Across-cancer specific immune responses induced by nanovaccines or microvaccines to prevent different cancers and cancer metastasis

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Highlights
Nanovaccines and microvaccines made from whole tumor tissue prevent different cancers
Across-cancer immune responses exist among different types of cancers
Many neoantigens are shared by melanoma cells and lung cancer cells
Allotransplanting T cells from immunized healthy individuals to cancer patients

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Across-cancer specific immune responses induced by nanovaccines or microvaccines to prevent different cancers and cancer metastasis

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SUMMARY
Metastatic cancers and recurrent cancers are diverse, different from primary cancers, and organ-dependent. However, how strong are across-cancer immune responses among different types of cancers remain unclear. Herein, vaccines-encapsulated-whole-components-of-tumor-tissue (VEWCOTT) were applied to demonstrate the across-cancer immune responses, thanks to inducing pan-clones T-cell immune responses. Either lung-cancer-tissue- or melanoma-tissue-based VEWCOTT simultaneously prevented melanoma, lung cancer, hepatoma, and metastatic cancer, which showed that strong across-cancer immune responses were induced. Both nanovaccines and microvaccines showed potent across-cancer prevention efficacy. VEWCOTT induced tumor-specific T cells in peripheral immune organs and major organs, and adjusted the immune-microenvironment of cancer-colonized organs. In addition, the allograft of T cells from VEWCOTT immunized mice to allogeneic naive mice efficiently prevent various cancers. Many neoantigens are shared by melanoma cells and lung cancer cells. Across-cancer immune responses exist among different types of cancers, and thus VEWCOTT has the advantage of simultaneously preventing cancer metastasis and cancers in different organs.

INTRODUCTION
Cancer metastasis in different organs and cancer recurrence are the major reasons for the death of patients with cancer after excision or other treatments. However, because metastatic cancers and recurrent cancers are normally different from the primary cancer and are organ-dependent, preventing cancer metastasis and cancer recurrence in multiple organs is challenging. Besides, because of the heterogeneity and complexity of cancer antigens, how strong are the across-cancer immune responses and the extent of cross-over of antigens among different types of cancers remain unclear. No studies have systematically investigated these yet because proper cancer vaccines loaded with whole-tumor-antigens were lacking, and vaccines-encapsulated-whole-components-of-tumor-tissue (VEWCOTT) is ideal to be applied to illustrate the cross-reaction among different cancers, thanks to VEWCOTT that can induce pan-clones T-cell immune responses.

Cancer vaccine can stimulate tumor-specific immune responses, induced by antigens and mutations in cancer cells, and thus is a good tool to illustrate such cross-talk among different types of cancers. In order to efficiently and optimally prove the cross-talk among different types of cancers, pan-spectrum antigens are needed to be included in the formulation of cancer vaccines and thus to be uptake by antigen-presenting cells (APCs), especially dendritic cells (DCs), for optimal tumor-specific T cell activation. Given that tumor tissues contain whole-tumor-cell antigens to induce pan-spectrum cancer-specific immune responses, tumor tissue itself is the best source to prepare cancer vaccines. To what extent do cancer antigens overlap among different types of cancers? Can cancer vaccines, made from tumor tissue, have extra benefits in inducing across-cancer immune responses? Do the cancer-specific immune responses have across-cancer-prophylactic effects among different types of cancers? We have demonstrated that our vaccine can induce memory T cells in peripheral immune organs, and can such vaccines also induce memory T cells located in major organs, such as the lung? These questions need to be illustrated by utilizing a cancer vaccine loading with pan-spectrum cancer antigens and no studies have done so yet.
Though tumor tissue is the best antigen source to prepare cancer vaccines, injecting lysates of tumor tissues directly back into patients induced very limited immune responses and didn’t show good efficacy, due to most cell lysate components don’t have the proper size for endocytosis and thus can not induce strong cancer-specific immune responses. Therefore, to be efficiently uptake by APCs and subsequently activate the tumor-specific T cells, the tumor tissue lysates need to be encapsulated into vaccines with proper size. We have proved that nano-sized cancer vaccines are preferentially uptake by APCs. However, whether micron-sized cancer vaccine can achieve similar efficacy is unknown. Therefore, nano-sized nano-vaccines (NVs) and micron-sized microvaccines (MVs), loaded with the whole components of tumor tissue, need to be compared in terms of inducing tumor-specific immune responses.

In clinics, many patients can’t avail the benefits of immunotherapy or metastasis prevention, because of the poor physical conditions, such as elder patients with weak immune abilities. Because the immune systems of such patients are too weak, cancer vaccines might not be suitable for injecting directly. Are there any alternative strategies to solve this problem?

In order to answer the above questions, we designed and conducted this study. Considering that the higher the diversity of the tumor antigens in cancer vaccines, the better is the vaccine, we loaded whole-components of tumor tissues to PLGA nanoparticles (NPs) or microparticles (MPs). The sizes of nano-vaccines (NVs) are approximately 300 nm and the sizes of microvaccines (MVs) are approximately 1.0 μm. Besides, we co-encapsulated immune adjuvants into the NVs or MVs to facilitate the efficient activation of extensive tumor-specific immune responses. Furthermore, both lung cancer tumor tissues and melanoma tumor tissues were loaded into the vaccines for cross-prophylactic investigations. It was found that such VEWCOTT, either melanoma- or lung-cancer-based, exhibited excellent cross-prevention efficacy in preventing melanoma, lung cancer, hepatoma, and melanoma metastasis in the lung. VEWCOTT induced memory T cells in the lung, besides peripheral immune organs, which is beneficial for protecting the lung from the invasion of cancer cells. In addition, transferring adoptive T cells from VEWCOTT immunized mice to allogeneic naive mice efficiently prevent various cancers. Proteomics studies showed that melanoma tissue-based vaccines or lung cancer tissue-based vaccines shared many neoantigens, and this is probably the major reason why cross-react immune responses are strong among different types of cancers. These cross-react immune responses are meaningful, considering the diversity of metastatic cancers in different organs and recurrent cancers.

RESULTS
Characterization of NV and MV
In order to re-assemble tumor tissues into nano-sized vaccines or micro-sized vaccines, the water-soluble components and water-insoluble components were loaded into PLGA NPs or MPs to prepare NV A or MV A and NV B or MV B (Figure S1A), and NV A (MV A) together with NV B (MV B) formed the VEWCOTT system. To maximize the loading capacity of NVs (MVs) and thus induce immune responses more efficiently, antigens were loaded both inside and on the surface of NV (MV). In addition, co-delivery of immune adjuvants and tumor antigens to APCs can activate tumor-specific immune responses more efficiently than delivery of tumor antigens alone. Therefore, poly(I:C), which can stimulate TLR3, was co-encapsulated with tumor antigens into NV/MV. Once the NVs/MVs were uptaken by APCs, poly(I:C) could be released together with the antigens in endosomes and stimulate TLR3 (located on endosomes membrane of DCs) to promote pro-inflammatory cytokine secretion and assist the induction of tumor-specific T cells (Figure S1B). In order to compare the impact of the location of an adjuvant target, Bacillus Calmette-Guérin (BCG), which stimulates the TLR4 on the surface of the cell membrane, was also investigated as an immune adjuvant.

The sizes of NVs after antigen coating were approximately 300 nm and the sizes of MVs after antigen coating were approximately 1.0 μm, and both NVs and MVs were slightly negatively charged (Figures S2A–S2E). In the morphologic studies, both NV and MV have spherical structures under the observation of transmission electron microscopy (TEM) and confocal microscopy (Figures S2C and S2F).

Both NV and MV could be efficiently uptake by DC2.4 cells or APCs in lymph nodes (Figures S3A–S3E). Confocal microscopy analysis was applied to investigate the cross-presentation induced by NVs and MVs, and the involvement of MHC I and MHC II pathways in antigen presentation. In this study, we encapsulated FITC-labeled OVA into PLGA NPs to study the release of antigens in endosome-lysosomes after
Figure 1. Analysis of the cross-prophylactic and immunotherapy efficacy of nanovaccines (NVs) made from lung cancer tumor tissues in various cancers

(A) Timeline (days) of vaccine immunization and lung cancer tumor inoculation.
(B–D) Lung cancer tumor growth curves and survival curves of mice treated with NV (n ≥ 8).
(E) Timeline (days) of vaccine immunization and melanoma tumor inoculation.
(F–H) Melanoma tumor growth curves and survival curves of mice treated with NV (n ≥ 8).
(I and J) Tumor growth curves and survival curves of recovered mice rechallenged with melanoma (n ≥ 8).
(K) Timeline (days) of vaccine immunization and hepatoma tumor inoculation.
(L–N) Hepatoma tumor growth curves and survival curves of mice treated with NV (n ≥ 8).
(O and P) Tumor growth curves and survival curves of recovered mice rechallenged with hepatoma (n ≥ 8).
(Q) Timeline (days) of melanoma tumor inoculation and NV immunotherapy.
being uptake by APCs. FITC-OVA is used to replace tumor antigens in order to observe the uptake of antigens by cells and release in cells. The fluorescence signal of FITC in endosome-lysosomes represents the antigens released in endosome-lysosomes and will be presented by the MHC II pathway; the fluorescence signal of FITC in cytoplasm represents the antigens escaped from endosome-lysosomes and will be presented by the MHC I pathway. It was found that, in both NV and MV, payloads were partly released in the endosome-lysosome system and partly escaped from the endosome-lysosome system into the cytosol for cross-presentation (Figures S3C and S3D). This result demonstrated that antigens in both NVs and MVs can be presented by MHC I and MHC II pathways, and thus stimulate both CD4+ and CD8+ cancer-specific T cells4,13,14,23–27 (Figure S1B).

The cross-presentation within DCs is critical for presenting antigens through both MHC I and MHC II pathways. The maturation of DCs verified that cross-presentation was induced by NVs and MVs. Herein, we induced bone marrow dendritic cells (BMDCs) from bone marrow cells (Figures S4A and S4B) and the studies showed that both NVs and MVs significantly increased the amount of MHC I molecules and MHC II molecules on the surface of DCs, indicating that NVs and MVs induced strong cross-presentation (Figures S4C–S4F). In addition, both NVs and MVs promoted the maturation of DCs, which illustrated that NVs and MVs could activate the immune responses efficiently.

**Lung cancer tumor tissues based NVs demonstrated across-cancer immune responses**

In order to demonstrate the across-cancer immune responses induced by tumor-tissue-based vaccines, prevention studies of NVs, made from lung cancer tumor tissues, were first conducted in lung cancer, melanoma, and hepatoma (Figures 1 and S5). NVs were administrated multiple times ahead of tumor-inoculation in the study (Figures 1A, 1E, and 1K).

In the prophylactic studies conducted in a subcutaneous lung cancer model, all mice in control groups, including PBS control, BCG control, and blank NP (containing poly (I:C)) control, developed lung cancer and died within 42 days. The mice, immunized with NVs containing lung cancer tumor tissue components but without immune adjuvants, displayed delayed tumor growth and prolonged survival time, including 30% of mice that became tumor-free. However, 100% mice, immunized with NVs made from lung cancer tumor tissues and immune adjuvants, became tumor-free after tumor-inoculation (Figures 1A–1D and S5A–S5F). These data showed that either poly(I:C) or BCG, when applied as immune adjuvants, improved the prophylactic efficacy of NVs.

In the studies conducted on melanoma and hepatoma, immunization mice with such NVs prevented 75% of the occurrence of melanoma and hepatoma, when immunized mice were across-cancer challenged with melanoma and hepatoma (Figures 1E–1H, 1K–1N, S5S–S5K, and S5I–S5H). In addition, when these complete response immunized mice were rechallenged with double amounts of cancer cells injection, all of these mice became tumor-free again after tumor-inoculation (Figures 1I, 1J, 1O, and 1P). These implied that tumor-specific immune memories were induced and existed in these treated mice.

In the above studies, both poly (I:C) and BCG showed high adjuvant efficacy in facilitating antigens to activate immune responses. Poly (I:C) performed better than BCG, and this is probably because the receptors of poly(I:C) localize in endosomes of DCs28–35 which facilitate the co-release of antigens and adjuvants in the endosomes of DCs.

To further verify the across-cancer immunotherapy efficacy, lung-cancer-based NVs were applied to treat the melanoma-bearing mice (Figures 1Q) and the results demonstrated that administration of lung cancer NVs can inhibit the melanoma tumor growth and prolong the survival time of mice (Figures 1R and 1S). These results indicated that the treatment with VEWCOTT has the benefits of killing different types of cancer cells, thanks to VEWCOTT-loaded whole-tumor cell neo-antigens and there are some overlaps of these neo-antigens among different types of cancer.
These results proved that NVs made from one type of tumor tissue had the cross-prophylactic effect among different types of cancers, indicating that sharing-antigens exist among different types of cancer. Thus, when NVs, made from whole-cell components of tumor tissues, were applied to prevent the occurrence of homologous cancer, or to prevent cancer relapse after surgical excision of cancer lesions, they have the advantage of preventing heterogeneous cancers. This is crucial in preventing cancer metastasis, given that metastatic cancers in different organs are different from the primary cancer because organs can impact the properties of cancers.

Memory T cells induced by prophylactic immunization in lung and immune organs

Long-term immune memories are critical in controlling containing and eliminating cancer. In previous cancer immunotherapy studies, we have reported that treated mice bearing melanoma with NVs (loaded with whole-cell components of melanoma cancer cell lines) induced memory T cells in the lymph node, spleen, and blood.\textsuperscript{15} However, memory T cells induced by prophylactic administration of NVs, loaded with whole-cell components of lung cancer tumor tissues, have not been investigated yet. In addition, the memory T cells in the major organ lung, induced by NVs made from tumor tissues, have not been studied yet in any previous studies.

To verify NVs made from lung cancer tumor tissues can also induce potent tumor-specific immune memories in preventative-immunized mice in lung and other peripheral immune organs, the amount of memory T cells in NV-immunized mice was investigated by flow cytometric analysis of sub-groups of memory T cells in the lung, lymph node, spleen, and peripheral blood (Figures 2 and S5).

Various CD8\textsuperscript{+} (Figure 2A) or CD4\textsuperscript{+} (Figure 2B) memory T cells were analyzed in mice immunized multiple times with NV made from lung cancer tumor tissues. It was observed that CD8\textsuperscript{+} central memory T cells (Tcm) significantly increased in the lung, lymph node, and blood, and CD4\textsuperscript{+} Tcm increased in the spleen. Besides, CD4\textsuperscript{+} Tem significantly increased in splenocytes. CD8\textsuperscript{+} tissue-resident memory T cells (Trm) increased in the lymph node and spleen, and CD4\textsuperscript{+} tissue-resident memory T cells (Trm) increased in the spleen and blood.
Tcm, Tem, and Trm are all playing roles in anti-tumor immune responses and Tcm, especially CD8\(^+\) Tcm, probably are the most important population among all memory T cells in anti-tumor immunity. CD8\(^+\) Tcm increased in the lung, lymph node, and peripheral blood indicated that, when encountering tumor cells, the immune system could react very rapidly to produce a large amount of tumor-specific T cells to kill the tumor cells. The results above illustrated that prophylactic injection of NVs efficiently induced memory T cells, especially Tcm, in major organ lung and peripheral immune organs, which is beneficial for cancer prevention in organs. Especially the increase of Tcm in the lung can provide special protection for the major organ lung from cancer cell invasion. These data indicated that prophylactic administration of NVs not only increases the memory T cells in peripheral immune organs, but also increases the immune memory in major organs.

**NVs made from melanoma tumor tissues exhibited strong cross-react immune responses**

The cross-prophylactic efficacy of NVs, made from melanoma tumor tissues, was further investigated in melanoma, lung cancer, and hepatoma (Figures 3 and S7). The prevention scheme was the same as above. In the preventative studies conducted in melanoma, 75% of the immunized mice (NVs applied poly(I:C) as adjuvants) became tumor-free after tumor-inoculation (Figures 3A–3D), which is a significant improvement when comparing with mice injected with PBS, blank NP (containing poly (I:C))+tumor tissue lysates or BCG. When comparing the immune adjuvants, it was found that poly (I:C) showed a better effect than BCG, probably because of the receptor of poly(I:C) co-located with antigens in endosomes of DCs.

NVs, made from melanoma tumor tissues, also showed potent prophylactic efficacy in lung cancer and liver cancer (Figures 3E–3L), indicating that across-cancer immune memories were induced and existed in these vaccine-treated mice. The fact that melanoma tumor tissues-based vaccines could prevent melanoma, lung cancer, and hepatoma verified that across-cancer antigens and across-cancer immune responses exist among different types of cancers, which is beneficial for the VEWCOTT to prevent metastatic cancer in different organs.

The same as above, the memory T cells induced by NVs were investigated (Figure S8). It was observed that both CD8\(^+\) Tcm and CD4\(^+\) Tcm significantly increased in the lung and blood. Meanwhile, both CD8\(^+\) Tem and CD4\(^+\) Tem significantly increased in the lymph node, spleen, lung, and blood. CD8\(^+\) tissue-resident memory T cells (Trm) significantly increased in the lung, blood, and spleen, and CD4\(^+\) tissue-resident memory T cells (Trm) significantly increased in the lung, spleen, and lymph node. The increase of such memory T cells in major organ lung and peripheral immune organs is helpful in controlling and eliminating cancer cells circulating into major organs.

By comparing the prophylactic efficacy of NVs, either applied poly (I:C) or BCG as immune adjuvants, in preventing various types of cancers, we discovered that lung cancer tumor tissue-based NVs showed better preventative efficacy than melanoma tumor tissue-based NVs, in preventing lung cancer, melanoma, and hepatoma (Figures 1, 3, S5, and S7). Impressively, in the prevention of melanoma, lung cancer tumor-tissue NVs showed similar efficacy with melanoma tumor-tissue NVs, demonstrating that antigens in vaccines obtained across types of cancer are also helpful.

**Prevention with VEWCOTT-induced tumor-specific T cells**

Our previous studies discovered that treating mice bearing melanoma with VEWCOTT expanded melanoma-specific T cells in the periphery and stronger immunotherapy responses are related to the increase of melanoma-specific CD8\(^+\) and CD4\(^+\) T cells in peripheral tissues.\(^{15,36}\) However, the amounts of tumor-specific CD8\(^+\) and CD4\(^+\) T cells induced by preventative administration of VEWCOTT have not been investigated yet. Besides, such T cells induced by different tumor tissues-based VEWCOTT have not been compared yet. Therefore, we investigated different tumor-specific T cells activated by preventative administration of different tumor-tissue-based NVs, by analyzing tumor-specific T cells induced in peripheral tissues after immunizing with NVs. Tumor-specific T cells were analyzed by mixing VEWCOTT with splenocytes (containing APCs such as B cells and DC) and thus tumor-specific T cells could be activated by pan-clone cancer antigens loaded in VEWCOTT. The activated tumor-specific T cells can then secrete cytotoxic IFN-\(\gamma\) and then the amount of tumor-specific T cells can be quantitatively analyzed by measuring IFN-\(\gamma\)+ T cells through flow cytometry. It was discovered that both lung cancer tumor tissues-based NV and melanoma tumor-tissue-based NV significantly increased tumor-specific T cells, both CD8\(^+\) and CD4\(^+\), in peripheral tissues (Figures 3M, 3N, and S9A), indicating that preventative administration of NVs induced tumor-specific T cells in peripheral tissues. Besides,
lung cancer tumor tissue-based NVs induced more tumor-specific T cells in peripheral tissues than melanoma tumor tissue-based NVs (Figures 3M, 3N, and S9B), which indicated that the amounts of tumor-specific T cells induced by different tumor-tissue-based VEWCOTT are different because of the neo-antigens in different cancers are variational.
In addition, these data provided one of the reasons why lung cancer tissue-based NVs showed stronger prevention efficacy than melanoma tissue-based NVs—lung cancer tissue-based NVs stimulated more tumor-specific T cells.

Comparing NVs and MVs in preventing cancer

The comparison of NVs and MVs, made from melanoma tumor tissues, was investigated in a melanoma mouse model (Figures 3O, 3P, and S7I–S7K). According to previous studies, NVs are uptake by APCs both in the lymph node and recruited to the injecting site, whereas MVs are uptake by APCs recruited to injecting site. The results demonstrated that both NVs and MVs showed potent efficacy, though NVs performed better than MVs, in preventing melanoma. These data implied that both NVs and MVs have the potential to prevent the occurrence of various cancers, or to prevent cancer relapse after surgical excision of cancer lesions.

Investigation of biodistribution of NPs and MPs

Fluorescent dye Dir (Ex754/Em778) was loaded into NPs and MPs to investigate the biodistribution of NPs and MPs with in vivo imaging methods44 (Figure 4). The results showed that NVs entered and accumulated at the draining lymph nodes from the injection site faster than MPs. MPs stayed at injection site longer time than NPs. These data suggested that MPs migrate to lymph nodes more slowly than small NPs.

Multiple doses of NVs showed better prevention efficacy than one dose

In order to investigate the impact of administration times of VEWCOTT on prevention efficacy, we compared the prophylactic efficacy of one dose and five doses of NVs (Figures 5A and S10). As shown in Figures 5B–5D, treating 5 times with NVs showed significant improvement in prevention efficacy, when comparing only administrating once (administered on day 49). These data illustrated that multiple doses with boosters are needed in preventing cancers with VEWCOTT.

VEWCOTT can prevent cancer metastasis

Melanoma is a type of cancer that tend to have metastases, especially transferring to the lung. Based on the fact that VEWCOTT, made from either melanoma tumor tissues or lung cancer tumor tissues, could prevent the occurrence of various cancers, we further investigated the prevention of melanoma metastasis by VEWCOTT made from melanoma tumor tissues or lung cancer tumor tissues. According to the results, VEWCOTT, prepared from both lung cancer tumor tissues and melanoma tumor tissues, efficiently prevented metastatic melanoma and prolonged the survival time of mice (Figures 5E–5K).

Furthermore, the memory T cells in mice, immunized with melanoma VEWCOTT followed by intravenously injecting with B16-F10 cancer cells, were analyzed with flow cytometry (Figure S11). It was observed that CD8+ central memory T cells (Tcm) increased in the lung and three immune organs, and CD4+ Tcm increased in the lung, lymph node, and spleen. Besides, CD8+ Tem increased in the lung and blood, and CD4+ Tem increased in the lung, lymph node, and blood. CD8+ Trm and CD4+ Trm didn’t increase in the lymph node, lung, spleen, and blood. These results demonstrated that preventative immunization with melanoma VEWCOTT efficiently induced immune memory not only in immune organs, but also in major organ lungs. The memory T cells in the lung are more important for protecting organs from cancer cells.

Both MVs and NVs showed potent efficacy in preventing melanoma metastasis in the lung. However, NVs (either made from melanoma tumor tissues or lung cancer tumor tissues) with a size of approximately 300 nm showed better prophylactic efficacy in preventing metastasis than MVs (made from melanoma tumor tissues) with a size of 1.0 μm. In addition, lung cancer tumor tissue-based NVs, showed similar prophylactic efficacy with melanoma tumor tissue-based NVs, in preventing metastatic melanoma. The results indicated that VEWCOTT could crossly prevent metastatic cancers and this is meaningful for preventing metastasis, given that metastatic cancers are different from the primary cancers because of the differences of colonized organs and microenvironment.

VEWCOTT can prevent orthotopic lung cancer

In order to evaluate the prophylactic efficacy of VEWCOTT in preventing orthotopic lung cancer, we immunized mice with VEWCOTT, either made from melanoma tumor tissues or lung cancer tumor tissues, and
then transferred cancer cells into the lung of mice. With the grow of tumor tissues in the lung, the mice became sick and the body weight significantly decreased. The mice in the PBS control group without immunization all died within 18 days. However, most mice in VEWCOTT immunized group are still healthy on day 36 and they were sacrificed on day 36 to observe the state of lung tissues (Figure 6). By comparing the survival time and tumor tissues at the lung site, it was discovered that both melanoma-based NVs and lung-cancer-based NVs prolonged the survival time and inhibited the tumor growth at lung site. These data confirmed that VEWCOTT could induce a cross-prophylactic effect, and both lung-cancer- and melanoma-based NVs could prevent orthotopic lung cancer. In addition, lung-cancer-based NVs performed better than melanoma-based NVs, given that approximately 80% mice survived in the group immunized with lung-cancer-based NVs and approximately 50% mice survived in the group immunized with melanoma-based NVs. The fact, that melanoma tumor tissue-based or lung cancer tumor tissue-based VEWCOTT crossly prevented melanoma metastasis and orthotopic lung cancer, further demonstrated that administrating VEWCOTT after the excision of tumor tissue potentially has the advantages of preventing cancer metastasis (though the metastatic cancer maybe different with the primary cancer) and cancer relapse in clinical.

Figure 4. Analysis of biodistribution of nanoparticles and microparticles with imaging in vivo method
Mice were pictured at 6, 24, and 48 h from both back and abdomen position, followed by imaging the organs after sacrificing the mice.
A - Melanoma vaccine

B - Tumour volume (mm³) over time

C - Tumour volume (mm³) over time (Day 15)

D - Percent survival over time

E - NVs and MVs

F - Number of tumour nodes on lung surface

G - Proportion of lung tumours (%)

H - Images of mice and tumours

I - High magnification images of tumours

J - % of CD8+ T cells

K - % of CD4+ T cells
Preventing cancer by allografting T cells from VEWCOTT immunized mice

It is well recognized that tumor-specific T cells are critical in killing cancer cells. To verify the function of tumor-specific T cells induced by VEWCOTT, we conducted the cancer cell-killing ability evaluation of spleenocytes and lymph node cells collected from VEWCOTT-immunized mice. The splenocytes and lymph node cells collected from NV-immunized mice were injected intravenous into allogeneic naive mice, pre-treated with cyclophosphamide to remove the immune cells of recipient mice (Figures 7A–7E and S12A–S12E). It was witnessed that the transplantation of T cells from NV-immunized mice to naive mice significantly retarded the tumor growth after tumor-inoculation in naive mice, both in lung cancer and melanoma. Besides, both T cells from splenocytes and lymph nodes showed such efficacy. In addition, the splenocytes collected from these treated naive mice (accepted T cells from VEWCOTT-immunized mice) possessed a higher amount of tumor-specific T cells (both CD8+ and CD4+) than that from untreated naive mice (Figure S13).

These data illustrated that induced tumor-specific T cells exist in both the lymph node and splenocytes in VEWCOTT-immunized mice, and such peripheral tumor-specific T cells are the main contributors to killing cancer cells. The phenomenon, that allograft with adoptive T cells from VEWCOTT immunized mice prevented cancer, potentially has practical significance, given that one obstacle to cancer immunotherapy or prevention is that a lot of patients with cancer are elder and have poor conditions. According to the data presented in our study, immunizing the young healthy relatives of patients with VEWCOTT and giving the VEWCOTT-activated T cells of immunized-relatives to the patients (with poor immune conditions) probably is one strategy to solve such problems.

Function of CD4+ and CD8+ T cells in preventing cancer

Though we have previously reported that CD8+ T cells played a critical role in cancer immunotherapy with VEWCOTT, however, the functions of CD8+ and CD4+ T cells have not been investigated in preventing cancer with VEWCOTT. Herein, we investigated the functions of T cell subtypes in anti-cancer immune responses by depleting CD4+ T cells or CD8+ T cells during our prophylactic studies (Figures 7F–7K and S12F–S12N). The study revealed that the depletion of CD8+ T cells eliminated most of the prophylactic efficacy. Meanwhile, the depletion of CD4+ T cells also eliminated some of the prophylactic efficacy (Figures 7F–7K). Meanwhile, CD4+ and CD8+ T cells double depletion eliminated almost all the benefits induced by NV immunization. These results indicated that both CD8+ T cells CD4+ T cells played important roles in killing cancer cells in our cancer-prophylactic method and CD8+ T cells are playing the dominant role in preventing cancer.

Neoantigens shared by lung cancer and melanoma

In previous studies, we have shown that melanoma-based VEWCOTT can load almost all detectable mutations and neoantigens in tumor tissues and the abundance of such mutations and neoantigens in VEWCOTT is similar to that in tumor tissue lysates. However, no studies have compared and systematically analyzed the difference of mutations and neo-antigens between different cancers and different cancer-based VEWCOTT. Herein, we compared the variety and detectable neoantigens between melanoma and lung cancer and corresponding cancer-based VEWCOTT by proteomics analyses. Proteomics analyses revealed that most neoantigens are shared by lung cancer tumor tissue and melanoma tumor tissue, and corresponding cancer-based VEWCOTT. As shown in Figure 8, the total number of proteins and some well-recognized neoantigens that can be detected were evaluated between melanoma and lung cancer. Many proteins and neoantigens, either neoantigen in melanoma or in lung cancer, were shared by lung cancer and melanoma tumor tissue, and corresponding cancer-based NVs (Figure 8).

**Figure 5. Analysis of the dose impact on the preventive efficacy of VEWCOTT and prevention of melanoma metastasis by NV or MV
(A) Timeline (days) of melanoma-based vaccine immunization and melanoma tumor inoculation.
(B–D) Melanoma tumor growth curves and survival curves of mice immunized with various melanoma-based NV doses (n ≥ 8).
(E) Timeline (days) of vaccine immunization and melanoma metastasis inoculation in the lung.
(F and G) Number of tumors on lung surface and the proportion of lung tumor in mice treated with various vaccines (n ≥ 5).
(H) Representative pictures of lung obtained from the sacrificed mice (n ≥ 5).
(I) Representative H&E staining pictures of lung obtained from the sacrificed mice (n ≥ 5).
(J and K), Flow cytometric analysis evaluating IFN-γ-secreting CD8+ T cells or CD4+ T cells among the splenocytes of mice treated with vaccines that were subsequently stimulated ex vivo with tumor antigens. **p < 0.005, ***p < 0.0005.
In terms of melanoma or lung cancer neoantigens, it seems melanoma tumor tissues have more water-soluble neoantigens than lung cancer tumor tissue. Whereas, lung cancer tumor tissues have more abundant water-insoluble neoantigens than melanoma tumor tissue.

Of course, these neoantigens we analyzed are only the ones already well identified by scientists. The results, to some extent, explained why cross-react responses exist among different types of cancer. The neo-antigens of cancer cells are highly heterogeneous and more than thousands, and thus several individual peptide neo-antigens induced very limited across-cancer immune responses because these several neo-antigens have small chances to be included in different types of cancer cells, whereas VEWCOTT loaded thousands of different kinds of neo-antigens and thus have more chances to cover the neo-antigens possessed by different types of cancer cells. Therefore, VEWCOTT-induced potent across-cancer immune response, thanks to including larger number of neo-antigens, is critical for across-cancer immune responses. A large number of neo-antigens are shared by different types of tumor tissues has a special advantage of a cancer vaccine made from whole components of cancer over several specific peptide neo-antigens, given that large number neoantigens can cover more diverse shared neoantigens and thus induce immune responses across different types of cancer.

Analysis of lung immune microenvironment after immunization and cancer challenge

The lung immune microenvironment was analyzed by fluorescent multiplex immunohistochemistry (mIHC)47 after immunization and cancer cell challenge. It was discovered that the abundance of various immune cells increased in the VEWCOTT-treated group after challenging with injection of cancer cells. Specifically, the levels of T cells, macrophages, NK cells, and B cells were increased in the lung in the VEWCOTT-treated group after orthotopic lung cancer and metastatic melanoma challenge (Figure S14), demonstrating that both adaptive immunity against cancer and innate immunity were activated by NVs at the lung site.48–50 In addition, stem-cell-like CD8+ T cells and CSCL13+ T cells increased at lung after tumor challenging (Figure 9). Given that TCF-1+ progenitor CD8+ T cells and CXCL13+ T cells are critical for cancer cell killing,51–54 the increase of such cells in the lung after tumor challenge verified that VEWCOTT efficiently induced cancer-specific immune responses. Because the existence of cancer after tumor is challenging, tumor-specific T cells will migrate to the tumor site, followed by recruiting some other immune cells to kill cancer cells.

Toxicity studies of NVs

In all studies conducted with mice, the body weight of mice in both MVs and NVs immunized mice did not decrease significantly (Figure S15). In addition, in the H&E study conducted on the sample of the heart, live, spleen, lung, and kidney, both MV- and NV-immunized mice didn’t show any abnormality (Figure S16), which indicated that NVs and MVs did not cause any major toxicity.

DISCUSSION

Studying across-cancer immune responses among different types of cancers may help us discover a better strategy to prevent and treat cancer metastasis and recurrence. VEWCOTT is a good tool to conduct such studies, given that such vaccines encapsulated more diverse tumor-specific antigens.

The VEWCOTT, loaded with specific tumor tissues, showed excellent efficacy in preventing corresponding homologous cancer in our previous studies.11 However, whether VEWCOTT could prevent different types of cancers and how strong are such cross-react immune responses among different types of cancers were not clear yet. In addition, whether VEWCOTT prevents cancer metastasis or not is unclear. To reveal these scotomas, we utilized VEWCOTT to prevent three different types of cancers and metastatic cancers. The results, that both melanoma-based VEWCOTT and lung-cancer-based VEWCOTT efficiently prevented all three types of cancer and cancer metastasis, illustrated that across-cancer immune responses exist strongly among different types of cancers and VEWCOTT has the advantage of simultaneously preventing various cancer, thanks to the extensive tumor-specific immune responses activated by diverse antigens in tumor tissues. Lung cancer
tumor-tissue VEWCOTT performed well because of the fact that lung-cancer-based NVs activated more tumor-specific T cells and the detailed mechanism behind this needs to be explored further in future studies. Simultaneously preventing different types of cancer is crucial in preventing cancer metastasis in different settings.

Figure 7. Prevention of cancer by allografting T cells from VEWCOTT immunized mice

(A) Timeline (days) of NVs (Poly (I:C)) immunization, allograft of T cells and tumor inoculation. (B and C) Lung cancer tumor growth curves and survival curves of naive mice allograft with splenic T cells or lymph node cells from mice immunized with lung cancer-based NVs (Poly (I:C)) \( (n \geq 5) \). (D and E) Melanoma cancer tumor growth curves and survival curves of naive mice allograft with splenic T cells from mice immunized with melanoma-based NVs (Poly (I:C)) \( (n \geq 5) \). (F) Timeline (days) of lung cancer-based NVs (Poly (I:C)) immunization, lung cancer tumor inoculation and T cell depletion. (G, and H) Lung cancer tumor growth curves and survival curves of NVs (Poly (I:C)) immunized mice when CD4+ or CD8+ or both positive cells were depleted \( (n \geq 5) \).

(I) Timeline (days) of melanoma-based NVs (Poly (I:C)) immunization, melanoma tumor inoculation, and T cell depletion. (J, and K) Melanoma tumor growth curves and survival curves of NVs (Poly (I:C)) immunized mice when CD4+ or CD8+ cells were depleted \( (n \geq 5) \). Data are presented as the mean ± SEM. * and ** indicate comparisons with PBS control group; ## and ### indicate comparisons with group treated with CD4+ and CD8+ cells’ double depletion; €€ and €€€ indicate comparisons with the group treated with CD8+ cells’ depletion; $\Psi$ indicates comparisons with the group treated with CD4+ cells’ depletion; $\Psi\Psi$ indicate comparisons with group allograft with T cells from untreated healthy mice. * and $\Psi$ indicate \( p < 0.05 \); **, ##, €€, and $\Psi\Psi$ indicate \( p < 0.005 \); ***, ###, and €€€ and $\Psi\Psi\Psi$ indicate \( p < 0.0005 \).

Tumor-tissue VEWCOTT performed well because of the fact that lung-cancer-based NVs activated more tumor-specific T cells and the detailed mechanism behind this needs to be explored further in future studies. Simultaneously preventing different types of cancer is crucial in preventing cancer metastasis in different settings.
organisms, given that metastatic cancers are normally multiple organs and the colonizing microenvironment and types of metastatic cancers are different from the primary cancer.

No studies on optimizing sizes of VEWCOTT have been reported. Therefore, 300-nm-sized NVs and 1.0-μm-sized MVs were compared in this study. The results discovered that both NVs and MVs could be uptake efficiently, and antigens in both NVs and MVs could be presented by both MHC I and MHC II pathways. Both of them showed potent efficacy in preventing various cancers, though NVs exhibited higher prophylactic efficacy than MVs. Theoretically, NVs could be uptake by APCs in lymph nodes and recruited to the injecting site, whereas MVs mainly be uptake by APCs recruited to the injecting site.

VEWCOTT prevents cancer mainly through inducing memory T cells (especially Tcm). These tumor-specific memory T cells, including Tcm, Tem, and Trm, are critical in controlling and eliminating tumors. The fact, that VEWCOTT induced multifarious tumor-specific T cells in peripheral tissues, ensured that sufficient candidates of tumor-infiltrating T cells exist in peripheral tissues, which is beneficial for rapid recognizing and killing tumor cells. However, no reports have studied the induction of memory T cells by VEWCOTT in major organs. This study demonstrated that besides inducing memory T cells in peripheral immune organs, VEWCOTT also induced memory T cells in the lung, which is one of the major organs. The induction of memory T cells by VEWCOTT in major organs is beneficial for protecting major organs from the invasion of cancer cells because they can respond faster once they encounter cancer cells.

By analyzing the antigens in tumor tissues and encapsulated in VEWCOTT, it was found that many identified neoantigens were shared by lung cancer tumor tissue and melanoma tumor tissue. This indicated that different types of cancer share many neoantigens, and these larger number of neoantigen-combinations can induce across-cancer immune response, which is beneficial for controlling cancer metastasis in different organs.55

One problem encountered in clinical is many patients with cancer are too weak and their immune system becomes too weak to stimulate sufficient immune responses. In such cases, alternative strategies are urgently needed. The results, that allogeneic transplantation of activated T cells from VEWCOTT-immunized mice to homologous naive mice can kill cancer cells, indicated that immunizing young healthy individuals (ideally relatives of the patients) for allogeneic transplantation is a potential strategy to solve this problem.

The points that across-cancer immune responses exist among different types of cancers provided important information for patients with cancer who did excision surgery and need to prevent cancer recurrence and cancer metastasis in different organs. Given that excision surgery is a widely applied cancer treatment method and VEWCOTT can be prepared from tumor tissues obtained from surgeries, the materials to prepare VEWCOTT are sufficient and easily obtained. VEWCOTT possesses the properties of inducing across-cancer specific immune responses, which ensured that it has a unique advantage in preventing cancer metastasis in different organs and cancer relapse, considering that metastatic cancers and recurrent cancers generally occur in multiple organs. All in all, strong across-cancer immune responses exist among different types of cancers and thus VEWCOTT has the advantage of simultaneously preventing multiple cancer occurrence, multiple cancer recurrence, and cancer metastasis in multiple organs.

In summary, this study revealed that strong across-cancer immune responses exist among different types of cancers. In addition, both NVs and MVs can induce a potent across-cancer immune response by stimulating tumor-specific immune responses and memory T cells, and these cross-react immune responses are helpful in preventing and potentially treating metastatic cancers in different organs and recurrent cancers.
Limitations of the study

A limitation of the study is only 300-nm NV and 1.0-µm MV were compared. Given that comparing comprehensive sized MVs is not the primary goal of the study, we didn’t compare different sized MVs. It would be ideal to compare more MVs with a larger size, such as 2.0-µm MV and 5.0-µm MV, which we will conduct in our future studies.

STAR METHODS

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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105511.

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AUTHOR CONTRIBUTIONS
M.L. conceived and designed the study. L.D. and L.M. conducted NV and MV preparation, animal studies, and flow cytometry studies. M.X. L.Z., Y.P., F.M., and Z.Z. contributed to characterization of NVs and MVs. L.M., L.D., Y.P., and Z.L. analyzed the data and drew the figures. L.M., H.J., and G.X. contributed to the proteomics studies. Z.P. and L.D. designed and conducted the H&E studies. M.L. and L.D. wrote the manuscript. All authors reviewed and approved the final version.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| PD-1 blocking antibody (Clone RMPI-1-14) | BioXcell | Cat.# BE0146; RRID: AB_2921514 |
| sCD8 antibody (Clone 2.43) | BioXcell | Cat.# BE0061; RRID: AB_125451 |
| sCD4 antibody (Clone GK1.5) | BioXcell | Cat.# BE0003-91; RRID: AB_1107636 |
| Purified anti-mouse CD16/32 (Clone 93) | Biolegend | Cat.# 101302; RRID: AB_312801 |
| Ms CD45R/B220 APC (Clone RA3-6B2) | BD Biosciences | Cat.# 553092; RRID: AB_398531 |
| Ms CD11c PerCP-Cy5.5 (Clone HL3) | BD Biosciences | Cat.# 560584; RRID: AB_1727422 |
| APC/CY7 anti-mouse F4/80 Antibody (Clone BM8) | Biolegend | Cat.# 123118; RRID: AB_893477 |
| FITC anti-mouse H-2kb (Clone AF6-88.5) | Biolegend | Cat.# 116506; RRID: AB_313733 |
| Brilliant Violet 510™ anti-mouse I-A/I-E Antibody (Clone m5/114.15.2) | Biolegend | Cat.# 107635; RRID: AB_2561397 |
| PE anti-mouse CD86 Antibody (Clone A17119A) | Biolegend | Cat.# 159203; RRID: AB_2832567 |
| APC anti-mouse CD80 Antibody (Clone 16-10A1) | Biolegend | Cat.# 104714; RRID: AB_313135 |
| APC/Cyanine7 anti-mouse CD3 Antibody (Clone 17A2) | Biolegend | Cat.# 100222; RRID: AB_2242784 |
| FITC anti-mouse CD3 Antibody (Clone 17A2) | Biolegend | Cat.# 100204; RRID: AB_312661 |
| Brilliant Violet 785TM anti-mouse CD3e antibody (Clone 145-2C11) | Biolegend | Cat.# 100355; RRID: AB_2565969 |
| PE/Cyanine7 anti-mouse CD4 Antibody (Clone GK1.5) | Biolegend | Cat.# 100422; RRID: AB_312707 |
| BV510 Rat Anti-Mouse CD45, (Clone 30-F11) | BD Biosciences | Cat.# 563891; RRID: AB_2734134 |
| PE anti-mouse CD8a Antibody (Clone 53-6.7) | Biolegend | Cat.# 100708; RRID: AB_312747 |
| FITC anti-mouse/human CD44 Antibody (Clone IM7) | Biolegend | Cat.# 103006; RRID: AB_312957 |
| PerCP/Cyanine5.5 anti-mouse CD62L Antibody (Clone MEL-14) | Biolegend | Cat.# 104432; RRID: AB_2285839 |
| APC anti-mouse CD69 Antibody (Clone H1.2F3) | Biolegend | Cat.# 104514; RRID: AB_492843 |
| APC anti-mouse IFN-γ Antibody (Clone XMG1.2) | Biolegend | Cat.# 505810; RRID: AB_315404 |
| Anti-mouse CD3 antibody | Abcam | Cat.# ab5690; RRID: AB_305055 |
| Anti-mouse CXCL13 antibody | Abcam | Cat.# ab199043 |
| Anti-mouse CD4 antibody | Abcam | Cat.# ab183685; RRID: AB_2686917 |
| Anti-mouse CD45R antibody | Abcam | Cat.# ab64100; RRID: AB_1140036 |
| Anti-mouse F4/80 antibody | Abcam | Cat.# ab100790; RRID: AB_10675322 |
| Anti-mouse CD49b antibody | Abcam | Cat.# ab115797; RRID: AB_10936458 |
| Anti-mouse CD8 antibody | Abcam | Cat.# ab217344; RRID: AB_2890649 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Resomer® RG 503 H, Poly(D,L-lactide-co-glycolide) acid terminated, lactide:glycolide 50:50, Mw 24000-38000 | Sigma Aldrich | Cat# 719870 |
| Rhodamine B modified PLGA (24K–38K, 50:50) | Xian ruixi Biological Technology Co., Ltd | Cat# R-L-38K |
| poly(vinyl alcohol) (PVA, 26 360627, MW: 9,000–10,000 Da) | Sigma Aldrich | Cat# 360627 |
| Collagenase | Stem cell Technologies | Cat# 07912 |
| hyaluronidase | Stem cell Technologies | Cat# 07461 |
| bovine pancreas DNase | Stem cell Technologies | Cat# 10608 |

(Continued on next page)
## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Mi Liu (mi.liu@suda.edu.cn).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The data supporting the findings of this study are included in the paper and its extended data. All other relevant data are available from the corresponding author upon reasonable request. The mass spectrometry proteomics data are available through the ProteomeXchange Consortium via the dataset identifier PXD037688.

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### Table: Reagents and Resources

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| BCG | Shanghai REBIO | Cat# R19019 |
| Urea | Thermo scientific | Cat# A12360.36 |
| D-()+-Trehalose Anhydrous | macklin | Cat# D807343 |
| OVA-FITC | Solarbio | Cat# SF21 |
| LysoTrackerTM Green | Invitrogen | Cat# L7526 |
| LysoTrackerTM deep red | ThermoFisher | Cat# L12492 |
| DAPI | ThermoFisher | Cat# D1306 |
| Murine GM-CSF | Pepro Tech | Cat# 315-03-250 |
| Antifade Mounting Medium | NobleRyder | I0052 |
| Matrigel | BD Biosciences | Cat# 356234 |

### Critical commercial assays

| Assay | SOURCE | IDENTIFIER |
|-------|--------|------------|
| LIVE/DEADTM Fixable Aqua Dead Cell Stain Kit | Life Technologies | Cat# 423102 |
| LIVE/DEADTM Fixable Violet Dead Cell Stain Kit | Life Technologies | Cat# 423113 |
| Micro BCA Protein Assay Kit | Thermo Scientific | Cat# 23235 |
| Pan T cell isolation Kit | Miltenyi Biotec | Cat# 130-095-130 |
| AlphaX 7-Color IHC Kit | AlphaBiol | Cat# AXT37100011 |

### Experimental models: Cell lines

| Cell line | SOURCE | IDENTIFIER |
|-----------|--------|------------|
| B16-F10 cell line | Cell Bank of the Chinese Academy of Sciences, Shanghai, China | CSTR:19375.09.3101MOUTCM36 |
| LLC cell line | Cell Bank of the Chinese Academy of Sciences, Shanghai, China | CSTR:19375.09.3101MOUTCM7 |
| Hepa 1–6 cell line | Cell Bank of the Chinese Academy of Sciences, Shanghai, China | CSTR:19375.09.3101MOUSCSP512 |
| DC 2.4 cell line | ATCC | Cat# HTX2245 |
| Mouse: C57BL/6 | Charles River | C57BL/6NCrI |
| Mouse: BALB/c | Charles River | BALB/cAnNCrI |

### Oligonucleotides

| Oligonucleotide | SOURCE | IDENTIFIER |
|----------------|--------|------------|
| poly(I:C) (vac-pic) | InvivoGen | Cat# tlrl-pic-5 |

### Software and algorithms

| Software | SOURCE | URL |
|----------|--------|-----|
| FlowJo v10.5.3 | BD Biosciences | https://www.flowjo.com/ |
| Leica LAS X | Leica | https://www.leica.com |
| GraphPad Prism 8.0 | GraphPad | https://www.graphpad.com |
| The mass spectrometry proteomics data | Proteome Xchange Consortium | The dataset identifier: PXD037688 (http://proteomecentral.proteomexchange.org) |
spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD037688.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

BMDCs were generated from bone marrow precursors of C57BL/6 WT, flushed from femurs, in Iscove’s modified Dulbecco’s medium (IMDM) (Euroclone) containing 10% heat-inactivated fetal bovine serum (Euroclone), 100 IU of penicillin, streptomycin (100 μg/mL⁻¹), 2 mM L-glutamine (Euroclone), and GM-CSF (10 to 20 ng mL⁻¹).

In vivo efficacy studies with mice

6–8 weeks old female C57BL/6 mice or BALB/c mice were used. All animal work was approved and monitored by the Animal Ethics Committee of Soochow University. All the mice were ordered from the animal facility platform of Soochow University.

METHOD DETAILS

Collection of cell components from tissue lysates

In the case of lysis of tumour tissues, mice bearing the corresponding tumour (500–2000 mm³) were sacrificed and tumour tissues were collected. Tumour tissues were ground and filtered through cell strainers, followed by obtaining single cell suspension and adding ultrapure water into the single cell suspensions. Then, we freeze-thawed the cells with sonication at least 5 times. Subsequently, the samples were centrifuged at 12,000 rpm for 5 min, and the supernatant was collected as the water-soluble components. The cell lysate precipitate was defined as the water-insoluble components and was solubilized with 8 M urea. For B16-F10 and LLC tumour tissues, the concentration of proteins/peptides in the water-soluble components or in the water-insoluble components was approximately 80 mg/mL.

Preparation of nanovaccine A (NV A) and nanovaccine B (NV B) loaded with lysis components and poly (I:C)

Nanovaccine A (NV A) was prepared by the double-emulsion method for B16-F10 and LLC tumour tissues. Briefly, to prepare the NV A, 300 μL of water-soluble components in endotoxin-free water (80 mg/mL, with 2.5 mg/mL poly(I:C) added) was added to 1 mL of PLGA (100 mg/mL) in dichloromethane and sonicated for 1 min. Then, the sample was added to 2.5 mL of PVA solution (20 mg/mL) and sonicated for 45 seconds. To solidify the nanoparticles (NPs), the sample was dropped into 50 mL of PVA solution (5 mg/mL) and stirred for 4 h at room temperature. The resultant NPs were collected by centrifugation for 25 min at 12,000 rpm and resuspended in 10 mL of 4% trehalose, followed by lyophilizing the NPs for 48 h. Before the administration of NV A, the nanoparticles were resuspended in 7 mL PBS and mixed with 3 mL of water-soluble components and 0.7 mg of poly(I:C). All the NVs were made under endotoxin-free conditions.

NV B was prepared by the procedure described above for both B16-F10 and LLC tumour tissues. Briefly, to prepare the NV B, 300 μL of water-insoluble components in 8 M urea (80 mg/mL containing 2.5 mg/mL poly(I:C)) was added to 1 mL of PLGA (100 mg/mL) in dichloromethane and sonicated for 1 min. Then, the sample was added to 2.5 mL of PVA solution (20 mg/mL) and sonicated for 45 seconds. To solidify the NPs, the sample was dropped into 50 mL of PVA solution (5 mg/mL) and stirred for 4 h at room temperature. The resultant nanoparticles were collected by centrifugation for 25 min at 12,000 rpm and resuspended in 10 mL of 4% trehalose, followed by lyophilizing the nanoparticles for 48 h. Before the administration of NV B, the NPs were resuspended in 7 mL of PBS and mixed with 3 mL of water-insoluble components and 0.7 mg of poly(I:C)) solubilized in 8 M urea. Then, the NPs were collected by centrifugation for 20 min at 12,000 rpm and resuspended in 9.5 mL PBS, followed by mixing with 0.5 mL of water-insoluble components (containing 0.7 mg of poly(I:C)) solubilized in 8 M urea. All the NVs were made under endotoxin-free conditions.

Preparation of microvaccine A (MV A) loaded with lysis components and poly (I:C)

Microvaccines A (MV A) was prepared by the double-emulsion method for B16-F10 tumour tissues. Briefly, to prepare the MV A, 300 μL of water-soluble components in endotoxin-free water (80 mg/mL, with 2.5 mg/mL poly(I:C) added) was added to 1 mL of PLGA (100 mg/mL) in dichloromethane and sonicated
for 30 seconds. Then, the sample was added to 2.5 mL of PVA solution (20 mg/mL) and sonicated for 4 seconds. To solidify the particles, the sample was dropped into 50 mL of PVA solution (5 mg/mL) and stirred for 4 h at room temperature. The resultant particles were collected by centrifugation for 25 min at 12,000 rpm and resuspended in 10 mL of 4% trehalose, followed by lyophilizing the particles for 48 h. Before the administration of MVs, the nanoparticles were resuspended in 7 mL PBS and mixed with 3 mL of water-soluble components and 0.7 mg of poly(I:C). All the MVs were made under endotoxin-free conditions.

MV B were prepared by the procedure described above. Briefly, to prepare the MV B, 300 µL of water-insoluble components in 8 M urea (80 mg/mL containing 2.5 mg/mL poly(I:C)) was added to 1 mL of PLGA (100 mg/mL) in dichloromethane and sonicated for 30 seconds. Then, the sample was added to 2.5 mL of PVA solution (20 mg/mL) and sonicated for 4 seconds. To solidify the particles, the sample was dropped into 50 mL of PVA solution (5 mg/mL) and stirred for 4 h at room temperature. The resultant oparticles were collected by centrifugation for 25 min at 12,000 rpm and resuspended in 10 mL of 4% trehalose, followed by lyophilizing the particles for 48 h. Before the administration of MVs, the particles were resuspended in 7 mL PBS and mixed with 3 mL of water-insoluble components solubilized in 8 M urea. Then, the particles were collected by centrifugation for 20 min at 12,000 rpm and resuspended in 9.5 mL PBS, followed by mixing with 0.5 mL of water-insoluble components (containing 0.7 mg of poly(I:C)) solubilized in 8 M urea. All the MVs were made under endotoxin-free conditions.

Preparation of NV A and NV B loaded with cell components and BCG

The concentration of water-soluble components in the cell lysates applied to make NPs (NV A) was approximately 80 mg/mL, including 4 mg/mL water-soluble components of BCG. The nanoparticles were prepared by the double-emulsion method, and the procedure was the same as the protocol described above. The resultant NPs were resuspended in 7 mL of PBS. Then, the NPs were mixed with 3 mL of water-soluble components and 5 mg of BCG.

The concentration of water-insoluble components in the cell lysates applied to make NPs (NV B) was 80 mg/mL, including 3 mg/mL 8 M urea-solubilized components of BCG. The nanoparticles were prepared as described above. The resultant NPs were resuspended in 9.5 mL of PBS. Then, the NPs were mixed with 0.5 mL of water-insoluble components solubilized in 8 M urea and 5 mg of BCG.

Preparation of blank NPs

The process to make blank NPs was the same as that to make NVs. Briefly, 300 µL of ultrapure water (or 8 M urea) containing 2.5 mg/mL poly(I:C) was added to 1 mL of PLGA (100 mg/mL) in dichloromethane and sonicated for 1 min or 30 seconds. Then, the sample was added to 2.5 mL of PVA solution (20 mg/mL) and sonicated for 45 seconds or 4 seconds. The sample was dropped into 50 mL of PVA solution (5 mg/mL) and stirred for 4 h at room temperature. The NPs were collected and lyophilized. Before the administration of blank NPs, they were resuspended in 10 mL of PBS.

Preparation of NPs modified with rhodamine B

Rhodamine B modified PLGA (24K–38K, 50:50) and PLGA (24K–38K, 50:50) were mixed at a molar ratio of 1:4 to prepare the NPs. The process to prepare NPs was the same as described above for NVs or MVs. Before the analysis of these NPs, the NPs were mixed with B16F10 tumor tissue components or LLC tumor tissue components.

Preparation of NPs encapsulated with a dye-conjugated protein

The concentration used to make NPs was 10 mg/mL for OVA-FITC. OVA-FITC in H2O or 8 M urea was encapsulated into NPs made from rhodamine B modified PLGA (24K–38K, 50:50) mixed with PLGA (24K–38K, 50:50) (molar ratio 1:4). The NP preparation procedure was the same as that described above. The acquired NPs were mixed with cancer tumor tissue lysates for confocal microscopy analysis.

Characterization of NVs, MVs and NPs

The size and zeta potentials of NVs, MVs and NPs were determined by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern instruments, Worcestershire, UK). In the size test, nanoparticles were dissolved in PBS and then evaluated before or after surface coating. In the zeta potential analysis, NVs, MVs and NPs were dissolved
in deionized water and assessed before or after surface coating. The amounts of proteins/peptides loaded within NVs, MVs were determined by microBCA assay.

The characterization of NPs made with dye or dye-conjugated OVA, including the sizes and zeta potentials of NPs, were determined by the protocols described above. The amounts of dye loaded into nanoparticles were quantified by a calibration curve for free dye or free dye-modified OVA.

The morphology of NVs or MVs was investigated by transmission electron microscopy (TEM, HT7700). 20 μL of 0.1 mg/mL PLGA particles was dropped onto a lacey copper grid coated with a continuous carbon film. The samples were dried and observed by using TEM.

**Uptake of NVs and MVs by APCs in vitro**

The splenocytes were collected and allowed to rest in plates for several hours at 37°C (5% CO2). Rhodamin B modified NPs or MPs were added to the splenocytes, followed by incubating at 37°C (5% CO2 and 95% humidity) for 4 h. Subsequently, the cells were collected and labelled by live/dead staining, followed by the staining of Fc block staining and the staining of anti-mouse antibodies against CD11c, F4/80 and B220. The stained cells were then analysed by flow cytometry analysis. Flow cytometric analysis was performed using a FACS AriaTMIII and the data were analysed by using FlowJo 10 software.

**Release of payloads and endosomal escape of payloads**

DC2.4 cells were collected and added to glass-bottom dishes. NPs/MPs loaded with dyes were added to the DC2.4 cells and co-incubated at 37°C (5% CO2) for 4 h. LysoTracker™ Green or LysoTracker™ deep red was added to the splenocytes 0.5–2 h prior to fixing the cells with 4% paraformaldehyde. Subsequently, the dishes and cells were washed several times to remove free NPs/MPs and dyes. Finally, VECTASHIELD anti-fade mounting medium with DAPI was added to the cells, and the cells were analysed by confocal microscopy.

**Uptake of NPs or MPs by APCs in lymph nodes**

NPs or MPs containing rhodamine B and coated with antigens were subcutaneously injected into the back of C57BL/6 mice. After 16 h, the nearby lymph nodes were collected, and the uptake of NPs/MPs by APCs was investigated by flow cytometry. APCs in lymph nodes were stained with anti-mouse antibodies against CD11c, F4/80 and B220. Flow cytometric analysis was performed using a FACS AriaTMIII and analysed using FlowJo 10 software.

**Cross-presentation and maturation of DC induced by NV and MV**

BMDCs were differentiated from bone marrow cells isolated from the femurs and tibias of 6-week-old C57BL/6 mice according to an established method. Non-adherent and loosely adherent cells with a maturation level of approximately 20% on day 7 were collected and used for the studies. To assess DC cross-presentation, BMDCs were pulsed with Free tumour lysate, Blank NP, melanoma NV and melanoma MV for 24 h. After various treatments, BMDCs were harvested, resuspended in FACS buffer (PBS that contained 1% FBS), incubated with Zombie NIR™ Fixable Viability and anti-CD16/32, and then stained with anti-MHCII-BV510, anti-MHCI-FITC and anti-CD11c-Percp-Cy5.5 before analysis by flow cytometry (BD FACSARiaTM III). In case of in vitro DC maturation experiments, generally, immature BMDCs were plated into 24-well and simulated with Free tumour lysate, Blank NP, melanoma NV and melanoma MV for 24 h. After various treatments, BMDCs were harvested, resuspended in FACS, incubated with Zombie NIR™ Fixable Viability and anti-CD16/32, and then stained with fluorophore-labelled antibodies against CD11c-Percp-Cy5.5, CD86-PE and CD80-APC for 30min before analysis by flow cytometry.20

**Analysis of biodistribution of nanoparticles**

The DiR dye (purchased from absin, abs45153692) was dissolved in water to prepare a concentration of 4 mg/mL and the DiR dye was encapsulated into PLGA nanoparticles or microparticles with the same methods as described above. The size of nanoparticle is around 300nm and the size of microparticle is around 1.0μm. The biodistribution of nanoparticles and microparticles were studied with Female C57BL/6 mice aged 6–8 weeks. Nanoparticles or microparticles loaded with DiR were subcutaneously injected at the right back of mice (0.5mg/mice), respectively. The biodistribution of nanoparticles and microparticles were analyzed with imaging in vivo at 6 h, 24 h and 48 h after injection. Meanwhile, the
heart, liver, spleen, lung, kidney and draining lymph nodes were taken out to observe the distribution of DiR loaded in 3 different lipid particles. Fluorescence signals (Ex740nm, Em790nm) were measured by IVIS® Spectrum (PerkinElmer, Waltham, USA).

**Tumour volume measurements**

Two perpendicular diameters were measured with a caliper and the tumour volumes were calculated using the formula $V = 0.52a \times b^2$, where $a$ and $b$ are the larger and smaller diameters, respectively. The tumour volumes were assessed every 3 days from day 3. Mice were sacrificed when tumour volume exceeded 2000 mm$^3$.

**Prevention of various cancers by NV or MV**

To induce tumour-specific immune responses before the occurrence of a tumour, 200 μL of NV/MV A (containing 2 mg of PLGA) and 200 μL of NV/MV B (containing 2 mg of PLGA) were injected subcutaneously at different sites on day $-49$, day $-42$, day $-35$, day $-28$ and day $-14$ ahead of tumour inoculation. In blank NP control group, to replace the injection of NV A or NV B, 200 μL of equal amounts of blank NP (containing immune adjuvants) and equal amounts of cell lysate components (containing immune adjuvants) were subcutaneously injected at different sites simultaneously on day $-49$, day $-42$, day $-35$, day $-28$ and day $-14$ ahead of tumour inoculation. In the PBS control group, 400 μL sterile PBS was injected subcutaneously on day $-49$, day $-42$, day $-35$, day $-28$ and day $-14$ ahead of tumour inoculation. Tumour inoculation was performed on day 0. A total of $1.5 \times 10^5$ B16-F10 cells were subcutaneously injected into the right back of C57BL/6 mice, or $2 \times 10^6$ LLC cells or $2 \times 10^6$ Hepa 1–6 cells were subcutaneously injected into the right armpit of C57BL/6 mice. Tumour volume was recorded every 3 days beginning on day 3.

**Immunotherapy of cancers by NV**

Tumour inoculation was performed on day 0 and $1.5 \times 10^5$ B16-F10 cells were subcutaneously injected into the right back of C57BL/6 mice. To induce tumour-specific immune responses after the occurrence of a tumour, 200 μL of NV A (containing 2 mg of PLGA) and 200 μL of NV B (containing 2 mg of PLGA) were injected subcutaneously on day 4, day 7, day $-10$, day 13, day 18 and day 23. Tumour volume was recorded every 3 days beginning on day 3.

**Prevention of cancer metastasis by NV or MV**

The administration of PBS or NV or MV was the same as above described. Tumour inoculation was performed on day 0. A total of $1.5 \times 10^5$ B16-F10 cells were intravenously injected into each C57BL/6 mouse. Mice were sacrificed and the lung were pictured and recorded for tumour numbers. The lungs from mice were fixed and then in formalin for 48 hours at room temperature. Then, the samples were processed for paraffin embedding. Formalin-fixed paraffin embedded (FFPE) samples were cut into sections, mounted on glass slides and dried. After that, the samples were stained with hematoxylin and eosin (H&E), followed by microscopy analysis.

**Prevention of orthotopic lung cancer by NV**

The administration of PBS or NV was the same as above described. Mice were anesthetized and a total of $1 \times 10^6$ LLC cells mixed with 20% of Matrigel (BD Biosciences) was injected to the lung of each mouse. The body weight of mice was monitored and the mouse was sacrificed when the body weight lost was over 20%. The lungs of different mice were collected and pictured, followed by being fixed in formalin for 48 hours at room temperature. Then, the samples were processed for paraffin embedding. Formalin-fixed paraffin embedded (FFPE) samples were cut into sections, mounted on glass slides and dried. After that, the samples were stained with hematoxylin and eosin (H&E) and analysed.

**Rechallenge mice with melanoma or hepatoma**

In the rechallenge experiments, $3 \times 10^5$ B16-F10 cells or $4 \times 10^6$ Hepa 1–6 cells were subcutaneously injected into the right back of cured C57BL/6 mice on day 0 and the growth of tumour was monitored.

**Depletion of CD4$^+$ or CD8$^+$ T cells during treatment**

200 μL of nanovaccine A (containing 2 mg of PLGA) and 200 μL of nanovaccine B (containing 2 mg of PLGA) were injected subcutaneously at different sites on day $-49$, day $-42$, day $-35$, day $-28$ and day $-14$ ahead of tumour inoculation. CD4$^+$ and CD8$^+$ T cell depletion assays were conducted with the melanoma and
lung cancer mouse model by administering an αCD8 or αCD4 antibody (10 mg/kg) two days prior to tumour inoculation, on the day of tumour inoculation and every four days after tumour inoculation for the duration of the experiment. The tumour inoculation was conducted on day 0 and the tumour inoculation methods were the same as those used for prevention study. The growth of tumour was monitored the same as above.

Proteomics analysis of components loaded in NVs

The proteins loaded in NV were analysed by mass spectrometer (MS). The NVs were first disassembled. And then, both free tumour tissue lysates or the disassembled components from NVs were investigated. The procedures of preparing the samples and MS data analysis were the same as described in previous studies.

Prevention of cancer by allograft of pre-stimulated T cells by cancer vaccines

To induce tumour-specific immune responses in donor mice, 200 μL of NV A (containing 2 mg of PLGA) and 200 μL of NV B (containing 2 mg of PLGA) were injected subcutaneously at different sites on day –35, day –28, day –21, day –14 and day –4 ahead of T cell transplantation. On day –1 prior the T cell transplantation, the recipient mice were intraperitoneal injected with cyclophosphamide (100 mg/kg). On day 0, pre-treated donor mice were sacrificed, followed by collecting splenocytes and lymph node cells. The T cells in splenocytes were isolated by Pan T cell isolation Kit II (Miltenyi Biotec). And then, 5 x 10^5 of T cells from splenocytes or lymph node cells were intravenously injected into each recipient mouse. Meanwhile, tumour inoculation was performed on day 0. A total of 1 x 10^6 B16-F10 cells were subcutaneously injected into the right back of C57BL/6 mice, or 2 x 10^6 LLC cells were subcutaneously injected into the right armpit of C57BL/6 mice. Tumour volume was recorded every 3 days beginning on day 3.

Flow cytometric analysis of the abundance of various memory T cells in different organs

On day 60 after first injection of NV, draining lymph nodes, splenocytes, and peripheral blood were collected for the flow cytometric analysis. Live/Dead Fixable VioBlue Cell Stain Kit (Life Technologies) was applied for live/dead cell discrimination. The samples were incubated with Fc Block for 5 min on ice, followed by surface staining with anti-mouse CD3, CD4, CD8, CD44, CD62L and CD69. Central memory T cells (Tcm) were phenotyped as CD3^+CD44^+CD62L^+, effector memory T cells (Tem) were identified as CD3^+CD44^+CD62L^- and tissue-resident memory T cells (Trm) as CD3^+CD44^+CD62L^-CD69^+.

Test of peripheral cancer-specific T cells

Splenocytes (containing APCs such as B cells and DC), collected after sacrificing mice, were co-cultured and stimulated with NVs loaded with tumour tissue lysates. After stimulation, the splenocytes were collected. Then the samples were incubated with Fc Block, followed by staining with anti-mouse antibodies against CD3, CD8 and CD4. The splenocytes were then fixed, permeabilized and stained for intracellular IFN-γ. Data are presented as the percentage of CD8^+IFN-γ^+ cells in the CD8^+ cell population or the percentage of CD4^+IFN-γ^+ cells in the CD4^+ cell population. Differences were compared by a t test.

Fluorescent multiplex immunohistochemistry (IHC), tissue imaging and analysis

Lung puncture FFPE tissue of mice were used as experimental sample. All the tissues were cut into sections of 4-μm thickness. The slides were deparaffinized in xylene for 30 mins and rehydrated in absolute ethyl alcohol for 5 mins (twice), 95% ethyl alcohol for 5 mins, 75% ethyl alcohol for 2 mins sequentially. Washed the slides with distilled water 3 times. A microwave-oven is used for heat-induced epitope retrieval, and during epitope retrieval the slides were immersed in boiling EDTA buffer (AlphaxBio, Beijing, China) for 15 mins. Antibody Diluent/Block (AlphaxBio, Beijing, China) was used for blocking. Two group of antibodies were use for mIHC staining. In the first group, primary antibodies performed as follows: CD3 (ab5690, Abcam, Cambridge, UK), CXCL13 (ab199043, Abcam, Cambridge, UK), CD4 (ab183685, Abcam, Cambridge, UK), TCF1 (CST2203, CST, Cambridge, UK) and CD8(ab217344, Abcam, Cambridge, UK). In another group, primary antibodies performed as follows: CD3 (ab5690, Abcam, Cambridge, UK), CD45R (ab64100, Abcam, Cambridge, UK), TCF1 (CST2203, CST, Cambridge, UK) and CD8(ab217344, Abcam, Cambridge, UK). All the primary antibodies were incubated for 1 h at 37°C. Then slides were incubated with Alpha Polymer HRP Ms+Rb (AlphaxBio, Beijing, China) or polymer HRP Rat (Zsbio, Beijing, China) for 10 min at 37°C correspondingly. AlphaX 7-Color IHC Kit (AlphaxBio, Beijing, China) was used for visualization. The correspondence between primary antibody and fluorophores was Alpha TSA 520 (CD3), Alpha TSA 570 (CXCL13), Alpha TSA 620 (CD4), Alpha TSA 650 (TCF1), Alpha...
TSA 690(CD8) for the first group and Alpha TSA 520 (CD3), Alpha TSA 570 (CD45R), Alpha TSA 620 (F4/80), Alpha TSA 650 (CD49b), Alpha TSA 690(CD8). After each cycle of staining, heat-induced epitope retrieval was performed to remove all the antibodies including primary antibodies and secondary antibodies. The slides were counterstained with DAPI for 5 mins and enclosed in Antifade Mounting Medium (I0052; NobleRyder, Beijing, China). Vectra 3 (Vectra 3.0.5; Akoya Biosciences, USA) was used for imaging the images captured were analyzed with specific spectral library build from single stained slides.

**Histopathology examination**

The major organ (heart, liver, spleen, lung, kidney) of mice in different groups were fixed in formalin for 48 hours at room temperature, followed by processing for paraffin embedding. Formalin-fixed paraffin embedded (FFPE) samples were cut into sections, mounted on glass slides and dried. After that, the samples were stained with hematoxylin and eosin (H&E), followed by microscopy analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical differences in the average tumour growth curves were determined by two-way ANOVA using the variables of time and volume. Differences in survival were determined for each group by the Kaplan-Meier method, and the overall p value was calculated by the log-rank test. Statistical differences in flow cytometric analysis were compared by a two-tailed t test. All the Statistics analysis was done by using GraphPad Prism 8.