2.5 μl).
▲ **CRITICAL STEP** The antibody used to analyze the cross-presentation should be chosen according to the antigen used in the nanovaccine.

46 Stain the BMDCs at 4 °C under dark conditions for 30 min, and add 500 µl PBS to stop staining and wash cells.

47 Collect the stained BMDCs through centrifugation at 800*g* and room temperature for 5 min, discard the supernatant and resuspend cells with 200 µl PBS.

48 Perform the flow cytometry evaluation according to the manufacturer's protocols within 1 h.
▲ **CRITICAL STEP** Using the CD80 or CD86 as the maturation marker, the $CD80^+$ or $CD86^+$ cells are gated in the $CD11c^+$ cells. For the cross-presentation assay, the MHCI-OVA^+ cells are gated in all BMDCs.

Evaluation of OMV-based nanovaccines in vivo

49 Evaluation of OMV-based nanovaccines in vivo can be achieved using one or more of the following options: option A to evaluate OMV-based nanovaccines displaying one tumor antigen (TRP2) in the pulmonary metastatic melanoma model; option B to evaluate OMV-based nanovaccines displaying two model antigens (OTI and OTII) in the pulmonary metastatic melanoma model; option C to evaluate OMV-based nanovaccines displaying one tumor antigen (Adpgk) in the subcutaneous colon cancer model; or option D to evaluate long-term immune memory elicited by OMV-based nanovaccines displaying model antigen OTI.

(A) **Evaluation of OMV-based nanovaccines displaying one tumor antigen (TRP2) in the pulmonary metastatic melanoma model** ● **Timing ~20 d**

(i) To prepare the pulmonary metastatic melanoma model on day 0, inject 100 µl PBS containing 200,000 B16-F10 cells into the C57BL/6 mice (6–8 weeks old) via tail vein. Set the total number of mice according to the number of control groups, with at least four mice in each group to complete the statistical analysis.
? TROUBLESHOOTING

(ii) Immunize the mice with 50 µg CC-SnT-TRP2 OMVs in 50 µl saline by subcutaneous injection into the tail base on days 3, 6 and 11. Keep the mice in a room at 20–22 °C with a 12-h light/dark cycle and a humidity of 30–70% during the time. Provide food and water ad libitum.
▲ **CRITICAL STEP** Set up necessary control groups including the antigen-only group (SnT-TRP2, 50 µg), the OMVs-only group (CN OMVs, 50 µg) and the mixture group (SnT-TRP2 + CN OMVs, 50 µg + 50 µg). There are at least four mice in each group, and all the mice receive immunization with the different formulations. There are 50 µg SnT-TRP2 and 50 µg CC OMVs in the final CC-SnT-TRP2 OMVs. In addition, CC OMVs displaying another irrelevant antigen (such as OTI, CC-SpT-OTI OMVs) can be used as a control.

(iii) Kill the C57BL/6 mice by cervical dislocation on day 17. Dissect the mice, and collect the lungs and spleens.

(iv) Fix the lungs using the Fekete's buffer (3 ml for each lung) for 2 d. During the fixation, replace the buffer every 2 h.

(v) Count the melanoma nodules in each lung. If the number is greater than 200 in one lung, the number is calculated as 200.

(vi) To prepare a single-cell suspension of splenocytes, put a 70 µm cell strainer into one well of a six-well plate and add 3 ml RPMI-1640 medium supplemented with 2% FBS, 100 U/ml penicillin G sodium and 100 µg/ml streptomycin.

(vii) Put one spleen into the cell strainer, and gently grind to form cell suspension.

(viii) Filter the cell suspension through a 70 µm cell strainer again.

(ix) Collect the splenocytes through centrifugation at 800*g* and room temperature for 5 min.

(x) Discard the supernatant, and resuspend the precipitated cells with 1 ml ACK Lysis Buffer to lyse the red blood cells. Incubate at room temperature for 90 s.

(xi) Collect the splenocytes through centrifugation at 800*g* and room temperature for 5 min.

(xii) Discard the supernatant, and resuspend the splenocytes with 5 ml RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin G sodium and 100 µg/ml streptomycin, then quantify the cell density using the Automated Cell Counter.

(xiii) To analyze the antigen-specific T cells in the splenocytes using flow cytometry, plate the splenocytes from Procedure 1, Step 49A(xii) into the 48-well plate. There are 5,000,000 cells and 500 µl culture medium in one well.

(xiv) Add 10 µg/ml TRP2 peptide to restimulate the splenocytes overnight.
▲ **CRITICAL STEP** Set up the negative control group without adding antigen peptide and the positive control group in which the antigen peptide is replaced by the 1:1,000 PI solution.

(xv) Add 2 μM monensin at 5 h before flow cytometry.

(xvi) Collect the splenocytes through centrifugation at 800*g* and room temperature for 5 min.

(xvii) Discard the supernatant, and resuspend the precipitated splenocytes with 1 ml RPMI-1640 medium containing 2% FBS. Use 200 μl cell suspension (~1,000,000 cells) to perform the cell staining of surface markers CD3 and CD8 using the FITC-anti-mouse CD3 (1:50, 4 μl) and APC-anti-mouse CD8 (1:80, 2.5 μl) following the protocol provided by the manufacturer, respectively.

(xviii) Perform the cell staining of intracellular marker IFNγ using the PE/Cy7-anti-mouse IFNγ (1:20, 10 μl) following the protocol provided by the manufacturer (Intracellular Flow Cytometry Staining Protocol provided by BioLegend).

(xix) Collect the stained splenocytes through centrifugation at 800*g* and room temperature for 5 min, and discard the supernatant and resuspend cells with 200 μl PBS.

(xx) Perform the flow cytometry evaluation according to the manufacturer's protocols within 1 h.

▲ **CRITICAL STEP** The $IFN\gamma^+$ cells in $CD3^+CD8^+$ T lymphocytes in splenocytes are considered as the antigen-specific T cells.

? TROUBLESHOOTING

(xxi) To analyze the antigen-specific T cells in the splenocytes using ELISPOT assay, plate the splenocytes from Procedure 1, Step 49A(xii) into the precoated ELISPOT 96-well plate of mouse IFNγ. There are 1,000,000 cells and 100 μl culture medium in one well.

(xxii) Add 10 μg/ml TRP2 peptide to restimulate the splenocytes for 20 h, and set up the same negative and positive control groups as that in Procedure 1, Step 49A(xiv).

(xxiii) Perform the ELISPOT assay detection according to the protocol provided by the manufacturer.

(xxiv) Quantify the brown spots in every well.

(B) **Evaluation of OMV-based nanovaccines displaying two model antigens (OTI and OTII) in the pulmonary metastatic melanoma model** ● **Timing ~20 d**

(i) To prepare the pulmonary metastatic melanoma model on day 0, inject 100 μl PBS containing 200,000 B16-OVA cells into the C57BL/6 mice (6–8 weeks old) via tail vein. Set the total number of mice according to the number of control groups, with at least four mice in each group to complete the statistical analysis.

(ii) Immunize the mice with 50 μg CC-SpT-OTI/SnT-OTII OMVs in 50 μl saline by subcutaneous injection into the tail base on days 3 and 7. Keep the mice in a room at 20–22 °C with a 12-h light/dark cycle and a humidity of 30–70% during the time. Provide food and water ad libitum.

▲ **CRITICAL STEP** Set up necessary control groups including the antigen-only groups (SpT-OTI, 50 μg; SnT-OTII, 50 μg; SpT-OTI + SnT-OTII, 25 μg + 25 μg), the mixture group (SpT-OTI + CN OMVs, 50 μg + 50 μg; SnT-OTII + CN OMVs, 50 μg + 50 μg; SpT-OTI + SnT-OTII + CN OMVs, 25 μg + 25 μg + 50 μg) and the single-antigen groups (CC-SpT-OTI OMVs, 50 μg SpT-OTI + 50 μg CC OMVs; CC-SnT-OTII OMVs, 50 μg SnT-OTII + 50 μg CC OMVs). There are 25 μg SpT-OTI, 25 μg SnT-OTII and 50 μg CC OMVs in the final CC-SpT-OTI/SnT-OTII OMVs. There are at least four mice in each group, and all the mice receive immunization with one of the different formulations. The two antigens are linked to CC OMVs by the two different molecular pairs, so they do not affect each other. According to the load rates calculated previously in Procedure 1, Step 18, there are 2.1 μg SpT-OTI and 4.9 μg SnT-OTII bound to the CC OMVs in the final CC-SpT-OTI/SnT-OTII OMVs.

▲ **CRITICAL STEP** When the OMVs display two antigens that have a synergistic immune activation effect, such as the combination of antigens that stimulates $CD4^+$ and $CD8^+$ T cells, respectively, we recommend a two-dose immunization regimen to reflect the differences between different control groups. Preliminary experiments should be conducted to determine the final experimental protocol. However, at least two immunizations are required.

(iii) Kill the C57BL/6 mice by cervical dislocation on day 17. Dissect the mice, and collect the lungs and spleens.

(iv) Evaluate the antitumor effect using the similar approach as in Procedure 1, Steps 49A(iv–v).

(v) Prepare the single-cell suspension of splenocytes using the similar approach as in Procedure 1, Step 49A(vi–xii).

(vi) Analyze the antigen-specific T cells in the splenocytes by flow cytometry using a similar approach as in Procedure 1, Steps 49A(xiii–xx). The only differences are restimulating cells using 5 μg/ml OTI and 5 μg/ml OTII peptides and staining cells using the FITC-anti-mouse CD3 (1:50, 4 μl), PE-anti-mouse CD4 (1:160, 1.25 μl), APC-anti-mouse CD8 (1:80, 2.5 μl) and PE/Cy7-anti-mouse IFNγ (1:20, 10 μl).
▲ **CRITICAL STEP** The $IFN\gamma^+$ cells in $CD3^+CD8^+$ or $CD3^+CD4^+$ T lymphocytes in splenocytes are considered as the antigen-specific T cells.

(vii) Analyze the antigen-specific T cells in the splenocytes by ELISPOT assay using the similar approach as in Procedure 1, Steps 49A(xxi–xxiv). The only difference is using 5 μg/ml OTI and 5 μg/ml OTII peptides to restimulate the cells.

(C) **Evaluation of OMV-based nanovaccines displaying one tumor antigen (Adpgk) in the subcutaneous colon cancer model** ● **Timing ~50 d**

(i) To prepare the subcutaneous colon cancer model on day 0, inject 100 μl PBS and Matrigel mixture (1:1, vol/vol) containing 1,000,000 MC38 cells into the right back of C57BL/6 mice (6–8 weeks old) subcutaneously. Set the total number of mice according to the number of control groups, with at least 14 mice in each group, in which at least 10 mice are used to analyze the tumor growth and survival and at least 4 mice are used for the statistical analysis of cells in tumor microenvironment.

(ii) Immunize the mice with 50 μg CC-SpT-Adpgk OMVs in 50 μl saline by subcutaneous injection into the tail base on days 3, 6 and 11. Keep the mice in a room at 20–22 °C with a 12-h light/dark cycle and a humidity of 30–70% during the time. Provide food and water ad libitum.
▲ **CRITICAL STEP** Set up necessary control groups including the mixture group (SpT-Adpgk + CN OMVs, 50 μg + 50 μg) and the positive control group [Poly (I:C) + SpT-Adpgk, 50 μg + 50 μg]. There are at least 14 mice in each group, and all the mice receive immunization with different formulations. There are 50 μg SpT-Adpgk and 50 μg CC OMVs in the final CC-SpT-Adpgk OMVs.

(iii) Record the tumor volume of at least ten mice in each group every other day; calculate tumor volume using the following equation: tumor volume = length × $width^2$ × 0.5.

(iv) Record the survival of these mice until the tumor volume reached 1,500 mm^3. Once the tumor volume exceeds 1,500 mm^3, kill the mice by cervical dislocation and record as dead in the survival statistics.

(v) Kill at least four mice in each group (different individuals than the mice used in the previous two steps) by cervical dislocation on day 29, and dissect the mice and collect the tumors.

(vi) To prepare the single-cell suspension of tumor tissues, put the tumor tissues in 1 ml tissue digestion solution and cut up into ~2 mm^3 pieces.

(vii) Digest the tissue in a shaker at 150 rpm and 37 °C for 30 min.

(viii) Filter the digestion suspension through a 70 μm cell strainer again.

(ix) Collect the cells through centrifugation at 800*g* and room temperature for 5 min.

(x) Discard supernatant and resuspend the cells with RPMI 1640 medium supplemented with 2% FBS. Using the Automated Cell Counter, adjust the cell density to 100,000 cells per 200 μl.

(xi) Stain the cells using the following antibodies: (1) APC-anti-mouse CD3 (1:40, 5 μl), PE-anti-mouse CD4 (1:80, 2.5 μl) and FITC-anti-mouse CD8 (1:100, 2 μl) in 200 μl cell suspension (100,000 cells); (2) APC-anti-mouse CD3 (1:40, 5 μl), FITC-anti-mouse CD4 (1:200, 1 μl) and PE-anti-mouse Foxp3 (1:20, 10 μl) in 200 μl cell suspension (100,000 cells); (3) FITC-anti-mouse Ly6G (1:200, 1 μl) and APC-anti-mouse CD11b (1:80, 2.5 μl) in 200 μl cell suspension (100,000 cells); (4) PE/Cy7-anti-mouse F4/80 (1:80, 2.5 μl) in 200 μl cell suspension (100,000 cells); (5) FITC-anti-mouse CD11c (1:200, 1 μl) in 200 μl cell suspension (100,000 cells); (6) PE-anti-mouse CD11b (1:80, 2.5 μl) and APC-anti-mouse Gr1 (1:80, 2.5 μl) in 200 μl cell suspension (100,000 cells) following the protocol provided by the manufacturer.

(xii) Collect the stained cells through centrifugation at 800*g* and room temperature for 5 min, and discard supernatant and resuspend cells with 200 μl PBS to perform the flow cytometry evaluation within 1 h. Evaluate the following immune cells infiltrating tumor tissues,

following the manufacturer's instructions: $CD3^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD4^+Foxp3^+$ regulatory T cells (Tregs), $CD11b^+Ly6G^+$ activated neutrophils, $F4/80^+$ macrophages, $CD11c^+$ DCs and $CD11b^+Gr1^+$ myeloid-derived suppressor cells (MDSCs).

(D) **Evaluation of long-term immune memory elicited by OMV-based nanovaccines displaying model antigen OTI** ● Timing ~80 d

(i) Immunize the healthy C57BL/6 mice (6–8 weeks old) with 50 μg CC-SpT-OTI OMVs in 50 μl saline by subcutaneous injection into the tail base on days 0, 3 and 8. Keep the mice in a room at 20–22 °C with a 12-h light/dark cycle and a humidity of 30–70% during the time. Provide food and water ad libitum. Set the total number of mice according to the number of control groups, with at least 14 mice in each group, in which at least 10 mice are used to analyze antitumor effect of immune memory after tumor challenge and at least 4 mice are used for the statistical analysis of immune memory cells.

▲ CRITICAL STEP Set up necessary control groups including the mixture group (SpT-OTI + CN OMVs, 50 μg + 50 μg) and the positive control group (Poly (I:C) + SpT-OTI, 50 μg + 50 μg). There are at least 14 mice in each group, and all the mice receive immunization with different formulations. There are 50 μg SpT-Adpgk and 50 μg CC OMVs in the final CC-SpT-Adpgk OMVs.

(ii) Kill at least four mice in each group by cervical dislocation on day 60, and dissect the mice and collect the spleen.

(iii) Prepare a single-cell suspension of splenocytes using the similar approach as in Procedure 1, Step 49A(vi–xii).

(iv) Analyze the memory T cells in the splenocytes by flow cytometry using the similar approach as in Procedure 1, Step 49A(xiii–xx). The only difference is staining cells using the APC-anti-mouse CD3 (1:40, 5 μl), FITC-anti-mouse CD8 (1:100, 2 μl), PE-anti-mouse CD44 (1:80, 2.5 μl) and PE/Cy7-anti-mouse CD62L (1:80, 2.5 μl).

(v) To evaluate the specific killing against tumor cells, plate the splenocytes from Procedure 1, Step 49D(iii) into a 48-well plate. There are 5,000,000 cells and 500 μl culture medium in one well.

(vi) Restimulate these splenocytes with 10 μg/ml OTI peptide overnight.

(vii) Quantify the cell density using the Automated Cell Counter, and culture 5,000 B16-OVA or MC38 cells and 50,000 restimulated splenocytes from the previous step in a well of 96-well plate for 24 h.

(viii) Remove the non-adherent cells, and wash the adherent cells with 100 μl PBS two times.

(ix) Perform the CCK8 assay following the protocol provided by the manufacturer, and calculate the percent of specific killing.

(x) Challenge the at least ten vaccinated mice from Procedure 1, Step 49D(i) (ten mice in each group) through injecting 100 μl PBS containing 200,000 B16-OVA cells into mice via tail vein on day 60.

(xi) Kill the mice by cervical dislocation on day 80, and dissect the mice and collect the lungs.

(xii) Fix the lungs and count the melanoma nodules using a similar approach as in Procedure 1, Step 49A(iv–v).

Procedure 2: isolation of cytoplasmic membranes of *E. coli* (EM) ● Timing ~40 h

1 Streak one loopful of *E. coli* DH5α stocks (~1×10^8 cells/ml) on the LB agar plate, applying the loop lightly. Incubate the plate at 37 °C for 16–18 h to obtain single colonies.

■ PAUSE POINT *E. coli* colonies on an LB agar plate can be stored at 4 °C for 1 month.

2 Pick a single colony of *E. coli*, and inoculate it into a sterile tube containing 5 ml LB medium. Incubate the culture overnight (12–16 h) on a shaker at 180 rpm and 37 °C.

3 Add 1 ml overnight culture into 100 ml fresh LB medium, and incubate at 37 °C for 4–6 h.

4 Collect the *E. coli* by centrifugation at 3,000*g* and 4 °C for 20 min. Discard the supernatant, wash the *E. coli* pellet with 100 ml ice-cold PBS and vortex gently. Centrifuge the solution at 3,000*g* and 4 °C for 20 min, discard the supernatant and repeat the PBS wash twice to remove the medium component. Finally, discard the supernatant and suspend the generated cell pellet with 80 ml PBS.

5 Add lysozyme into the *E. coli* PBS solution to a final concentration of 2 mg/ml. Incubate the mixture with shaking at 180 rpm and 37 °C for 1 h to digest the cell wall of *E. coli*.

6 Collect the protoplasts by centrifuging at 3,000*g* and 4 °C for 20 min.
PAUSE POINT The pellet containing the protoplasts should be used immediately for experiments but can be stored at 4 °C for 12 h.
? TROUBLESHOOTING

7 Discard supernatant, and add 10 ml PBS to the protoplast pellet to wash. Collect by centrifugation at 3,000*g* and 4 °C for 20 min. Discard supernatant, and wash the pellet with PBS twice.

8 Add 10 ml extraction buffer to protoplast pellet and use a pipette to suspend the protoplast until the suspension is homogeneous. Incubate the suspension on ice for 30 min, shake or vortex the suspension gently every 5 min.

9 Centrifuge the suspension at 3,000*g* and 4 °C for 5 min. Transfer the supernatant into a new tube, and discard the undigested pellet.
? TROUBLESHOOTING

10 Collect cytoplasmic membrane by centrifuging at 20,000*g* and 4 °C for 30 min. Discard supernatant, and resuspend cytoplasmic membrane with 0.5 ml sterile PBS. Evaluate the total protein concentration using the BCA.
▲CRITICAL STEP Repeated freeze–thaw cycles degrade proteins. Aliquot the cytoplasmic membrane, and store at −80 °C.
PAUSE POINT The obtained cytoplasmic membrane can be stored for 1 year at −80 °C.

Isolation of autologous TM ● Timing ~9 d

11 Inject CT26 or B16-F10 tumor cells (2×10^5 per mouse) into the left back of the BALB/c or C57BL/6 mice (6–8 weeks old).
▲CRITICAL STEP The number of mice in this step depends on the experiment purpose. For characterization the physicochemical property of nanovaccine, a few mice is preferred. For tumor therapy, the total number of mice depends on the group setting (usually 10–12 mice per group for survival statistics).

12 Measure the tumor length and width with electronic calipers every other day. Calculate the tumor volume on the basis of the following formula: volume = 0.5 × length × width^2.

13 Allow the average tumor volume to reach ~300 mm^3 for each group, then anesthetize the mice and resect tumor tissue.
PAUSE POINT Resected tumor tissue can be stored at 4 °C for 12 h.

14 Transfer the whole-tumor tissue (200–300 mg) to one well of a six-well plate containing 1.5 ml tissue dissociation solution. Use scissors to cut the tumor tissue into 1–2 mm^3 pieces, and incubate them at 37 °C for 10 min.
▲CRITICAL STEP Cut the tumor tissue as small as possible. Incubation time can be extended to 20 min.

15 Repeat the dissociate Step 14 twice to prepare a single-cell suspension.
▲CRITICAL STEP For the B16-F10 melanoma tissue that has less extracellular matrix than CT26 tumor, the 10 min incubation in Step 14 can be skipped, otherwise the excessive digestion of the tissue may lead to a loss of tumor membranes.

16 Filter the mixture with a 70 μm cell strainer to obtain the cell suspension, and collect cells by centrifuging at 300*g* and 4 °C for 5 min.

17 Discard supernatant, and resuspend cells with 1 ml ACK lysing buffer for 1 min.
▲CRITICAL STEP Lysis time should not be >1 min to avoid any unwanted degradation of protein on TM.

18 Add 10 ml PBS to stop lysis.

19 Collect tumor cells by centrifuging at 300*g* and 4 °C for 5 min. Discard supernatant, and resuspend cells with 10 ml IB buffer.
▲CRITICAL STEP Resuspension and subsequent steps must be performed at 4 °C to minimize the activation of proteases and phospholipases.

20 Break cells by sonication using a probe ultrasonicator at 35% power for 5 min.
▲CRITICAL STEP Place the mixture on the ice bath, and the ultrasonicator should pulse on and off cycle every 1–2 s to avoid overheating.

21 Centrifuge the suspension at 3,000*g* and 4 °C for 5 min. Transfer the supernatant into a new tube, and discard the pellet.

22 Centrifuge the collected supernatant at 10,000*g* and 4 °C for 10 min. Transfer the supernatant into a new tube, and discard the pellet.

▲ **CRITICAL STEP** The supernatant of Steps 21 and 22 contains microsomes, and cytosolic proteins cannot be frozen before further centrifugation because freezing will cause damage to microsomal integrity and contaminate the plasma membrane fraction.

23 Centrifuge the collected supernatant at 100,000*g* and 4 °C for 2 h. Discard the supernatant, and collect membrane pellet.

▲ **CRITICAL STEP** Complete filling of the tubes and balancing the weights of tubes placed on opposite sides are important to maintain safety during centrifugation.

24 Resuspend cytoplasmic membrane pellet with 0.5 ml sterile PBS. Evaluate the total protein concentration using the BCA.

▲ **CRITICAL STEP** Repeated freeze–thaw cycles degrade proteins. Aliquot the tumor membrane, and store at −80 °C.

■ **PAUSE POINT** The obtained tumor membrane can be store for 1 year at −80 °C.

Preparation of PLGA nanoparticle core ● Timing ~1.5 h

25 Dissolve 10 mg PLGA in 1 ml DCM.

■ **PAUSE POINT** PLGA in DCM can be stored for 2 weeks at 4 °C.

26 Add 0.2 ml deionized water into PLGA solution.

▲ **CRITICAL** Replace 0.2 ml deionized water with 0.2 ml rhodamine B (0.5 mg/ml) for preparation of the rhodamine B-loaded HM-NPs in Step 40A(i).

27 Emulsify the mixture by sonication using a probe ultrasonicator at 25% power for 3 min.

▲ **CRITICAL STEP** Place the mixture on the ice bath, and the ultrasonicator should pulse on and off cycle every 1–2 s to avoid overheating.

28 Add 2 ml 1% (wt/vol) sodium cholate solution to the mixture.

29 Emulsify the solution again by sonicating at 35% power for another 5 min.

▲ **CRITICAL STEP** Place the mixture on the ice bath, and the ultrasonicator should pulse on and off cycle every 1–2 s to avoid overheating.

30 Add the mixture into 10 ml 0.5% (wt/vol) sodium cholate solution, and stir for 10 min.

31 Remove the organic solvent in the mixture by rotary evaporation under reduced pressure.

32 Collect the nanoparticles by centrifugation at 11,000*g* and 4 °C for 15 min, and resuspend them in 500 μl deionized water. This solution can be stored for 1 week at 4 °C.

33 Measure the size and PDI of PLGA nanoparticles by DLS following the manufacturer's specifications.

▲ **CRITICAL STEP** Avoid using a phosphate buffer system to resuspend PLGA nanoparticles; otherwise, PLGA nanoparticles would easily precipitate. The PDI of PLGA nanoparticles should be <0.3.

Preparation of HM ● Timing ~0.5 h

34 Quantify the total protein concentration of the EM from Step 10 and TM from Step 24 using a BCA Protein Quantitation Kit according to the instructions.

35 Add 1.5 mg EM and 0.5 mg TM into the tube. Use PBS to adjust the total volume to 500 μl. Incubate it at 37 °C for 15 min of gentle oscillation at 50 rpm.

36 Extrude the mixture 13 times through the 400 nm cutoff sterile extruder.

▲ **CRITICAL STEP** Prewash the extruder with deionized water. Do not overtighten the extruder, as this can cause the polycarbonate film to tear, leading to film leakage and ineffective sizing. The odd number of passes is necessary to obtain size-selected vesicles without too many unextruded vesicles or other contaminants. Use the HM immediately after preparation.

Preparation of HM-coated PLGA nanoparticles (HM-NPs) ● Timing ~0.5 h

37 To prepare HM-NPs, mix 2 mg HM from Step 36 (500 μl) and 10 mg PLGA nanoparticles (500 μl) from Step 32 in a tube. Sonicate the mixture for 10 min by a probe ultrasonicator at 30% power on the ice bath. Similarly, mix 2 mg TM (use PBS to adjust the volume to 500 μl) from Step 24 or 2 mg EM (use PBS to adjust the volume to 500 μl) from Step 10 with 10 mg PLGA nanoparticles (500 μl) from Step 32, and sonicate these mixtures for 10 min to prepare TM-coated PLGA nanoparticles (TM-NPs) and EM-coated PLGA nanoparticles (EM-NPs), respectively.

▲ **CRITICAL STEP** Place the mixture on the ice bath, and the ultrasonicator should pulse on and off cycle every 1–2 s to avoid overheating.

38 Co-extrude the mixtures from Step 37 at least 13 times through a 200 nm cutoff sterile extruder. ▲CRITICAL STEP For the Mix-NPs, directly mix 1.5 mg TM-generated TM-NPs (750 μl) and 0.5 mg EM-generated EM-NPs (250 μl) from Step 38 without any other extrude procedures.
▲CRITICAL STEP Prewash the extruder with deionized water. Do not overtighten the extruder, as this can cause the polycarbonate film to tear, leading to film leakage and ineffective sizing. The odd number of passes is necessary to obtain size-selected vesicles without too many unextruded vesicles or other contaminants. Use the vaccine formulations immediately after preparation.

Physicochemical characterization of HM-NPs ● Timing ~9 h

39 Various options are available to characterize the finial nanoparticle product. Use option A for TEM characterization, option B for DLS characterization and option C for immunogold staining.

(A) **TEM imaging ● Timing ~2 h**

(i) Drop 10 μl HM-NPs from Step 38, at a concentration of 1 mg/ml, onto carbon-coated copper 200-mesh grids, and incubate for 10 min at room temperature.
(ii) Drain the solvent with filter paper.
(iii) Stain the sample using 1% (wt/wt) phosphotungstic acid stain solution (pH 6.0) for 10 min.
(iv) Measure the morphology and size of HM-NPs using a TEM according to the instructions[15].

(B) **DLS characterization ● Timing ~1 h**

(i) Dilute 10 μl HM-NPs from Step 38, at a concentration of 1 mg/ml, into 1 ml of ultrapure water in a centrifuge tube.
(ii) Mix the solution by vortex, and transfer it to a DLS cuvette.
(iii) Measure the zeta potential and size distribution using a Malvern Zetasizer according to the instructions[15].

(C) **Immunogold staining ● Timing ~6 h**

(i) Deposit 10 μl HM-NPs from Step 38 onto a glow-discharged carbon-coated grid. Ensure the HM-NPs solution totally covers the grid for 20 min in the dry environment.
(ii) Wash it with 50 μl PBS three times.
(iii) Block the grid with 1% (wt/vol) BSA solution for 15 min, and stain it with 10 μl (0.5 mg/ml) of anti-mouse Na^+/K^+ ATPse (to label the TM) and 10 μl (0.5 mg/ml) of anti-Ftsz (to label the EM) for 1 h incubation.
(iv) Wash it with 1% BSA solution six times, 3 min every time.
(v) Stain the grid with 10 μl anti-rabbit IgG-gold conjugate (5 nm, 1:20) and anti-mouse IgG-gold conjugate (10 nm, 1:20) diluted in 1% BSA solution for 1 h.
(vi) Wash it with 50 μl 0.5% (wt/vol) BSA solution.
(vii) Fix samples with 50 μl 1% (wt/vol) glutaraldehyde in PBS for 5 min.
(viii) Wash it with water eight times, 2 min every time.
(ix) Stain the sample using 1% (wt/wt) phosphotungstic acid stain solution (pH 6.0) for 10 min.
(x) Confirm that both the 5 nm and 10 nm gold nanoparticles are present on the HM-NPs by TEM according to the instructions[15].

In vitro experiments with HM-NPs ● Timing ~7 d

40 To assess the cellular uptake efficiency of HM-NPs by BMDCs, follow option A. To examine the TLRs activation efficiency of HM-NPs in BMDCs, follow option B. To use HM-NPs to induce BMDCs activation and maturation, follow option C.

(A) **Flow cytometry analysis of cellular uptake ● Timing ~1 d**

(i) Prepare rhodamine B-loaded PLGA nanoparticle core (Rho-Core) as described in Procedure 2, Steps 25–33, then utilize Rho-Core to generate rhodamine B-loaded EM-NPs, TM-NPs, Mix NPs or HM-NPs, as described in Procedure 2, Steps 37–38.
(ii) Collect BMDCs from C57BL/6 mice (6–8 weeks old) using the similar approach as in Procedure 1, Steps 31–40.
(iii) Add BMDCs to a 1.5 ml centrifuge tube at a density of 4×10^4 cells per tube with 400 μl RPMI-1640 medium supplemented with 100 U/ml penicillin G sodium and 100 μg/ml streptomycin. Add 100 μl rhodamine B-loaded EM-NPs, TM-NPs, Mix NPs and HM-NPs from Procedure 2, Step 40A(i) (total membrane protein amount is 2 mg/ml) to the tube, and incubate for 8 h at 37 °C in 5% CO_2.
▲CRITICAL STEP Do not add BMDCs into a plate or dish for incubation with nanoparticles in this step. BMDCs, especially the matured BMDCs, easily adhere to the surface-modified plate, but they are less adhering on tube surface.

(iv) Collect the cells by centrifuging at 300*g* and 4 °C for 5 min. Aspirate the supernatant, and wash the cells once with 1 ml PBS with 2% FBS.

(v) Collect the cells by centrifuging at 300*g* and 4 °C for 5 min, discard the supernatant and resuspend the cell pellet using 500 μL PBS with 2% FBS.

(vi) Evaluate the fluorescence intensity of rhodamine B in the collected cells using flow cytometry to detect the amount of EM-NPs, TM-NPs, Mix NPs or HM-NPs taken up by BMDCs.

(B) **TLR activation** ● Timing ~3 d

(i) Collect BMDCs from C57BL/6 mice (6–8 weeks old) using the similar approach as in Procedure 1, Steps 31–40.

(ii) Seed BMDCs in a six-well plate at a density of 1×10^6 cells per well with 2 ml RPMI-1640 medium supplemented with 100 U/ml penicillin G sodium and 100 μg/ml streptomycin.

(iii) Add 500 μl EM-NPs, TM-NPs, Mix NPs and HM-NPs from Procedure 2, Steps 37–38 (total membrane protein amount is 2 mg/ml) to the wells, and incubate for 24 h at 37 °C in 5% CO_2.

(iv) Aspirate the medium, and add 3 ml PBS. Gently shake the plate for 3 min to wash the cells and aspirate the PBS.

(v) Extract the total cellular protein, and evaluate the expression level of different TLRs using standard western blot analysis techniques[15], and use β-actin as an internal control. Incubate the blot membranes with primary antibodies against TLR1 (1:1,000), TLR2 (1:100), TLR4 (1:200), TLR6 (1:200), NF-κB (1:2,000) and β-actin (1:5,000), and then add HRP-conjugated secondary antibodies (1:10,000) before visualization.

(C) **DC activation and maturation** ● Timing ~3 d

(i) Collect BMDCs from C57BL/6 mice (6–8 weeks old) using a similar approach as in Procedure 1, Steps 31–40.

(ii) Add BMDCs to a 1.5 ml centrifuge tube at a density of 4×10^4 cells per tube with 400 μl RPMI-1640 medium supplemented with 100 U/ml penicillin G sodium and 100 μg/ml streptomycin.

▲ CRITICAL STEP Do not add BMDCs into plate or dish for incubation with nanoparticles in this step. BMDCs, especially the matured BMDCs, are easily adhere to the surface-modified plate, but they are less adhering on tube surface.

(iii) Add 100 μl EM-NPs, TM-NPs and HM-NPs from Procedure 2, Steps 37–38 (total membrane protein amount is 2 mg/ml) to the tube, and incubate for 24 h at 37 °C in 5% CO_2.

(iv) Centrifuge the cells at 300*g* and 4 °C for 5 min. Collect the supernatant, and use corresponding ELISA kits to analyze the concentrations of IL-6, TNF-α and IL-1β.

(v) Resuspend the cell pellet in 100 μl RPMI-1640 medium containing 2% FBS. Add FITC-anti-mouse CD11c (1:100, 1 μl), PE-anti-mouse CD80 (1:200, 0.5 μl) or PE-anti-mouse CD86 (1:200, 0.5 μl) to cells, and incubate at 4 °C for 20 min for fluorescence labeling.

(vi) Collect cells by centrifuging at 300*g* and 4 °C for 5 min. Discard the supernatant, and resuspend the cells with 1 ml PBS with 2% FBS for washing.

(vii) Collect cells by centrifuging at 300*g* and 4 °C for 5 min. Discard the supernatant, and resuspend the cell pellet using 300 μl PBS with 2% FBS.

(viii) Detect the fluorescence intensity of cells using a flow cytometer to evaluate the expression of CD80 and CD86 in $CD11c^+$ cells according to the manufacturer's protocols.

In vivo experiments with HM-NPs ● Timing ~90 d

41 To use HM-NP vaccination for postoperative immunotherapy in a CT26 tumor and B16-F10 tumor model, follow option A. To use HM-NPs to provide long-term protection in a CT26 tumor rechallenge model, follow option B.

(A) **Postoperative immunotherapy** ● Timing ~60 d

(i) Establish the CT26 and B16-F10 tumor models in BALB/c and C57BL/6 mice (6–8 weeks old), respectively, following Procedure 2, Steps 11–12. Allow the average tumor volume to reach ~300 mm^3 for each group, then anesthetize the mice and resect tumor tissue. Use the excised tumor to isolate the tumor membrane following Procedure 2, Steps 13–24. Aliquot tumor membrane and store at −80 °C. Use aliquot tumor membrane for the vaccine formulations preparation following Procedure 2, Steps 37–38 before immunization.

▲ CRITICAL STEP Most but not all of the tumor tissues should be excised.

▲ CRITICAL STEP Use the vaccine formulations from Procedure 2, Steps 37–38 immediately after preparation.

? TROUBLESHOOTING

(ii) Randomize the mice into the different experimental groups after surgery (at least ten mice per group for survival statistics). Immunize mice with different formulations (including PBS as control, Mix NPs and HM-NPs) via subcutaneous injection (100 μg of membrane protein per mouse) on days 3, 5 and 9 after surgery.

(iii) Monitor the tumor volumes in the different groups over 60 d since tumor inoculation.

(B) **Rechallenge model** ● Timing ~90 d

(i) Establish the HM-NPs vaccination for postoperative immunotherapy in the CT26 tumor models by following Procedure 2, Step 41A.

(ii) Randomize the HM-NPs-treated, postoperative mice into three groups (at least ten mice per group) on 60 d after the first CT26 tumor inoculation, and inoculate with CT26 colon adenocarcinoma cells or 4T1 breast tumor cells (2×10^5 per mouse) through injection into the right back.

(iii) Monitor the tumor volumes in the different groups over 90 d since the first CT26 tumor inoculation.

Troubleshooting

Troubleshooting advice can be found in Table 4.

Table 4 | Troubleshooting table

Procedure 1

Step	Problem	Possible reason	Solution
6	There are very few bacterial colonies on the plate (Supplementary Fig. 9a–c)	The antibiotics are not being used in the right way	Choose the right antibiotics according to the resistance gene in the backbone plasmid; use the antibiotic-free LB medium in Step 4 during competent cell resuscitation
		The activity of competent cells is insufficient	Store the competent cells at −80 °C until use, and avoid repeated freezing and thawing
15	The amount of extracted OMV is very low (Supplementary Fig. 10a,b)	The lack of bacterial activity leads to a decrease in OMV secretion	Pick the single bacteria colony on the antibiotic plate as the culture source, not from the cryopreserved bacteria in glycerin
49A(i)	Pulmonary metastases are rare (Supplementary Fig. 11)	There is something wrong with the tumor cellular viability	Collect the tumor cells at a culture density of 70%, and keep on ice until injection
49A(xx)	The number of antigen-specific T cells is very low	There are problems with the antibody using in the detection by flow cytometry	Store the antibody in a 4 °C and dark environment, and set up the positive control group using a PI solution for quality control

Procedure 2

Step	Problem	Possible reason	Solution
6	The protoplasts pellet is much smaller than the original *E. coli* pellet in Step 4 or not visible	The time of digest is too long, or the container is contaminated with denaturant or detergent	Shorten the treatment time for cell wall digestion, or remove the denaturant or detergent contaminated from the container
9	The volume of the resident pellet is too high, or volume is similar to that of the original *E. coli* pellet in Step 4	The time of digest time is too short, or lysozyme is inactive	Prolong the digest time or prepare a new lysozyme solution
41A(i)	No recurrence occurred in the mice without the therapeutic agent treatment	All the tumor tissues are removed	If the tumors have smooth peripheral rim and a capsule is present, remove all the tumor tissue; then cut one small piece (4–6 mm^3 or half-rice-grain-sized blocks) out, and put it back in the resection site before suturing

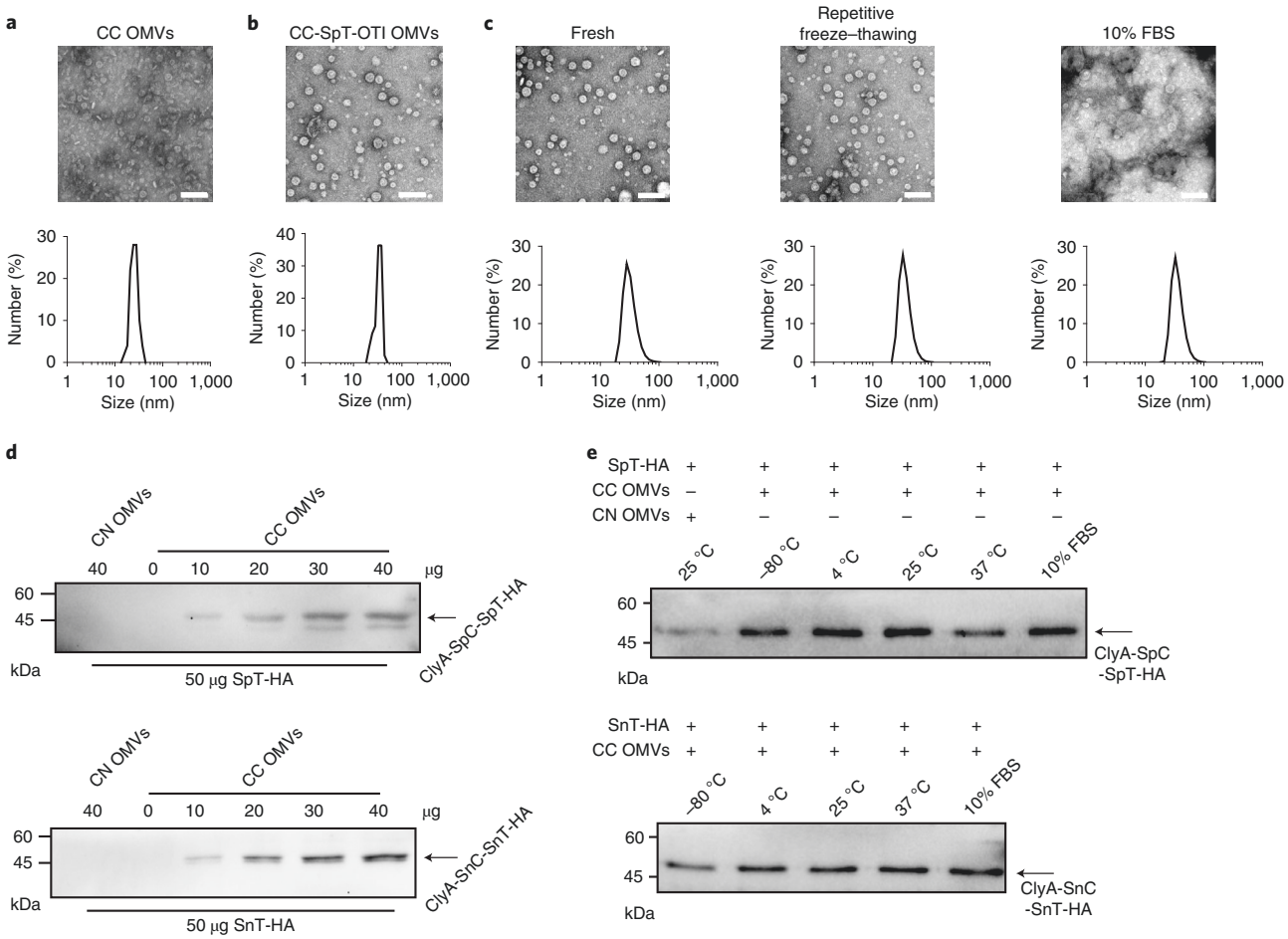


Fig. 2 | Characterization of OMV-based nanovaccines. a, TEM image and DLS analysis (below) of CC OMVs. Scale bar, 100 nm. The CC OMVs exhibit a typical bilayer structure and uniform spherical morphology with an average diameter of ~30 nm. **b**, Representative TEM image and DLS analysis (below) of CC-SpT-OTI OMVs. Scale bar, 100 nm. The antigen display of SpT-OTI has no obvious effect on the morphology and size of CC-SpT-OTI OMVs, compared with those of CC OMVs (**a**). **c**, TEM images and DLS analyses (below) of fresh CC OMVs, CC OMVs after five freeze–thaw cycles (−80 °C) and CC OMVs after incubation in 10% FBS for 24 h. Scale bar, 100 nm. The morphology and size of CC OMVs are unaffected after repeated freezing and thawing or incubation in 10% FBS. **d**, The conjugation of SpT-HA or SnT-HA to ClyA-Catchers on the CC OMV surface verified by western blot analysis using an anti-HA antibody. **e**, Examination of ClyA-SpC-SpT-HA or ClyA-SnC-SnT-HA complexes by western blot analysis to evaluate the connection stability. CC OMVs are incubated with SpT-HA or SnT-HA for 1 h, then stored at different temperatures or treated with 10% FBS for 24 h. CN OMVs are from the bacteria expressing ClyA without the fused Catchers (ClyA-none). Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0.

Timing

Procedure 1: OMV-based nanocarriers

Steps 1–8, plasmid transformation and bacterial culture: ~2 d
Steps 9–16, OMV extraction: ~7 h
Steps 17–18, antigen display: ~1 h
Steps 19–30, characterization of OMV-based nanovaccines: ~2 d
Steps 31–48, immune stimulation evaluation of OMV-based nanovaccines in vitro: ~8 d
Step 49A, evaluation of OMV-based nanovaccines displaying one tumor antigen (TRP2) in the pulmonary metastatic melanoma model: ~20 d
Step 49B, evaluation of OMV-based nanovaccines displaying two model antigens (OTI and OTII) in the pulmonary metastatic melanoma model: ~20 d
Step 49C, evaluation of OMV-based nanovaccines displaying one tumor antigen (Adpgk) in the subcutaneous colon cancer model: ~50 d
Step 49D, evaluation of long-term immune memory elicited by OMV-based nanovaccines displaying model antigen OTI: ~80 d

Procedure 2: HM-NPs

Steps 1–10, isolation of cytoplasmic membranes of *E. coli* (EM): ~40 h
Steps 11–24, isolation of autologous TM: ~9 d
Steps 25–33, preparation of PLGA nanoparticle core: ~1.5 h
Steps 34–36, preparation of HM: ~0.5 h
Steps 37–38, preparation of HM-coated PLGA nanoparticles (HM-NPs): ~0.5 h
Step 39, physicochemical characterization of HM-NPs: ~9 h
Step 40, in vitro experiments with HM-NPs: ~7 d
Step 41, in vivo experiment with HM-NPs: ~90 d

Anticipated results

Procedure 1: OMV-based nanocarriers

The CC OMVs prepared in Procedure 1 exhibit a typical bilayer structure and uniform spherical morphology with a diameter of ~30 nm (Fig. 2a). The antigen display of SpT-OTI has no obvious effect on the morphology and size of CC-SpT-OTI OMVs (Fig. 2b). After repeated freezing and thawing, the morphology and size of CC OMVs remain unchanged, indicating that CC OMVs can be stored at −80 °C (Fig. 2c). The morphology and size are also unaffected by incubation in 10% FBS for 24 h, suggesting that CC OMVs are likely to remain stable in the body long enough for vaccination to be effective (Fig. 2c).

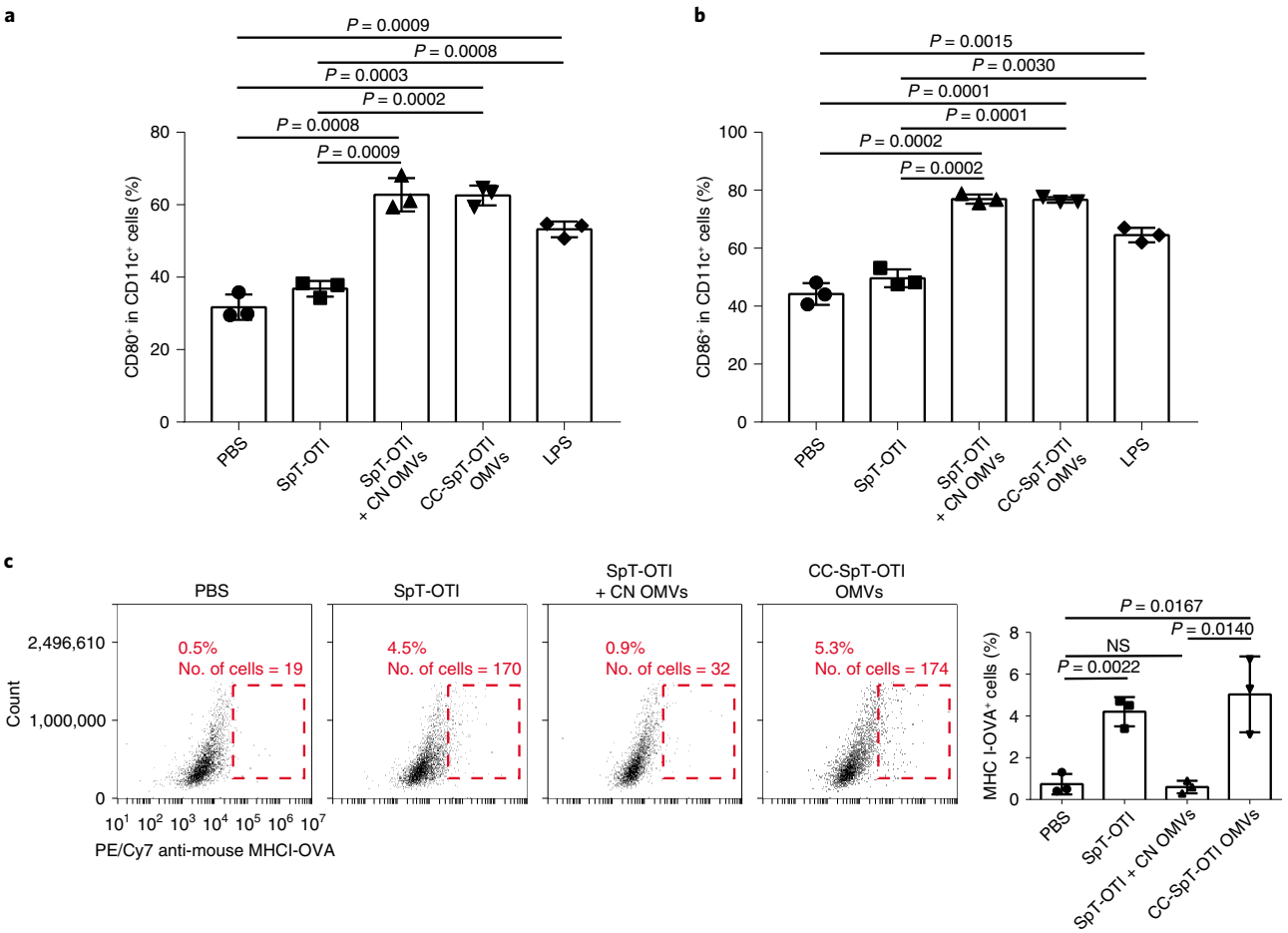


Fig. 3 | Immune stimulation evaluation of OMV-based nanovaccines in vitro. a,**b**, Maturation of BMDCs after the indicated treatments. The expression of the maturation markers $CD80^+$ (**a**) or $CD86^+$ (**b**) is examined as a percentage in $CD11c^+$ cells by flow cytometry. LPS is used as positive control. PBS is used as control, and CN OMVs are from the bacteria expressing ClyA without the fused Catchers (ClyA-none). **c**, Representative flow dot plots and corresponding quantitative data of MHI-OVA^+ cells in BMDCs treated with the indicated formulations. All data are presented as mean ± standard deviation (SD) (n = 3 independent experiments). Statistical analysis is performed by two-tailed unpaired t-test. NS, no significance ($P > 0.05$). Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0.

To verify the antigen display on the CC OMVs, the HA-tagged SpyTag (SpT-HA) and SnoopTag (SnT-HA) are synthesized, respectively. The linkage between SpT-HA and SpC on CC OMVs, or SnT-HA and SnC on CC OMVs, is verified by western blot analysis using an anti-HA antibody, indicated by a concentration-dependent increase in the appearance of the ClyA-SpC-SpT-HA or ClyA-SnC-SnT-HA conjugate (45 kDa) (Fig. 2d). The connection between SpC and SpT or SnC and SnT is stable and is unaffected by storage at different temperatures or treatment with 10% FBS for 24 h (Fig. 2e).

In the immune stimulation evaluation in BMDCs in Procedure 1, Steps 31–48, the CC-SpT-OTI OMVs induce notable maturation and antigen presentation, indicated by the upregulation of the surface expression of CD80, CD86 and MHCI-OVA (Fig. 3a–c and Supplementary Fig. 2a–c). In the evaluation of CC OMVs displaying one tumor antigen (TRP2) in the pulmonary metastatic melanoma model, Procedure 1 Step 49A, immunization with CC-SnT-TRP2 OMVs almost eliminates B16-F10 tumor metastasis in the pulmonary metastatic melanoma model (Fig. 4a,b). All other formulations including the SnT-TRP2, CN OMVs and SnT-TRP2 + CN OMVs are less effective. Flow cytometry and ELISPOT analyses indicate that the CC-SnT-TRP2 OMVs elicit a strong increase in the numbers of $IFN\gamma^+$ cytotoxic T lymphocytes (Fig. 4c and Supplementary Fig. 3a,b) and IFNγ production (Fig. 4d,e) in the splenocytes after restimulation with TRP2 antigen peptide. These results indicate that the CC OMVs displaying a specific tumor antigen can induce a strong antigen-specific immune response.

In the evaluation of CC OMVs displaying two model antigens (OTI and OTII) in the pulmonary metastatic melanoma model, Procedure 1 Step 49B, the strongest antitumor effect is found in the mice immunized with CC-SpT-OTI/SnT-OTII OMVs (Fig. 5a,b). Splenocytes from animals in the

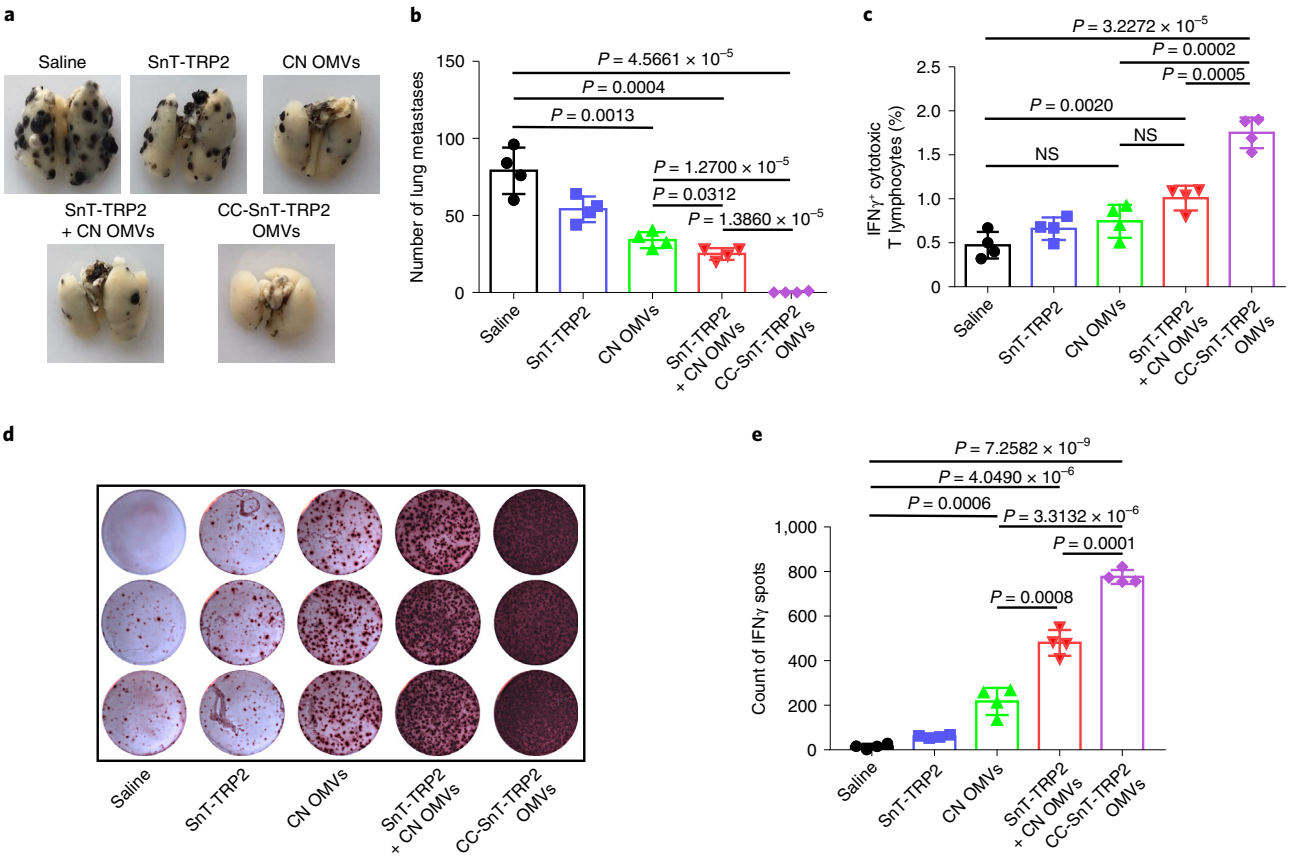


Fig. 4 | Evaluation of OMV-based nanovaccines displaying one tumor antigen (TRP2) in the pulmonary metastatic melanoma model. **a**,**b**, Representative images of lungs collected at the end of the treatment period (**a**) and the counted tumor nodules in the lungs from four mice for each treatment ($n = 4$) (**b**). **c**, Flow cytometry analysis of $IFN\gamma^+$ cytotoxic T lymphocytes in splenocytes restimulated with $TRP2_{180–188}$ antigen peptide. The percentage of $IFN\gamma^+$ cells in the $CD3^+CD8^+$ T-cell subpopulation is shown ($n = 4$). **d**,**e**, IFNγ secretion from splenocytes (as determined by the ELISPOT assay) restimulated with $TRP2_{180–188}$ antigen peptide (**d**). Quantitative analysis of the ELISPOT data is shown in **e** ($n = 4$). The data are shown as mean ± SD. Statistical analysis is performed by a two-tailed unpaired *t*-test. NS, no significance ($P > 0.05$). Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0.

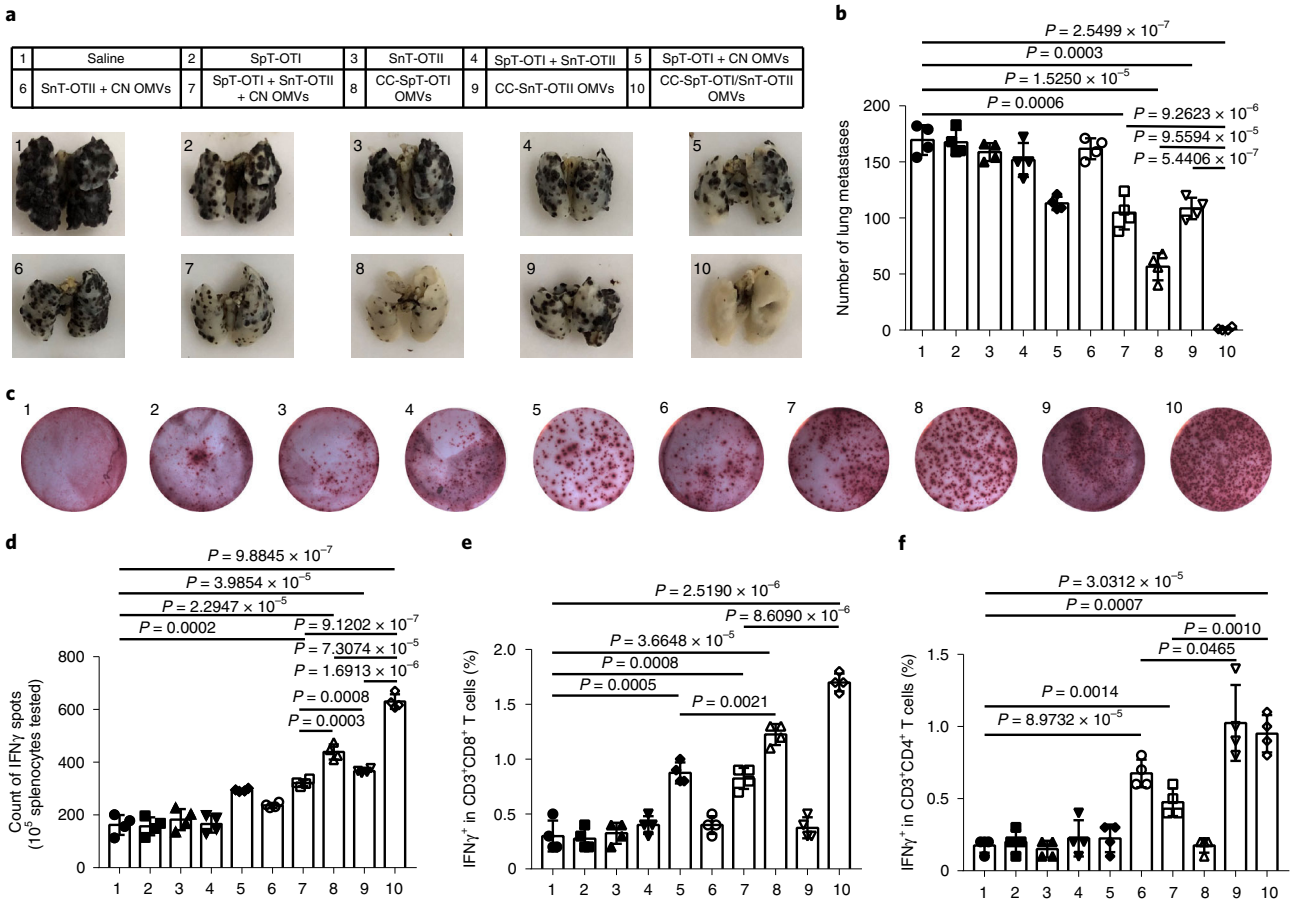


Fig. 5 | Evaluation of OMV-based nanovaccines displaying two model antigens (OTI and OTII) in the pulmonary metastatic melanoma model. **a**,**b**, Representative images of lungs collected at the end of the treatment period (**a**) and the counted tumor nodules in the lungs from four mice for each treatment ($n = 4$) (**b**). **c**,**d**, IFNγ secretion from splenocytes (as determined by the ELISPOT assay) restimulated with OTI and OTII antigen peptides (**c**). Quantitative analysis of the ELISPOT data is shown in **d** ($n = 4$). **e**,**f**, Flow cytometry analysis of IFNγ$^+$ cells in the CD3$^+$CD8$^+$ T-cell subpopulation (**e**) or the CD3$^+$CD4$^+$ T-cell subpopulation (**f**) in splenocytes restimulated with OTI and OTII antigen peptides ($n = 4$). The data are shown as mean ± SD. Statistical analysis is performed by a two-tailed unpaired *t*-test. Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0.

CC-SpT-OTI/SnT-OTII OMV group also secrete the most IFNγ when restimulated with the OTI and OTII antigen peptides (Fig. 5c,d). Interestingly, the increased proportions of IFNγ$^+$ in the CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ cells are detected in the mice immunized with the formulations containing OTI and OTII, respectively (Fig. 5e,f and Supplementary Fig. 4a–c). These data demonstrate that the OMV-based nanocarriers can simultaneously display different types of antigens to trigger a multiple T-cell-mediated, synthetic antitumor immunity.

In the immune stimulation evaluation in the subcutaneous tumor model, Procedure 1, Step 49C, CC-SpT-Adpgk OMVs exhibit the strongest inhibition effects on tumor growth, even stronger than the mixture of SpT-Adpgk antigen with the clinically approved adjuvant Poly (I:C) (Fig. 6a). On day 50, 70% of mice in the CC-SpT-Adpgk group still survive, which is much more than the survivors in the Poly (I:C) + SpT-Adpgk group (30%) (Fig. 6b). Meanwhile, all mice die before day 43 in the saline and SpT-Adpgk + CN OMV groups. The results of tumor immune microenvironment analysis on day 29 show that the infiltration of CD3$^+$ T cells, CD3$^+$CD8$^+$ T cells, CD3$^+$CD4$^+$ T cells, CD11b$^+$Ly6G$^+$ activated neutrophils and CD11c$^+$ DCs are all significantly elevated in MC38 tumor tissues after immunization with the CC-SpT-Adpgk OMVs (Fig. 6c and Supplementary Figs. 5a–d, 6a,b and 7a,b), and the immunosuppressive microenvironment mediated by CD3$^+$CD4$^+$Foxp3$^+$ Tregs is alleviated effectively by CC-SpT-Adpgk OMV treatment (Fig. 6c).

The long-term immune memory evaluation results from Procedure 1, Step 49D, reveal that the immunization of CC-SpT-OTI OMVs induces an obvious increase of CD3$^+$CD8$^+$CD62L$^+$CD44$^+$ central memory T cells (T_{cm}) and CD3$^+$CD8$^+$CD62L$^-$CD44$^+$ effector memory T cells (T_{em}) over 60 d (Fig. 7a and Supplementary Fig. 8a,b). The splenocytes collected on day 60 exhibit a greater cytotoxic effect against B16-OVA cells in the CC-SpT-OTI OMV group than in other groups

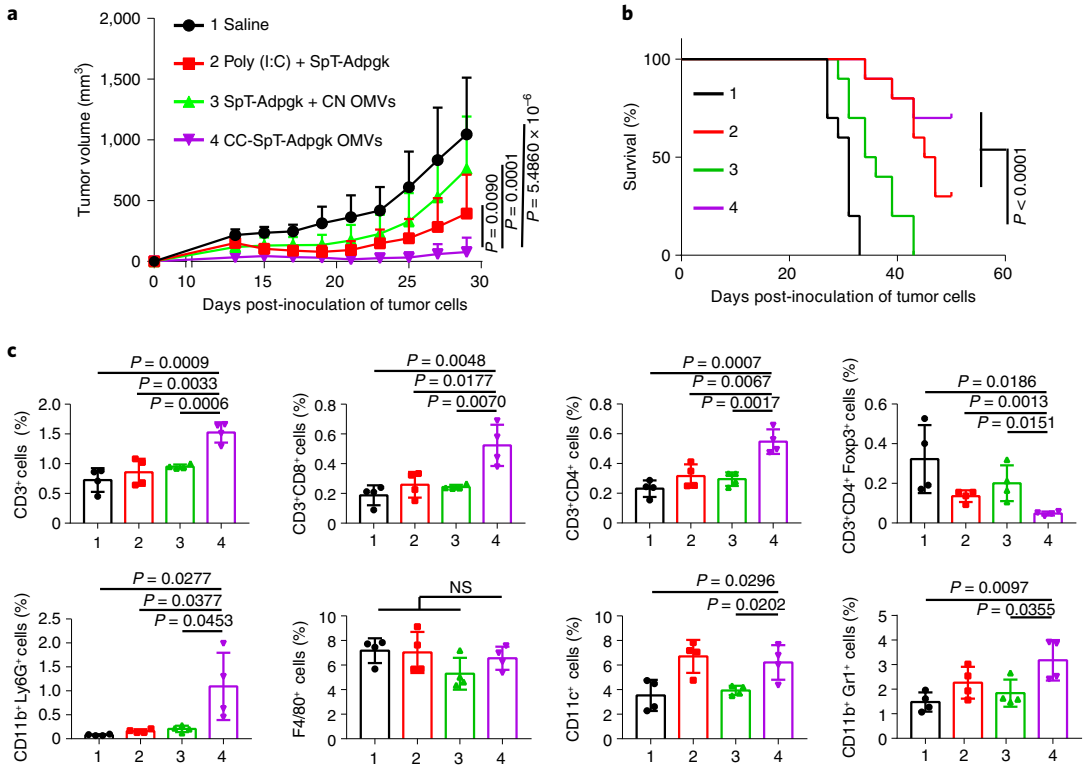


Fig. 6 | Evaluation of OMV-based nanovaccines displaying one tumor antigen (Adpgk) in the subcutaneous colon cancer model. a, Tumor volumes (n = 10). **b**, Mouse survival (n = 10). **c**, Flow cytometry analysis (n = 4) of the following immune cells: $CD3^+$, $CD3^+CD8^+$, $CD3^+CD4^+$, $CD3^+CD4^+Foxp3^+$ T lymphocytes, activated neutrophils ($CD11b^+Ly6G^+$ cells), macrophages ($F4/80^+$ cells), DCs ($CD11c^+$ cells) and MDSCs ($CD11b^+Gr1^+$ cells). The data are shown as mean ± SD. Statistical analysis is performed by a two-tailed unpaired t-test. NS, no significance ($P > 0.05$). Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0.

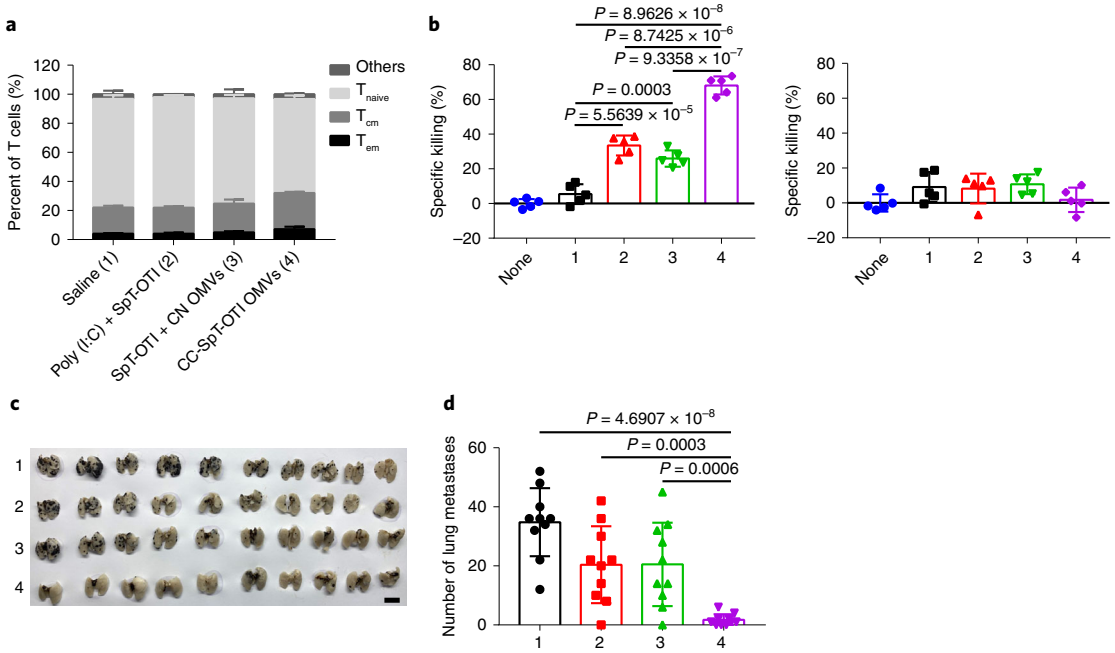
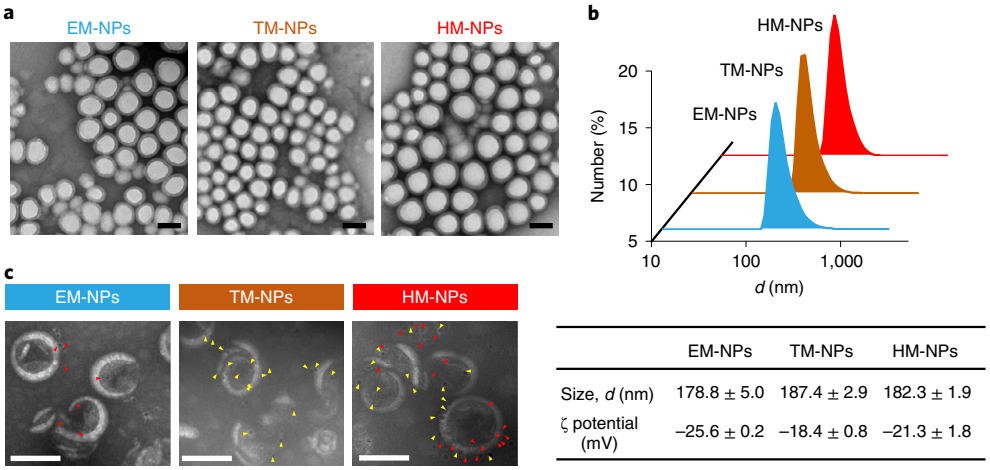


Fig. 7 | Evaluation of long-term immune memory elicited by OMV-based nanovaccines displaying model antigen OTI. a, Quantitative flow cytometry analysis of effector memory T cells (T_{em}, $CD3^+CD8^+CD62L^-CD44^+$), central memory T cells (T_{cm}, $CD3^+CD8^+CD62L^+CD44^+$) and naive T cells (T_{naive}, $CD3^+CD8^+CD62L^+CD44^-$) in splenocytes on day 60, showing CC-SpT-OTI OMV-induced T-cell memory (n = 5). **b**, Specific killing ability of splenocytes collected on day 60 toward B16-OVA cells with OTI antigen (left) and MC38 cells without OTI antigen (right) analyzed by CCK-8 assay (n = 5). **c**,**d**, Lungs collected on day 80 (**c**) and the counted tumor nodules in the lungs (**d**) (n = 10). The data are shown as mean ± SD. Statistical analysis is performed by a two-tailed unpaired t-test. NS, no significance ($P > 0.05$). Figure adapted from ref. [14] under a Creative Commons licence CC BY 4.0.



	EM-NPs	TM-NPs	HM-NPs
Size, *d* (nm)	178.8 ± 5.0	187.4 ± 2.9	182.3 ± 1.9
ζ potential (mV)	−25.6 ± 0.2	−18.4 ± 0.8	−21.3 ± 1.8

Fig. 8 | Characterization of the HM-NPs. a, TEM images of EM-NPs, TM-NPs and HM-NPs. Scale bars, 100 nm. **b**, Size distribution (*d*, diameter; nm, nanometers) and ζ potentials of EM-NPs, TM-NPs and HM-NPs; data in table are presented as mean ± SD ($n = 3$ independent experiments). **c**, TEM images of EM-NPs, TM-NPs and HM-NPs with immunogold labeling of FtsZ (red arrowheads, 5 nm gold nanoparticles) and Na^+/K^+-ATPase (yellow arrowheads, 10 nm gold nanoparticles), followed by negative staining with uranyl acetate. White scale bars, 100 nm. The experiments are performed at least three times. Figure adapted with permission from ref. [15], AAAS.

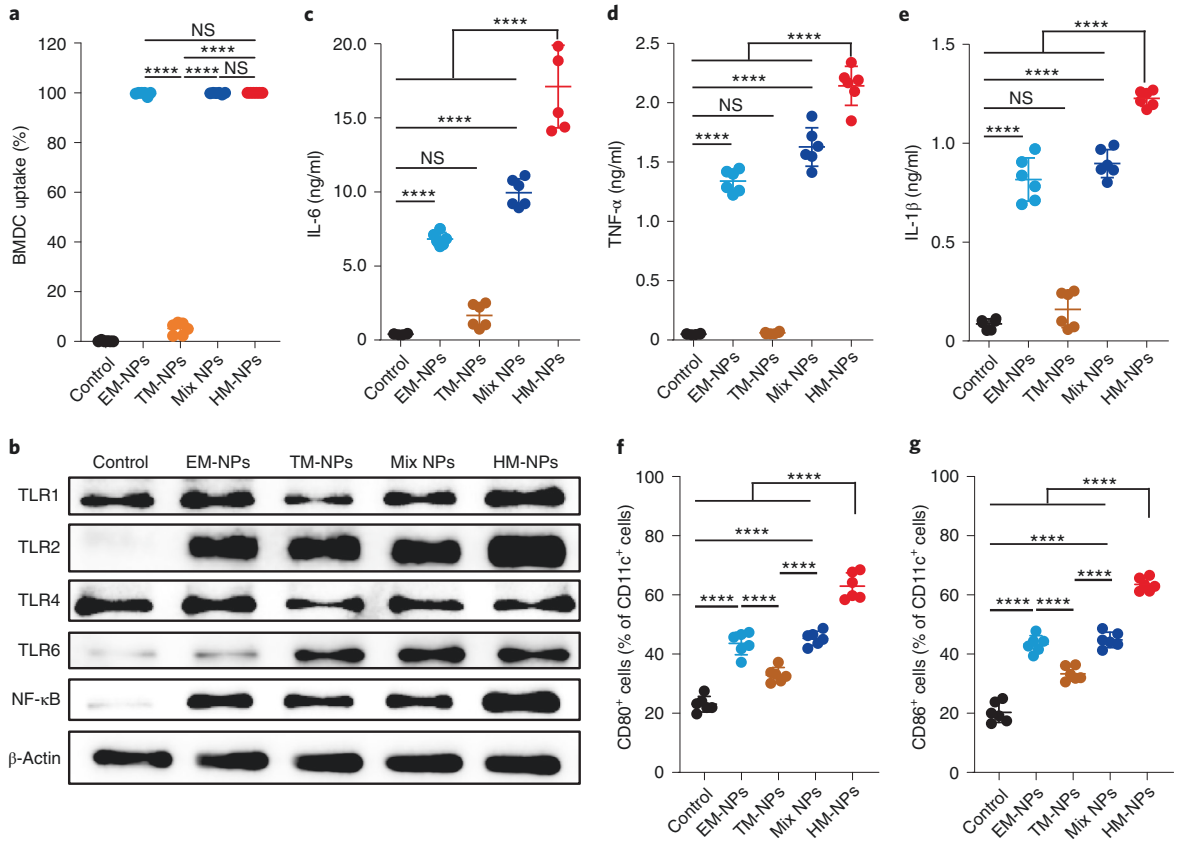


Fig. 9 | BMDC activation and maturation in vitro. a, Cellular uptake of rhodamine B-loaded membrane NPs after an 8-h incubation with BMDCs, as assessed by flow cytometry ($n = 6$, biologically independent samples). **b**, Western blot analysis of a series of membrane TLR proteins and NF-κB in membrane-coated nanoparticles treated BMDCs. The gels are loaded with equal amounts of the proteins (10 μg). **c**–**e**, Proinflammatory cytokine concentrations in BMDC supernatants after incubation with membrane NPs for 24 h, including IL-6 (**c**), TNF-α (**d**) and IL-1β (**e**) ($n = 6$, biologically independent samples). **f**,**g**, Flow cytometry analysis of $CD80^+$ (**f**) or $CD86^+$ (**g**) BMDCs after incubation with membrane NPs for 24 h ($n = 6$, biologically independent samples). Data are presented as mean ± SD. Statistical significance is calculated by one-way ANOVA with Bonferroni's multiple comparisons test or unpaired Student's *t*-test. $^{****}P < 0.0001$; NS denotes no significant difference ($P > 0.05$). Figure adapted with permission from ref. [15], AAAS.

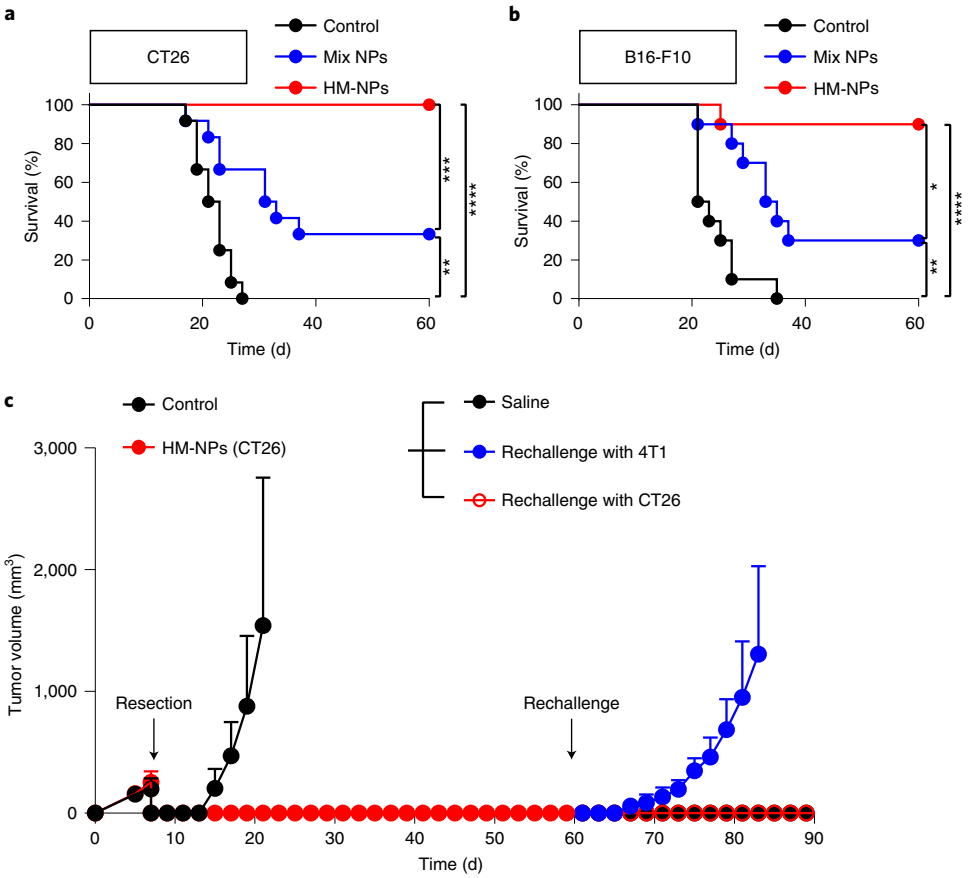


Fig. 10 | Inhibition of tumor reoccurrence by HM-NP vaccination in multiple murine tumor models. a, The survival curves for each group in the CT26 tumor model ($n = 12$). **b**, The survival curves for each group in the B16-F10 tumor model ($n = 10$). **c**, Tumor growth curves after tumor rechallenge. Postoperative mice with CT26 tumors are immunized with HM-NPs. On day 60, the mice treated with HM-NPs are randomized into different groups and inoculated with saline, CT26 or 4T1 cells (2×10^5 tumor cells per mouse; $n = 12$). Statistical significance is analyzed by two-way ANOVA with Bonferroni's multiple comparisons test for growth curves and log-rank (Mantel–Cox) test for comparing survival curves. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. NS denotes no significant difference ($P > 0.05$). Figure adapted with permission from ref. [15], AAAS.

(Fig. 7b). This effect disappears in the experiments using MC38 cells without OTI antigen (Fig. 7b). On day 60, the immunized mice are challenged with i.v. injection of B16-OVA cells. Compared with the obvious lung metastasis in the mice immunized with other formulations, there is almost no lung metastasis in mice in CC-SpT-OTI OMV group (Fig. 7c,d).

Procedure 2: HM-NPs

HM-NPs display a typical spherical structure at the nanoscale, the diameter of HM-NPs is ~180 nm and the average surface charge is ~20 mV (Fig. 8a,b). The immunogold staining followed by TEM imaging provides evidence that FtsZ and Na^+/K^+-ATPase, specific markers of EM and TM, respectively, are both present on the HM-NPs (Fig. 8c).

Flow cytometry results reveal the cellular uptake of HM-NPs by BMDCs (Fig. 9a), and all bacterial cytoplasmic membrane-derived formulations (EM-NPs, Mix NPs and HM-NPs) exhibit ~20-fold greater cell-associated fluorescence than the TM-NP group. After entering BMDCs, the HM-NPs induce the greatest proinflammatory cytokine concentration (Fig. 9c–e) and DC maturation (Fig. 9f,g).

Finally, we evaluated the ability of the HM-NP vaccine for postoperative immunotherapy in the CT26 and B16-F10 tumor models. In total, 100% and 90% of the mice vaccinated with HM-NPs after surgical treatment exhibit no tumor relapse within 60 d in CT26 and B16-F10 tumor models, respectively, whereas 100% tumor relapse is observed in the control groups (Fig. 10a,b). Meanwhile,

the protection provided by HM-NPs is a specific and long-term immune response. The previously described HM-NPs-vaccinated mice in the CT26 tumor model are randomized into three groups and inoculated with saline, CT26 cells or 4T1 cells after 60 d. As shown in Fig. 10c, the mice inoculated with CT26 tumor cells show complete tumor elimination and a tumor inhibition rate of 100%. The tumor volumes in the mice inoculated with 4T1 cells reach up to 1,000 cm^3 after 3 weeks.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The main data discussed in this protocol are available in the supporting primary research papers (refs. [14,15]). Source data for Figs. 2–10 and Supplementary Fig. 1 are provided as Supplementary information. Source data are provided with this paper.

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Author contributions

X.Z. and G.N. originated the method of preparing OMV-based nanocarriers; R.Z. and G.N. originated the method of preparing HM-NPs. All authors wrote the manuscript and approved the contents of the protocol.

Competing interests

G.N. and X.Z. are inventors on a filed provisional application China patent no. CN202011407688.7 (Bacterial outer membrane vesicle, a general nanovaccine containing bacterial outer membrane vesicle, preparation method and application thereof), submitted by the National Center for Nanoscience and Technology that covers the potential diagnostic and therapeutic uses of OMV-based nanovaccine for cancer immunotherapy. G.N. and R.Z. are inventors on a filed provisional application China patent no. CN202010418720.5 (Nanovaccine to prevent cancer recurrence), submitted by the National Center for Nanoscience and Technology that covers the potential diagnostic and therapeutic uses of HM-NP vaccine for cancer immunotherapy. The authors declare that they have no other competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-022-00713-7.

Correspondence and requests for materials should be addressed to Guangjun Nie.

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Key references using this protocol

Cheng, K. et al. *Nat. Commun.* **12**, 2041 (2021): https://doi.org/10.1038/s41467-021-22308-8
Chen, L. et al. *Sci. Transl. Med.* **13**, eabc2816 (2021): https://doi.org/10.1126/scitranslmed.abc2816

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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

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Software and code

Policy information about availability of computer code

Data collection	BD Accuri C6 Software (version number, 1.0.264.21) were used to collect data of flow cytometry.
Data analysis	ImageJ (version number, 1.8.0) was used to analyze the data of western blot analysis and Coomassie blue staining. BD Accuri C6 Software (version number, 1.0.264.21) and FlowJo (version number, 10.0.0.0) were used to analyze the data of flow cytometry. GraphPad Prism (version number, 5.0 for Procedure 1 and 8.0 for Procedure 2) and IBM SPSS Statistics (version number, 19.0) were used for the statistical analysis.

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- A description of any restrictions on data availability

The main data discussed in this protocol are available in the supporting primary research papers (ref.14,15). Source data for Figures 2-10 and Supp. Figure 1 is provided as Supplementary information.

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Sample size	No statistical methods were used to determine sample size. In vitro experiments were performed with at least 3 biologically independent samples. All in vivo experiments were performed with at least 3 independent animals. Sample sizes were sufficient to perform statistical analyses.
Data exclusions	No samples were excluded.
Replication	All attempts at replication were successful. Experimental repeat numbers are also reported in Figure Legends.
Randomization	All samples/organisms were numbered and randomly grouped by random number table method.
Blinding	All experimental procedures and quantification of results, including injections, isolation of the tumors or organs, tissue histological analysis and flow cytometry, were done by two independent researchers. Meanwhile, all researchers were blinded to group allocation.

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Procedure 1:
Anti-HA tag antibody (Abcam, cat. no. ab236632, Clone: EPR22819-101, RRID: AB_2864361)
HRP-conjugated secondary antibody (goat anti rabbit IgG, Invitrogen, cat. no. A16096, Clone: polyclone, RRID: AB_2534770)
FITC-anti-mouse CD11c (BioLegend, cat. no. 117306, Clone: N418, RRID: AB_313775)
PE/Cy7-anti-mouse CD80 (BioLegend, cat. no. 104734, Clone: 16-10A1, RRID: AB_2563112)
APC-anti-mouse CD86 (BioLegend, cat. no. 105012, Clone: GL-1, RRID: AB_493342)
PE/Cy7-anti-mouse H-2Kb bound to SIINFEKL (BioLegend, cat. no. 141608, Clone: 25-D1.16, RRID: AB_11218593)
FITC-anti-mouse CD3 (BioLegend, cat. no. 100204, Clone: 17A2, RRID: AB_312661)
APC-anti-mouse CD8 (BioLegend, cat. no. 100712, Clone: 53-6.7, RRID: AB_312751)
PE/Cy7-anti-mouse IFNγ (BioLegend, cat. no. 505826, Clone: XMG1.2, RRID: AB_2295770)
PE-anti-mouse CD4 (Invitrogen, cat. no. 2013481, Clone: RM4-5, RRID: unknown)
APC-anti-mouse CD3 (BioLegend, cat. no. 100236, Clone: 17A2, RRID: AB_2561456)
FITC-anti-mouse CD8 (Invitrogen, cat. no. 2002714, Clone: 53-6.7, RRID: unknown)
FITC-anti-mouse CD4 (BioLegend, cat. no. 100406, Clone: GK1.5, RRID: AB_312691)
PE-anti-mouse Foxp3 (BioLegend, cat. no. 126404, Clone: MF-14, RRID: AB_1089117)
FITC-anti-mouse Ly6G (BioLegend, cat. no. 127606, Clone: 1A8, RRID: AB_1236494)
APC-anti-mouse CD11b (BioLegend, cat. no. 101212, Clone: M1/70, RRID: AB_312795)
PE/Cy7-anti-mouse F4/80 (BioLegend, cat. no. 123114, Clone: BM8, RRID: AB_893478)
PE-anti-mouse CD11b (BioLegend, cat. no. 101208, Clone: M1/70, RRID: AB_312791)
APC-anti-mouse Gr1 (BioLegend, cat. no. 108412, Clone: RB6-8C5, RRID: AB_313377)
PE-anti-mouse CD44 (BioLegend, cat. no. 103008, Clone: IM7, RRID: AB_312959)
PE/Cy7-anti-mouse CD62L (BioLegend, cat. no. 104418, Clone: MEL-14, RRID: AB_313103)

Procedure 2:
Anti-mouse IgG-gold conjugate (10 nm) (Fitzgerald, cat. no. 43R-IG084GD, Clone: unknown, RRID: AB_1286630, 1:20).
Anti-rabbit IgG-gold conjugate (5 nm) (Fitzgerald, cat. no. 43R-IG100GD, Clone: unknown, RRID: AB_1286646,1:20).
Anti-mouse Na+/K+ ATPse (Abcam, cat. no. ab76020, Clone: polyclone, RRID: AB_1310695, undiluted for Immunogold staining).
Anti-Ftsz (Agrisera, cat. no. AS10-715, Clone: polyclone, RRID: AB_10754647, https://www.agrisera.com/en/artiklar/ftsz-procaryotic-cell-division-gtpase-2.html).
Anti-mouse TLR4 (Abcam, cat. no. ab13867, Clone: polyclone, RRID: AB_300696, 1:200 for western blot)
Anti-mouse TLR1 (Abcam, cat. no. ab180798, Clone: polyclone, RRID: unknown, 1:1,000 for western blot)
Anti-mouse TLR2 (Abcam, cat. no. ab209216, Clone: EPR20302-119, RRID: unknown, 1:100 for western blot)
Anti-mouse TLR6 (CST, cat. no. 12717, Clone: D1Z8B, RRID: AB_2798005, 1:200 for western blot)
Anti-mouse NF-κB (Abcam, cat. no. ab16502, Clone: polyclone, RRID: AB_443394, 1:2,000 for western blot)
Anti β-actin (Abcam, cat. no. ab8226, Clone: Abcam 8226, RRID: AB_306371,1:5,000 for western blot)
FITC-anti-mouse CD11c (Biolegend, cat. no. 117305, Clone: N418, RRID: AB_313774, 1:100)
PE-anti-mouse CD80 (Biolegend, cat. no. 104707, Clone: 16-10A1, RRID: AB_313128,1:200)
PE-anti-mouse CD86 (Biolegend, cat. no. 105007, Clone: GL-1, RRID: AB_313150, 1:200)

Validation

All primary antibodies were purchased from the supplier as noted above and used without additional validation. The validation of all the antibodies could be found from manufacturers online:
Procedure 1:
Anti-HA tag antibody (ab236632): https://www.abcam.cn/ha-tag-antibody-epr22819-101-ab236632.html
HRP-conjugated secondary antibody (A16096): https://www.thermofisher.cn/cn/zh/antibody/product/Goat-anti-Rabbit-IgG-H-L-Secondary-Antibody-Polyclonal/A16096
FITC-anti-mouse CD11c (117306): https://www.biolegend.com/en-us/products/fitc-anti-mouse-cd11c-antibody-1815
PE/Cy7-anti-mouse CD80 (104734): https://www.biolegend.com/en-us/products/pe-cyanine7-anti-mouse-cd80-antibody-9320
APC-anti-mouse CD86 (105012): https://www.biolegend.com/en-us/products/apc-anti-mouse-cd86-antibody-2896
PE/Cy7-anti-mouse H-2Kb bound to SIINFEKL (141608): https://www.biolegend.com/en-us/products/pe-cyanine7-anti-mouse-h-2kb-bound-to-siinfekl-antibody-7883
FITC-anti-mouse CD3 (100204): https://www.biolegend.com/en-us/products/fitc-anti-mouse-cd3-antibody-45
APC-anti-mouse CD8 (100712): https://www.biolegend.com/en-us/products/apc-anti-mouse-cd8a-antibody-150
PE/Cy7-anti-mouse IFNγ (505826): https://www.biolegend.com/en-us/products/pe-cyanine7-anti-mouse-ifn-gamma-antibody-5865
PE-anti-mouse CD4 (2013481): https://assets.thermofisher.cn/TFS-Assets/LSG/certificate/Certificates-of-Analysis/12004285_2013481.PDF
APC-anti-mouse CD3 (100236): https://www.biolegend.com/en-us/products/apc-anti-mouse-cd3-antibody-8055
FITC-anti-mouse CD8 (2002714): https://assets.thermofisher.cn/TFS-Assets/LSG/certificate/Certificates-of-Analysis/11008185_2002714.PDF
FITC-anti-mouse CD4 (100406): https://www.biolegend.com/en-us/products/fitc-anti-mouse-cd4-antibody-248
PE-anti-mouse Foxp3 (126404): https://www.biolegend.com/en-us/products/pe-anti-mouse-foxp3-antibody-4660
FITC-anti-mouse Ly6G (127606): https://www.biolegend.com/en-us/products/fitc-anti-mouse-ly-6g-antibody-4775
APC-anti-mouse CD11b (101212): https://www.biolegend.com/en-us/products/apc-anti-mouse-human-cd11b-antibody-345
PE/Cy7-anti-mouse F4/80 (123114): https://www.biolegend.com/en-us/products/pe-cyanine7-anti-mouse-f4-80-antibody-4070
PE-anti-mouse CD11b (101208): https://www.biolegend.com/en-us/products/pe-anti-mouse-human-cd11b-antibody-349
APC-anti-mouse Gr1 (108412): https://www.biolegend.com/en-us/products/apc-anti-mouse-ly-6g-ly-6c-gr-1-antibody-456
PE-anti-mouse CD44 (103008): https://www.biolegend.com/en-us/products/pe-anti-mouse-human-cd44-antibody-2206
PE/Cy7-anti-mouse CD62L (104418): https://www.biolegend.com/en-us/products/pe-cyanine7-anti-mouse-cd62l-antibody-1922

Procedure 2:
Anti-mouse IgG-gold conjugate (43R-IG084GD): https://www.fitzgerald-fii.com/goat-anti-mouse-igg-10-nm-gold-colloid-43r-ig084gd.html
Anti-rabbit IgG-gold conjugate (43R-IG100GD): https://www.fitzgerald-fii.com/goat-anti-rabbit-igg-5-nm-gold-colloid-43r-ig100gd.html
Anti-mouse Na+/K+ ATPse (ab76020): https://www.abcam.com/sodium-potassium-atpase-antibody-ep1845y-plasma-membrane-loading-control-ab76020.html
Anti-Ftsz (AS10-715): https://www.agrisera.com/en/artiklar/ftsz-procaryotic-cell-division-gtpase-2.html
Anti-mouse TLR4 (ab13867): https://www.abcam.com/tlr4-antibody-ab13867.html
Anti-mouse TLR1 (ab180798): https://www.abcam.cn/tiltlr1-antibody-ab180798.html
Anti-mouse TLR2 (ab209216): https://www.abcam.com/tlr2-antibody-epr20302-119-ab209216.html
Anti-mouse TLR6 (12717): https://www.cellsignal.com/products/primary-antibodies/toll-like-receptor-6-d1z8b-rabbit-mab/12717
Anti-mouse NF-κB (ab16502): https://www.abcam.cn/nf-kb-p65-antibody-ab16502.html
Anti β-actin (ab8226): https://www.abcam.cn/beta-actin-antibody-mabcam-8226-loading-control-ab8226.html
FITC-anti-mouse CD11c (117305): https://www.biolegend.com/ja-jp/products/fitc-anti-mouse-cd11c-antibody-1815?GroupID=BLG11937
PE-anti-mouse CD80 (104707):https://www.biolegend.com/en-us/products/pe-anti-mouse-cd80-antibody-43
PE-anti-mouse CD86 (105007): https://www.biolegend.com/en-us/products/pe-anti-mouse-cd86-antibody-256?GroupID=BLG10719

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Procedure 1:
The B16-F10 murine melanoma cell line (ATCC, Manassas, USA Cat. no. CRL-6475, RRID: CVCL_0159, sex chromosomes: XY), American Type Culture Collection (ATCC, Manassas, USA).
The MC38 murine colon cancer cell line (CRC cat. no. 1101MOU-PUMC000523, RRID: CVCL_B288, sex chromosomes: XX), Cell Resource Center, IBMS, CAMS/PUMC.

	The B16-OVA cell is generously provided by Prof. Wang Hao at the National Center for Nanoscience and Technology, which was constructed from B16-F10 that was originally purchased from American Type Culture Collection (Manassas, VA, USA) through stable transfection of the ovalbumin gene (Gene ID: 396058).. Procedure 2: Murine 4T1 breast tumor cell (ATCC, Manassas, USA, cat. no. CRL-2539, RRID: CVCL_0125, sex chromosomes: XX) Murine B16-F10 melanoma cell (ATCC, Manassas, USA cat. no. CRL-6475, RRID: CVCL_0159, sex chromosomes: XY) Murine CT26 colon carcinoma cell (ATCC, Manassas, USA cat. no. CRL-2638, RRID: CVCL_7256, sex chromosomes: unknown)
Authentication	All cell lines were validated with cell line authentication by the short tandem-repeat DNA profiling.
Mycoplasma contamination	All cell lines were carried out with mycoplasma detection and were negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	All cell lines are not listed in the database.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	All animal studies were performed in accordance with ARRIVE guidelines. The mice were fed in a room at 20-22 °C with a 12-h light/dark cycle and a humidity of 30–70%. Provide food and water ad libitum. Procedure 1: C57BL/6 mice (6-8 week old), Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Procedure 2: C57BL/6 and BALB/c Mice (6-8 week old), Vital River Laboratory Animal Technology Co. Ltd (Beijing, China).
Wild animals	This study did not involve wild animals.
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	All animal studies were approved by the Institutional Animal Care and Use Committee of National Center for Nanoscience and Technology.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	The sample preparation method was described in the Protocol.
Instrument	BD Accuri C6 (BD Biosciences, USA) was used to analyze samples.
Software	BD Accuri C6 Software (version number, 1.0.264.21) and FlowJo (version number, 10.0.0.0) were used to analyze the data of flow cytometry.
Cell population abundance	Over 10000 cells were analyzed for fluorescent intensity in the defined gate.
Gating strategy	A gate is drawn around the cells. Single cells are determined with the area and the height of the side scatter (SSC). The analysis was carried out in this gate.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.