Converting Immune Cold into Hot by Biosynthetic Functional Vesicles to Boost Systematic Antitumor Immunity

Cold tumor

Apoptosis

ICD

Hot tumor

Convert

BFVs/PD1-TRAIL-CAT

H2O2

O2

Memory T cell

Infiltration

Naïve T cell

Tumor-draining lymph nodes

TRAIL induces cancer cell immunogenic death, ectogenic PD1 blocks checkpoint signal

Catalase reduces TME hypoxia to enhance effector T cell infiltration and activation

BFVs boost systematic antitumor immunity and achieve long-term immune memory
Converting Immune Cold into Hot by Biosynthetic Functional Vesicles to Boost Systematic Antitumor Immunity

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SUMMARY

Immune cold tumor characterized by low immunogenicity, insufficient and exhausted tumor-infiltrating lymphocytes, and immunosuppressive microenvironment is the main bottleneck responsible for low patient response rate of immune checkpoint blockade. Here, we developed biosynthetic functional vesicles (BFVs) to convert immune cold into hot through overcoming hypoxia, inducing immunogenic cell death, and immune checkpoint inhibition. The BFVs present PD1 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on the surface, whereas load catalase into their inner core. The TRAIL can specifically induce immunogenic death of cancer cells to initiate immune response, which is further synergistically strengthened by blocking PD1/PDL1 checkpoint signal through ectogenic PD1 proteins on BFVs. The catalase can produce O2 to overcome tumor hypoxia, in turn to increase infiltration of effector T cells while deplete immunosuppressive cells in tumor. The BFVs elicit robust and systematic antitumor immunity, as demonstrated by significant regression of tumor growth, prevention of abscopal tumors, and excellent inhibition of lung metastasis.

INTRODUCTION

Immune checkpoint blockade (ICB) therapy has shown great therapeutic potential in various cancer types, including melanoma, renal carcinoma, head and neck squamous cell carcinomas, non-small cell lung cancer, urothelial carcinoma, and bladder cancer (Tang et al., 2018; Tumeh et al., 2014). In particular, ICB therapy by monoclonal antibodies specific for the immune checkpoint proteins of programmed cell death receptor-1 (PD1) or its predominant ligand (PDL1) has achieved tremendous success in clinic (Boussiotis, 2016; Zou et al., 2016). However, only a small portion of patients benefit from current ICB therapies (Robert et al., 2015; Topalian et al., 2012), mainly ascribed to many tumors inherently featured as immune cold with low level and exhausted tumor-infiltrating lymphocytes (TILs), insufficient tumor antigen burden, and immunosuppressive microenvironment caused by tumor hypoxia, which does not respond well to ICB therapies (Galon and Bruni, 2019; Huang et al., 2019). Although combination of ICB with various other therapeutic modalities (i.e., radiotherapy [Chao et al., 2018], chemotherapy [Wang et al., 2018], phototherapy [He et al., 2016], and other immunotherapies [Wolchok et al., 2013]) has achieved a synergistic effect to some degree, the clinical outcome is still not satisfactory as these therapeutic modalities are also very difficult to convert immune cold into hot as well as they have their own intrinsic defects, such as radiation of radiotherapy, severe systemic toxicity of chemotherapy, shallow tissue penetration of phototherapy and serious cumulative immune-related toxicities of combinational immunotherapies, all of which inevitably hinder their further clinical efficacy (Yue et al., 2019). Therefore, more rational synergistic approaches that can robustly convert the immune cold tumor into hot are extremely on demand to improve the therapeutic potential of ICB.

Up to now, several strategies have been developed to amplify immunomodulatory effects (Chen et al., 2019; Chiang et al., 2018; Galstyan et al., 2019; Mi et al., 2018; Ruan et al., 2019; Wang et al., 2016, 2019). Among them, biosynthetic functional vesicles (BFVs) with surface presenting PD1 proteins hold great promise to improve cancer immunotherapy efficiency, owing to their unique advantages including excellent biocompatibility and high fidelity of surface markers (Liu et al., 2019; Zhang et al., 2018a, 2018b, 2018c). However, other typical immunosuppressive features involved in immune cold tumor, for example, low...
Figure 1. BFVs Synergistically Improve Immunotherapy by Converting Immune Cold into Hot

(A) Schematic illustration of the mechanism of BFVs to generate robust antitumor immune responses. TRAIL can specifically induce immunogenic cancer cell death (ICD) to initiate immune responses. Ectogenic PD1 proteins on BFVs can block the PD-1/PDL1 immune checkpoint to further synergistically strengthen the immune responses. CAT can alleviate tumor hypoxia to enhance intra-tumoral infiltration of effector T cells and weaken immunosuppression.

(B) Confocal images of HEK293 FT cells stably expressing mouse PD1 and mouse TRAIL on cell membranes, respectively. DiO and Dil were used to stain cell membrane (scale bar: 10 μm).

(C) The representative flow cytometric analysis of PD1-expressing (gated on mCherry+) and TRAIL-expressing (gated on EGFP+) HEK293 FT cells.

(D) Western blot assay to confirm the expression of mouse PD1 (indicated as PD1+) and mouse TRAIL (indicated as TRAIL+) in 293FT cells. Actin was used as the loading control.

(E) Confocal images to demonstrate the co-existence of PD1-mCherry and TRAIL-EGFP on the single vesicle by the overlap of red/green fluorescence (scale bar: 10 μm).

(F) The transmission electron microscopy image of BFVs (scale bar: 50 nm).
tumor associated antigen (TAA) burden that inhibits the recognition ability or activation of cytotoxic T cells in tumor, still extensively exist to significantly impair the immunotherapy effect. Thus, in addition to PDL1 blockade, the BFVs that can simultaneously induce immunogenic cell death (ICD) of cancer cells to increase tumor immunogenicity (such as TAA burden) are extremely needed to improve the ICB efficacy. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising biotherapeutic candidate that can specifically induce apoptosis of cancer cells without causing toxicity to normal cells (von Karstedt et al., 2017). Therefore, TRAIL might be a potential ICD inducer and could seamlessly cooperate with ectogenic PD1 when both of them are presented on BFVs.

Although the cooperation of TRAIL and PD1 can synergistically improve the tumor immunogenicity, the dynamic immune evasion mechanisms due to the complicated tumor microenvironment (TME) of solid cold tumor still often lead to therapeutic failure (Binnewies et al., 2018; Kim et al., 2018). Typically, solid tumors are characterized by a highly hypoxic TME, which contributes to immune resistance and immune suppression/tolerance through multi-pathways, including recruitment of immunosuppressive cells (i.e., myeloid-derived suppressor cells [MDSCs], tumor-associated macrophages [TAMs], and T-regulatory [Treg] cells), making cancer cells resistant to cytotoxic T lymphocyte (CTL)-mediated lysis, desensitization of chemokine receptors, and reduction of proinflammatory cytokines (Noman et al., 2015). Therefore, alleviating the hypoxia of TME would be a powerful strategy to reverse immune cold into hot for improving immunotherapy outcomes. Although respiratory hyperoxia has been applied to decrease the intra-tumoral hypoxia for reinforcing immunotherapy effect (Hatfield et al., 2015), this protocol also produced excessive amounts of reactive O2 species (ROS) that might overwhelm natural antioxidant defenses to destroy normal cellular structures (e.g., inducing hyperoxic acute lung injury [Kallet and Matthay, 2013]). Compared with such systemic O2 supply strategy, in situ intra-tumoral generation of O2 will be a more efficient, favorable, and safe approach for alleviating hypoxia but has rarely been reported to modulate the immune responses.

To overcome the low immune responses of ICB therapy in cold tumors, we herein proposed a proof-of-concept strategy by designing BFVs with surface presenting PD1 and TRAIL while encapsulating catalase (CAT) into the inner core to thoroughly convert tumor from immune cold into hot milieu for eliciting robust and systematic antitumor immunity (Figure 1A). We hypothesized that the TRAIL component on BFVs could initiate the immune responses by specifically inducing immunogenic cancer cell death, which could be further strengthened by collaborating with presented ectogenic PD1 proteins as the checkpoint blockade to reactivate the anergic tumor-specific CTLs; in addition, the CAT could catalyze highly abundant H2O2 in TME for in situ generation of O2, thus to ameliorate tumor hypoxia for systematically reversing the un-favorable environment of ICB therapy to ensure the trafficking and killing activities of CTLs in tumors. The comprehensive immuno-modulating ability and robust antitumor immunity of our BFVs have been clearly demonstrated in immune cold tumor models (4T1 breast tumor models [Sagiv-Barfi et al., 2015], including both subcutaneous and orthotopic models), through significant primary tumor growth regression, excellent metastasis prevention, obvious distal and immune-memory effects, and long-term survival benefits. Taken together, the here reported BFVs can elicit potent and durable immune responses by turning immune cold tumors into hot, and in principle it might be an universal immunotherapy paradigm for various other solid tumors and very promising for future translation.

RESULTS
Fabrication of BFVs

To bio-synthesize the functional vesicles (as depicted in Figure S1), we first established stable HEK293 FT cells expressing the proteins of mouse PD1 fused with mCherry (PD1-mCherry) and TRAIL fused with EGFP (TRAIL-EGFP) on the cell membrane, respectively, by using lentiviral vector for corresponding gene transfection (Figure S2). As shown in Figure 1B, the PD1 fusing proteins and the TRAIL fusing proteins were nicely expressed on the plasma membrane of transfected cells through confocal laser scanning microscopy (CLSM) observation, evidenced by their co-localization with cell membrane-staining dyes. Flow cytometry analysis further proved that nearly 90% of the genetically engineered cells were PD1 or TRAIL positive.
(Figure 1C), and the stable over-expression of the PD1 or TRAIL proteins can be further confirmed by western blot (WB) analysis (Figures 1D, S3A, and S3B). Collectively, these results clearly confirm the successful display of PD1 or TRAIL proteins on the membrane of HEK293 FT cells.

Then, the cell membranes of engineered HEK293 FT cells were extracted, and the BFVs were obtained by co-extrusion of PD1 anchoring membranes, TRAIL anchoring membranes, and commercially available CAT through 1, 0.4, 0.2, and 0.1 μm pore-sized polycarbonate membrane filters. To vividly observe the co-existence of PD1 and TRAIL on BFVs, the rough products extruded through 1-μm filters were imaged by CLSM (Figure 1E). As expected, the vesicles presented both green fluorescence of TRAIL-EGFP and red fluorescence of PD1-mCherry, demonstrating the successful fusion of both cell membranes. Next, the morphology and size of BFVs were characterized by transmission electron microscopy and dynamic light scattering analyses, which showed a uniform vesicular morphology with average diameter around 100 nm (Figures 1F, 1G, S4A and S4B). The zeta potential of the BFVs was determined as $-24.9 \pm 1.1$ mV (Figure S4C). The immunoprecipitation assay showed that the anti-PD1 antibody could pull down the majority of BFVs, demonstrating the PD1 protein had a right outside-out orientation on most of the BFVs (Figures 1Ha and S3C).

In addition, we fabricated BFVs with larger size by extruding cell membranes (pre-labeled with DiO) and CAT (pre-labeled with Cy5) through 10-μm filters, to intuitively demonstrate the CAT can be encapsulated inside the inner core of BFVs by using CLSM. As shown in Figure S5A, the physical mixture of CAT and cell membranes (without co-extrusion) that exhibited only DiO green fluorescence, implying the free CAT unloaded in BFVs, could be completely removed by repeated washing in the process of producing BFVs. However, BFVs/CAT obtained by co-extrusion of BFVs and CAT showed dual and merged fluorescence signals, demonstrating the co-existence of cell membranes and CAT. Furthermore, 3D reconstruction of z stack images (Figure S5A) and plot profile (Figure S5B) along a single merged fluorescence vesicle revealed that the CAT was successfully encapsulated inside the BFV. Furthermore, we also compared the enzyme activity of the final product from physical mixing or from the co-extrusion procedure through detecting the $H_2O_2$ scavenging ability by using TiOSO$_4$ as a color probe (from colorless to yellow after encountering $H_2O_2$ with maximum absorbance at 450 nm), to verify the encapsulation of CAT inside BFVs. As shown in Figure S5C, the product from physical mixture has negligible $H_2O_2$ scavenging ability, as demonstrated by nearly the same absorbance as control group. In sharp contrast, the TiOSO$_4$ absorbance in BFVs/CAT group was much lower. These results demonstrated the successful encapsulation of CAT in the BFVs by our co-extrusion procedure.

The TRAIL and PD-1 contents in BFVs were determined by ELISA (Figure S6), and the ratio to total proteins in BFVs was calculated to be 1.02% and 5.50%, respectively. To estimate the loading content of CAT, it was labeled with Cy5 prior to loading into BFVs; then, the CAT content was analyzed by fluorescence measurement (Figure S6), and the ratio to total proteins in BFVs was calculated to be 35.07%.

BFVs Block PDL1 on Cancer Cells to Increase Their Susceptibility to CTLs

The interaction between PD1 on activated CTLs (CD8+ T cells) and its ligand PDL1 on cancer cells can exhaust CTLs and inhibit antitumor immune responses. Conversely, blockade of PD1/PDL1 axis could reverse this resistance and profoundly enhance therapeutic efficacy (Hirano et al., 2005). We next investigated whether our BFVs could act as a PD1 checkpoint blockade. For this purpose, the 4T1 cells were incubated with FITC-labeled PD1 presenting vesicles (BFVs/PD1) and imaged by CLSM. As shown in Figure 2A, BFVs/PD1 but not the control vesicles (without PD1 presenting, free NVs) could effectively bind to and be internalized by 4T1 cells after 4 h of incubation, but such binding and internalization could be blocked by PD1 antibody pretreatment. To further intuitively demonstrate that the binding of BFVs/PD1 on 4T1 cells is mediated by the interaction between PD1 and PDL1, 4T1 cells were transfected with PDL1-EGFP plasmid to establish PDL1-EGFP expressing cells. Subsequently, the BFVs/PD1 with mCherry tag were incubated with these cells for 0.5 h at 4°C, then obvious co-localization with PDL1-EGFP on 4T1 cells was observed, in addition to some dissociative red fluorescence dots implying that BFVs/PD1 also might bind to endogenous PDL1 in 4T1 cells (Figure 2B). Similar results also could be observed in PDL1-EGFP-expressing H22 cells (Figure S7). Together, these results confirmed that the PD1-presenting BFVs could specifically interact with tumor cells through the binding between PD1 and PDL1.

We next attempted to explore whether the BFVs/PD1 could enhance the cytotoxicity of CTLs against cancer cells. As depicted in Figure 2C, naive mouse CD8+ T cells were first isolated from the spleen of BALB/c
mice by magnetic bead sorting, followed by CD3/CD28 antibody stimulation to obtain activated CD8\(^+\) T cells. Subsequently, cancer cells with various treatments, such as blocking or overexpressing PDL1 on cell membranes, were added to the activated CD8\(^+\) T cells to check the cytotoxic activity by using Annexin V-FITC and PI cell apoptosis (shortened as APCA) assay. The PDL1 overexpressing 4T1 cells were less susceptible to the activated CD8\(^+\) T cells than the parental 4T1 cells; vice versa, when 4T1 cells were pretreated

**Figure 2. In Vitro Immune Checkpoint Inhibition of BFVs**

(A) The CLSM images to present the internalization of FITC-labeled BFVs/PD1(50 \(\mu\)g/mL) by 4T1 breast cancer cells after 4 h of incubation (scale bar: 20 \(\mu\)m) at 37\(^\circ\)C. free NVs: blank vesicles without presenting PD1 and TRAIL, BFVs/PD1: vesicles with surface presenting PD1 proteins; BFVs/PD1 + anti-PDL1: 4T1 cells pretreated with PDL1 antibody before BFVs/PD1 incubation.

(B) CLSM images to demonstrate the binding of BFVs/PD1-mCherry to the PDL1-EGFP over-expressed on 4T1 cell membrane (scale bar: 10 \(\mu\)m). 4T1 cells were transfected with PDL1-EGFP plasmid for 24 h, then incubated with BFVs/PD1-mCherry (50 \(\mu\)g/mL) for 0.5 h at 4\(^\circ\)C. The lower panel is the enlarged image of the white dashed box on the upper panel.

(C) Representative procedure to schematically show the experimental design of the CTL cytotoxicity assay in vitro. Naïve CD8\(^+\) T cells extracted from mouse spleen were activated by incubation with IL2, anti-CD3, and anti-CD28 antibodies, then they were added into 4T1 cells that previously underwent various treatments indicated as below: 4T1 cells overexpressing PDL1 (PDL1 OE), or normal 4T1 cells treated with PBS (PBS), free NVs, BFVs/PD1, or PDL1 antibody (anti-PDL1). After 24 h of co-incubation, the apoptosis ratio of 4T1 cells was measured.

(D) Apoptosis of the 4T1 cells treated as mentioned above by using Annexin V-FITC and PI cell apoptosis assay.

(E) The Granzyme B secreted in the suspensions of co-culture system after 24 h of treatment. Data are expressed as min to max, show all points (ns, no significance; ***p < 0.001; n = 3).
BFVs Induce ICD Responses in Cancer Cells

ICD plays an important role in initiating immune responses against tumor-associated antigens. Here, we further explore the potential of TRAIL as an ICD inducer, since it has been reported to selectively induce apoptosis by binding to the death receptors DR4 or DR5 in many cancer cell types while leaving normal cells unharmed (Jiang et al., 2016). Accordingly, we demonstrated that BFVs with surface presenting TRAIL-EGFP (BFVs/TRAIL) could co-localize with the DR5 on DR5-mCherry artificially over-expressed 4T1 cells or H22 cells (Figures 3A and S9). Meanwhile, TdT-mediated dUTP Nick-End Labeling (TUNEL) staining (Figure 3B) and APCA assay (Figures 3C and S10) demonstrated that the TRAIL on BFVs played a crucial role in inducing apoptosis of cancer cells (4T1 and H22) but not normal cells (NIH 3T3), whereas the PD1 proteins on BFVs had negligible effect. Calreticulin (CRT) is considered as one of the danger-associated molecular patterns (DAMPs) involved in ICD (Obied et al., 2007). To confirm whether TRAIL on BFVs could promote ICD, the CRT expression of 4T1 cells with indicated treatments was analyzed by CLSM. As shown in Figure 3D, the CRT signals (green) dramatically increased in BFVs/PD1-TRAIL-treated group, compared with PBS or BFVs/PD1-treated groups.

The release of DAMPs together with tumor-associated antigens (TAAs) could promote the maturation of DCs and further enable the ability to prime T cell activation (Yue et al., 2019). To verify this, we carried out a series of experiments as illustrated in Figure 3E. The supernatant of 4T1 cells after above treatments were added into immature DCs collected from bone marrow cells after stimulating with granulocyte macrophage colony stimulating factor (GM-CSF). After 48 h of incubation, the DC maturation was evaluated by dual staining for CD80 and CD86, the typical markers on the surface of matured DCs. It showed that the supernatant of cancer cells treated with BFVs/PD1-TRAIL could significantly improve the degree of DC maturation, as demonstrated by the higher percentage of CD80/CD86-positive DC cells (Figure 3F). Consistently, the levels of DC-secreted immune cytokines, such as TNF-α, IFN-γ, and IL12-P70 in supernatant also significantly increased after the BFVs/PD1-TRAIL treatment (Figure 3G). Afterward, the matured DCs were incubated with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD8+ T cells to explore their ability to prime T cell activation. The FACS analysis demonstrated that the matured DCs triggered by BFVs/PD1-TRAIL-treated cancer cells could significantly improve T cell proliferation (Figures 3H, 3I, and S11), compared with other groups without TRAIL component. Taken by BFVs/PD1 or PDL1 antibody, they became significantly more susceptible (Figures 2D and S8). These results indicated that blocking of PDL1 on cancer cells by BFVs/PD1 could effectively reverse the inhibition of T cell-mediated cytotoxic activity harnessed by cancer cells. As a main route of carrying out immunosurveillance, CTLs can secrete granzymes to induce cancer cell death (Cullen et al., 2010). As shown in Figure 2E, it was clearly demonstrated that blocking PD1/PDL1 interactions by BFVs/PD1 could obviously induce tumor cell death through the release of Granzymes B from the activated CD8+ T cells. These results strongly suggest that the BFVs/PD1 can block the PDL1 functions on cancer cells to re-strengthen T cell-mediated cytotoxic activity.

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Figure 4. BFVs Alleviate Tumor Hypoxia to Enhance Intra-tumoral Infiltration of Effector T Cells and Relieve Immunosuppressive Tumor Microenvironment

(A) The schematic illustration of relieving tumor hypoxia through decomposing H$_2$O$_2$ into O$_2$ by CAT.

(B) Oxygen generation in H$_2$O$_2$ solutions (0.5 mM) with BFVs/PD1-TRAIL (without CAT) or BFVs/PD1-TRAIL-CAT (with CAT).

(C) Fluorescence microscopy images of 4T1 cells treated with BFVs/PD1-TRAIL and BFVs/PD1-TRAIL-CAT, whereas the cells treated with PBS were used as the control. The 4T1 cells were stained with Hydrogen Peroxide Assay Kit (green) or oxygen indicator of [Ru(dpp)$_3$]Cl$_2$ (red) (scale bar: 50 μm). The statistical analysis data are expressed as min to max, show all points (***p < 0.001; n = 3).

(D) Photoacoustic imaging to show the vascular saturated O$_2$ (sO$_2$) levels of tumor after i.t. injection of PBS, CAT, and BFVs/PD1-TRAIL-CAT at different time points. The black-white scale bar shows the ultrasound imaging signals and the blue-red scale bar shows the photoacoustic signals.

(E) Immunofluorescence staining of the tumor slices by using Hypoxyprobe-1 Plus Kits (green), scale bar: 50 μm.
together, these results suggest that the TRAIL rather than PD1 on BFVs is able to induce ICD responses in cancer cells to effectively mobilize host adaptive immunity, and it is an important aspect for changing the immune cold state of tumor.

BFVs Relieve Tumor Hypoxia to Enhance Intra-tumoral Infiltration of Effector T Cells and Deplete Immunosuppressive Cells

It has been reported that CAT could decompose H2O2 into O2 (Figure 4A) (Chao et al., 2018); therefore, we wonder whether our BFVs with CAT in the inner core could \textit{in situ} generate O2 in the tumor microenvironment (with relatively high level of H2O2) to relieve tumor hypoxia. We first evaluated the ability of CAT in BFVs to generate O2 in the presence of H2O2. As shown in Figure 4B, H2O2 and BFVs/PD1-TRAIL (without CAT loading) had negligible effect on O2 generation, whereas the BFVs/PD1-TRAIL-CAT (with CAT loading) yielded significantly higher amounts of O2 with H2O2 incubation. Afterward, we further explored the intracellular catalytic activity of BFVs/PD1-TRAIL-CAT by detecting H2O2 and O2 levels in cells with different treatments under hypoxic condition. As expected, the cells treated with BFVs/PD1-TRAIL-CAT exhibited much weaker fluorescence of H2O2 and hypoxia indicators as compared with other control groups, suggesting the loaded CAT kept excellent enzyme activity to catalyze H2O2 into O2 after internalization by cells (Figure 4C). For investigating the ability of relieving tumor hypoxia by BFVs/PD1-TRAIL-CAT \textit{in vivo}, the oxygen saturation of tumors after local injection of BFVs/PD1-TRAIL-CAT was determined by photoacoustic imaging (PAI). Compared with the PBS group, the vascular saturated O2 (sO2) levels in the BFVs/PD1-TRAIL-CAT or free CAT-treated tumors were significantly higher (Figure 4D). To further confirm the relief of tumor hypoxia, the Hypoxyprobe-1 Plus Kits were used to assess the oxygenation status of tumor tissues. As shown in Figure 4E, the tumor treated with free CAT or BFVs/PD1-TRAIL-CAT showed dramatically decreased green fluorescence signal of hypoxia indicator. Concomitantly, the intra-tumoral H2O2 concentration in free CAT or BFVs/PD1-TRAIL-CAT-treated tumors was significantly lower than in the PBS-treated group (Figure 4F).

Emerging evidences demonstrated that tumor hypoxia could impair the antitumor immune responses by impeding killing function of CTLs, while promoting local immune suppression (Noman et al., 2015). Then, the influence of O2 on the activation of CD8+ T cells was first studied \textit{in vitro}. After 48 h of culture, the primary CD8+ T cells were admitted to APCA assay. The ratio of apoptotic CD8+ T cells was significantly higher under hypoxic condition, compared with normoxic condition (Figures 4G, S12A, and S12B); the expression of TIGIT on CD8+ T cells, a T cell exhausting marker (Joller et al., 2011), was also significantly up-regulated under hypoxic environment (Figures 4G, S12C, and S12D). Therefore, we anticipated the relief of hypoxia in TME might reverse the inhibition of antitumor immunity of CD8+ effector T cells to some degree. To further explore the effects of relieving hypoxia on immune responses, we analyzed the infiltrated immune cell populations in tumors with different treatments after intra-tumoral (i.t.) injection by FACS analysis. The results showed that our BFVs/PD1-TRAIL-CAT not only could promote the intra-tumoral infiltration of CD8+ T cells (Figures 4H and S13 and Tables S1 and S2) but also could deplete the immunosuppressive cells like Treg cells (Figures 4I and S14 and Table S3) and MDSCs (Figures 4J and S15 and Table S4). These results suggest that BFVs can remodel hypoxia TME and reverse the hypoxia-associated immunosuppression, which is crucial for converting immune cold tumor into hot.

\textbf{In Vivo Antitumor Effect of BFVs}

To evaluate the \textit{in vivo} therapeutic effect of BFVs, a bilateral 4T1 tumor model was first established by subcutaneously (s.c.) injecting 1 x 10^6 and 5 x 10^5 4T1 tumor cells into the left and right flanks of mice as primary and abscopal tumors, respectively. The primary tumor received various treatments by i.t. injection at days 1, 4, 7, and 10 with the equivalent dosage (Figure 5A), while leaving the abscopal tumors untreated. Antitumor growth effects against the primary and abscopal tumors are summarized in Figures 5B and 5C.
The primary tumors in the group of anti-PDL1, BFVs/PD1, BFVs/TRAIL, and BFVs/PD1-TRAIL treatment displayed slower tumor growth compared with the mice treated with PBS and blank vesicles without any functional components (free NVs). Notably, our BFVs containing PD1, TRAIL, and CAT (BFVs/PD1-TRAIL-CAT) exerted the highest antitumor efficacy among all groups, suggesting their synergistic effects. For abscopal tumors without direct treatment, the therapeutic outcomes are consistent with the primary tumors, with the highest therapeutic efficacy in BFVs/PD1-TRAIL-CAT-treated mice, and 70% of mice in this group survived for over 60 days, whereas all the mice in other groups survived no longer than 40 days, which might be attributed to the systematic activation of antitumor immunity. To verify this explanation, we analyzed the infiltration of TILs and the pro-inflammatory cytokines in abscopal tumors of the treated mice. As expected, BFVs/PD1-TRAIL-CAT-treated mice showed significantly promoted CD8+$^+$ T cell infiltration (Figure 5D), as well as the highest level of TNF-$\alpha$, IFN-$\gamma$, and IL12-P70 compared with other groups (Figure 5E), implying the cooperation of PD1, TRAIL, and CAT to deeply convert immune cold into hot, which in turn boosts the efficacy of immunotherapy.

Furthermore, we employed the orthotopic 4T1 tumor model, as it is more clinically relevant, to investigate the therapeutic effect of BFVs with systematic administration. The experimental protocol is shown in Figure 6A, wherein the orthotopic tumor model was established by injecting $5 \times 10^5$ 4T1 cells into abdominal mammary fat pads of female BALB/c mice. Before therapeutic assessment, in vivo distribution of ICG-labeled vesicles was analyzed by NIR II fluorescence imaging system. It revealed that vesicles containing PD1 proteins (BFVs/PD1) had excellent tumor accumulation even after 48 h of injection, enabling their durable therapeutic effect (Figure S16). In addition, the tumor accumulation of BFVs/PD1 was significantly higher than the control vesicles (without PD1 presenting), ascribing to the targeting ability of PD1 to PD1 on cancer cells rather than the difference of Pharmacokinetics (Figure S17). In contrast, free ICG, which was easily cleared out from the body, had negligible fluorescence signal in major organs including tumor tissue compared with BFVs (Figure S16). With regard to antitumor efficacy (Figures 6B–6D, S18, and S19), the overall trends of tumor growth inhibition on the orthotopic tumor model were approximately the same as those on the subcutaneously xenografted tumor model, highlighting the distinctive antitumor ability of BFVs/PD1-TRAIL-CAT owing to the synergistic contributions from TRAIL, PD1, and CAT to convert immune cold into hot. Histological examinations of the tumor tissues stained with H&E, TUNEL, and Ki67 also confirmed that our BFVs/PD1-TRAIL-CAT induced the most extensive level of tumor cell necrosis and apoptosis (Figures 6E and S20).

We next further explored the mechanisms underlying the remarkable antitumor efficiency of our BFVs-mediated synergistic therapy. First, immunofluorescent examination of tumor sections proved that the BFVs/PD1-TRAIL-CAT significantly increased CRT expression compared with other groups (Figure 6E). Since the CRT together with other DAMPs would promote the DC maturation for antigen presenting to T lymphocytes in draining lymph nodes (LNs), we analyzed the levels of DC maturation in draining LNs by FACS after 3 days of the last injection (Figure S21). As expected, the BFVs/PD1-TRAIL-CAT therapy triggered the highest level of DC maturation (CD11c+$^+$CD80+$^+$CD86$^+$) among all groups. Meanwhile, the intra-tumoral infiltration of T lymphocytes and immune-related cytokines were examined by multispectral immunofluorescence staining (Figure 6F) and ELISA (Figure 6G), respectively. It showed that the BFVs/PD1-TRAIL-CAT treatment triggered a significantly higher degree of CD8+$^+$ T cell infiltration in tumors than other treatments. The cytokine secretion results also supported that BFVs/PD1-TRAIL-CAT treatment could elicit robust antitumor immune activity in tumor tissues, with the significantly increased secretion of pro-inflammatory cytokines (TNF-$\alpha$, IFN-$\gamma$, IL12-P70, Granzyme B) and decreased secretion of anti-inflammatory cytokines (TGF-$\beta$, IL10). We also analyzed, in addition to tumor tissues, the frequency of T lymphocytes in peripheral blood by using FACS, and the result showed the percentage of CD8+$^+$ T cells...
in BFVs/PD1-TRAIL-CAT-treated groups was the highest compared with other groups, indicating the enhanced systemic immune responses after BFVs treatment (Figures 6H and S22). Taken together, these data suggest that the BFVs integrated functional components of TRAIL, PD1, and CAT could significantly boost systematic antitumor immune responses through converting immune cold into hot by simultaneously overcoming hypoxia, increasing immunogenic cell death, and checkpoint inhibition.

As the distinct characteristics of adaptive immune responses, immune-memory effects can provide long-term prevention against tumor recurrence and distant metastasis. Thus, we further assessed the immune-memory effects of our BFVs by i.v. infusion of 4T1 cells into the mice after the aforementioned treatments at day 30, whereas the residual tumor mass of each group was surgically resected from each mouse at the day 23. The animal experimental design is shown in Figure 7A. From photographs of the lung and H&E images (Figures 7B–7D), the PBS and free NVs-treated mice displayed large amounts of lung metastasis, whereas other control vesicle (BFVs/PD1, BFVs/TRAIL and BFVs/PD1-TRAIL)-treated mice showed certain degree of lung metastasis inhibition; as a remarkable contrast, the BFVs/PD1-TRAIL-CAT-treated mice showed almost no detectable metastasis, confirming the excellent immune memory effects after converting immune cold into hot.

To further identify the possible mechanisms of the long-term immune memory post synergistic immunotherapy of BFVs, we analyzed the central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) in peripheral blood of mice after various treatments. Compared with T_{CM} only providing immunities in the condition of expansion, differentiation, and migration, T_{EM} can induce immediate protections upon secondary challenge (Ko and Formenti, 2018; McGranahan et al., 2016). As shown in Figures 7E–7I, S23, and S24, BFVs/PD1-TRAIL-CAT exhibited the highest frequency of CD8^{+} T_{EM} and CD4^{+} T_{EM} along with the lowest frequency of CD8^{-} T_{CM} and CD4^{-} T_{CM} in comparison with other groups, demonstrating the ability of our BFVs/PD1-TRAIL-CAT to shift systemic CD8^{+} and CD4^{+} T_{CM} cells toward a T_{EM} phenotype in the process of converting primary tumor from immune cold into hot, which is responsible for generating more-potent antitumor immune memory responses to prevent recurrence.

DISCUSSION

With a review of the recent developments of tumor immunotherapy, the combination paradigms of conventional therapeutic modalities with ICB have shown great potency to augment cancer immunotherapy efficacy, but their effectiveness largely depends on the baseline immune responses. In such a case, most of solid tumors featured as immune cold lacking pre-existing adaptive immunity do not respond well to the current combinational therapeutic strategies (Galon and Bruni, 2019). Another concern with the present approaches would be the concurrent increase in undesired side effects (Whiteside et al., 2016). The main mechanisms involved in cold tumor include lack of TAA burden, deficient and exhausted TILs, and hypoxic TME-driven immunosuppressive environment. Conventional therapeutic modalities (e.g., radiotherapy, chemotherapy) can augment the antigenicity of tumor cells through increasing the release of TAAs, whereas these released TAAs are often poorly processed, presented, and recognized by immune cells owing to the extensive existing immune-suppression mechanisms; thus, the immune responses in such case are generally very limited (McGranahan et al., 2016). Meanwhile, like a double-edged sword, radiotherapy or chemotherapy can also subvert tumor immunosurveillance through recruiting myeloid cells...
Figure 7. Long-Term Immune-Memory Effect of BFVs

(A) Schematic illustration of BFV treatment for lung metastasis inhibition of 4T1 tumor in BALB/c mice.

(B) Photographs of the lungs excised from sacrificed mice of each group.

(C) Statistical analysis of lung metastasis nodules (*p < 0.05, **p < 0.01; n = 3).

(D) H&E staining images of lung metastasis slices in different groups.

(E) Flow cytometry analysis of the TCM and TEM in peripheral blood isolated from sacrificed mice after various treatments by staining CD44-PE-Cy7 and CD62L-PerCP-Cy5.5.

(F–I) The statistical analysis for the percentage of the TCM and TEM in CD8+ or CD4+ T cells, data are expressed as min to max, show all points (**p < 0.01, ***p < 0.001; n = 3).
with distinct roles in T cell suppression and other side effects on normal tissue (Ko and Formenti, 2018). To restore the exhausted TILs, monoclonal antibody such as checkpoint inhibitor has gained considerable success in a subset of patients, but it also suffers from inefficient delivery into targeted site and severe immune-related adverse events (Naidoo et al., 2015). With respect to remodeling TME, respiratory hyperoxia (60% oxygen) has been verified to decrease intra-tumoral hypoxia for promoting antitumor immune response, whereas this systemic O₂ supply approach might cause acute lung injury (Kallet and Matthay, 2013). Thus, how to simultaneously, safely, and effectively overcome these obstacles (converting tumor from immune cold into hot) is the key challenge for boosting robust immune responses to fight against cold tumors.

Compared with the existing therapeutic approaches that failed to turn cold tumor into hot milieu, our BFVs integrating PD1, TRAIL, and CAT showed several distinct merits and could efficiently convert cold tumor into hot. In our design, the TRAIL could selectively elicit ICD of cancer cells, whereas it had limited toxicity on normal cells; the CAT in BFVs not only could in situ generate O₂ by virtue of the high levels of H₂O₂ in cancer cells without producing pulmonary O₂ toxicity but also could reduce the malignant biological roles caused by H₂O₂ including metastasis of cancer cells, angiogenesis, and resistance to therapies (López-Lázaro, 2007). Furthermore, all components in our BFVs belong to biomolecules whose biological functions are nicely preserved in TME and have excellent biocompatibility and biodegradability without any noticeable toxicities (Figures S25–S29). Therefore, our BFVs integrating PD1, TRAIL, and CAT could effectively convert immune cold into hot through simultaneously overcoming hypoxia, inducing ICD and immune checkpoint inhibition. As a result, our BFVs not only boosted robust and systemic antitumor immune responses that simultaneously inhibited primary and abscopal tumor growth but also achieved long-term immune memory effects to prevent tumor recurrence/metastasis. In principle, our reported BFVs also could be suitable for the immunotherapy of other solid tumors. Thus, our approach might be particularly meaningful and readily adapted to a broad diversity of immune cold tumors that do not respond well to ICB therapies.

Limitations of the Study
The BFVs proposed here have comprehensive immuno-modulating ability to elicit robust and systematic antitumor immunity, but their antitumor efficiency might be heavily affected by the heterogeneity of cancer cells, tumor microenvironment, and patients’ baseline immunity, which are very difficult to precisely measure and predict. Furthermore, the exact components of naturally obtained cell membranes are very difficult to accurately determine and quantify, which might be an obstacle for pharmaceutical translation, owing to difficulty in getting the same quality BFVs from batch to batch, and also lack of precise quality control strategy for large-scale production. These limitations should be further resolved for future translational applications.

Resource Availability
Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Xiaolong Liu (xiaoloong.liu@gmail.com).

Materials Availability
This study did not generate new unique materials.

Data and Code Availability
All data produced or analyzed for this study are included in the published article and its supplementary information files.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101341.
ACKNOWLEDGMENTS
This work was supported by the National Natural Science Foundation of China (Grant No. 61727823, 81671813, 81601538), the joint research projects of Health and Education Commission of Fujian Province (Grant No. 2019-WJ-20).

AUTHOR CONTRIBUTIONS
M.W., D.Zheng., and D.Zhang. contributed equally to this work; M.W., J.Liu., and X.Liu. designed the project; M.W., D.Zheng., D.Zhang., P.Y., L.P., F.C., Z.L., Z.C., J.Li., Z.W., and X.Lin. conducted the experiments; M.W., D.Zheng., and D.Zhang. analyzed and interpreted the data; X.Liu. and J.Liu. supervised the overall research; M.W., D.Zheng., and X.Liu. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Binnewies, M., Roberts, E.W., Kersten, K., Chan, V., Fearon, D.F., Merad, M., Coussens, L.M., Gabrilovich, D.I., Ostrand-Rosenberg, S., and Hedrick, C.C. (2016). Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat. Med. 24, 541–550.

Boussiotis, V.A. (2016). Molecular and biochemical aspects of the PD-1 checkpoint pathway. N. Engl. J. Med. 375, 1767–1778.

Chao, Y., Xu, L., Liang, C., Feng, L., Xu, J., Dong, Z., Tian, L., Yi, X., Yang, K., and Liu, Z. (2018). Combined local immunostimulatory radioisotope therapy and systemic immune checkpoint blockade imparts potent antitumor responses. Nat. Biomed. Eng. 2, 611.

Chen, Q., Chen, G., Chen, J., Shen, J., Zhang, X., Wang, J., Chan, A., and Gu, Z. (2019). Bioreactive protein complex of aPD1 and sCD47 antibodies for enhanced immunotherapy. Nano Lett. 19, 4879–4889.

Chiang, C.-S., Lin, Y.-J., Lee, R., Lai, Y.-H., Cheng, H.-W., Hsieh, C.-H., Shyu, W.-C., and Chen, S.-Y. (2018). Combination of fucoidan-based magnetic nanoparticles and immunomodulators enhances tumor-localized immunotherapy. Nano Letters 13, 746–754.

Cullen, S., Brunet, M., and Martin, S. (2010). Granymes in cancer and immunity. Cell Death Differ 17, 616–623.

Galon, J., and Bruni, D. (2019). Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. Nat. Rev. Drug Discov. 18, 197–218.

Galstyan, A., Markman, J.L., Shatalova, E.S., Chiechi, A., Korman, A.J., Patil, R., Klymyshyn, D., Tourtelotte, W.G., Israel, L.L., Braubach, O., et al. (2019). Blood-brain barrier permeable nano immunocongjutes induce local immune responses for glioma therapy. Nat. Commun. 10, 3850.

Hatfield, S.M., Kjaergaard, J., Lukashev, D., Schreiber, T.H., Belikoff, B., Abbott, R., Sethumadhavan, S., Philbrick, P., Ko, K., and Cannicci, R. (2015). Immunological mechanisms of the antitumor effects of supplemental oxygenation. Hatfield 7, 277ra230.

He, C., Duan, X., Guo, N., Chan, C., Poon, C., Weichselbaum, R.R., and Lin, W. (2016). Core-shell nanoscale coordination polymers combine chemotherapy and photodynamic therapy to potentiate checkpoint blockade cancer immunotherapy. Nat. Commun. 7, 12499.

Hirano, F., Kaneko, K., Tamura, H., Dong, H., Wang, S., Ichikawa, M., Rietz, C., Flies, D.B., Lau, J.S., Zhu, G., et al. (2005). Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res. 65, 1089–1096.

Huang, L., Li, Y., Du, Y., Zhang, Y., Wang, X., Ding, Y., Yang, X., Meng, F., Tu, J., Luo, L., et al. (2019). Mild photothermal therapy potentiates anti-PD-L1 treatment for immunologically cold tumors via an all-in-one and all-in-control strategy. Nat. Commun. 10, 4871.

Jiang, X., Fitch, S., Wang, C., Wilson, C., Li, J., Grant, G.A., and Yang, F. (2016). Nanoparticle engineered TRAIL-overexpressing adipose-derived stem cells target and eradicate glioblastoma via intracranial delivery. PNAS 113, 13857–13862.

Joller, N., Hafler, J.P., Brynedal, B., Kassam, N., Spoerl, S., Levin, S.D., Sharpe, A.H., and Kuchroo, V.K. (2011). Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J. Immunol. 186, 1338–1342.

Kallet, R.H., and Matthay, M.A. (2013). Hypoxic acute lung injury. Respir. Care 58, 123–141.

Kim, T.K., Herbst, R.S., and Chen, L. (2018). Defining and understanding adaptive resistance in cancer immunotherapy. Trends Immunol. 39, 624–631.

Ko, E.C., and Formenti, S.C. (2018). Radiotherapy and checkpoint inhibitors: a winning new combination? Ther. Adv. Med. Oncol. 10, https://doi.org/10.1177/1758835918768240.

Liu, X., Liu, C., Zheng, Z., Chen, S., Fang, X., Xiang, X., Tang, J., Ren, E., Chen, Y., You, M., et al. (2019). Vesicular antibodies: a bioactive multifunctional combination platform for targeted therapeutic delivery and cancer immunotherapy. Adv. Mater. 31, 1808294.

López-Lázaro, M. (2007). Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. Cancer Lett. 252, 1–8.

McGranahan, N., Furness, A.J., Rosenthal, R., Ramskov, S., Lyngaa, R., Sani, S.K., Jamal-Hanjani, M., Wilson, G.A., Birkbak, N.J., and Hiley, C.T. (2016). Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 351, 1463–1469.

Mi, Y., Smith, C.C., Yang, F., Qi, Y., Roche, K.C., Serody, J.S., Vincent, B.G., and Wang, A.Z. (2018). A dual immunotherapy nanoparticle improves T-cell activation and cancer immunotherapy. Adv. Mater. 30, 1706098.

Naidoo, J., Page, D., Li, B., Connell, L., Schindler, K., Lacouture, M., Postow, M., and Wolchok, J. (2015). Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies. Ann. Oncol. 26, 2375–2391.

Noman, M.Z., Hasmim, M., Messai, Y., Terry, S., Kieda, C., Janji, B., and Chouaib, S. (2015). Hypoxia: a key player in antitumor immune response. A review in the theme: cellular responses to hypoxia. Am. J. Physiol-cell Ph 309, C569–C579.

Obed, M., Tesniere, A., Ghiringhelli, F., Finia, G.M., Apetoh, L., Perfettini, J.-L., Costedo, M., Mignot, G., Panaretakis, T., and Casares, N. (2007). CaIretulin expression dictates the...
immunogenicity of cancer cell death. Nat. Med. 13, 54–61.

Robert, C., Schachter, J., Long, G.V., Arance, A., Grob, J.J., Mortier, L., Daud, A., Carlino, M.S., McNeil, C., and Lotem, M. (2015). Pembrolizumab versus ipilimumab in advanced melanoma. N. Engl. J. Med. 372, 2521–2532.

Ruan, H., Hu, Q., Wen, D., Chen, Q., Chen, G., Lu, Y., Wang, J., Cheng, H., Lu, W., and Gu, Z. (2019). A dual-bioresponsive drug-delivery depot for combination of epigenetic modulation and immune checkpoint blockade. Adv. Mater. 31, 1806957.

Sagiv-Barfi, I., Kohrt, H.E.K., Czerwinski, D.K., Ng, P.P., Chang, B.Y., and Levy, R. (2015). Therapeutic antitumor immunity by checkpoint blockade is enhanced by ibrutinib, an inhibitor of both BTK and ITK. PNAS 112, E966–E972.

Tang, J., Yu, J.X., Hubbard-Lucey, V.M., Neftelinov, S.T., Hodge, J.P., and Lin, Y. (2018). The clinical trial landscape for PD1/PDL1 immune checkpoint inhibitors. Nat. Rev. Drug Discov. 17, 854–855.

Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., Smith, D.C., McDermott, D.F., Powderly, J.D., Carvajal, R.D., Sosman, J.A., and Atkins, M.B. (2012). Safety, activity, and immune correlates of anti-CTLA-1 antibody in cancer. N. Engl. J. Med. 366, 2443–2454.

Tumeh, P.C., Hanvie, C.L., Yearley, J.H., Shintaku, I.P., Taylor, E.J., Robert, L., Chmielowski, B., Spasic, M., Henry, G., and Ciobanu, V. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 515, 568.

von Karstedt, S., Montinaro, A., and Walczak, H. (2017). Exploring the TRAILs less travelled. TRAIL in cancer biology and therapy. Nat. Rev. Cancer 17, 352.

Wang, C., Sun, W., Wright, G., Wang, A.Z., and Gu, Z. (2016). Inflammation-triggered cancer immunotherapy by programmed delivery of CpG and anti-PD1 antibody. Adv. Mater. 28, 8912–8920.

Wang, C., Wang, J., Zhang, X., Yu, S., Wen, D., Hu, Q., Ye, Y., Bomba, H., Hu, X., and Liu, Z. (2018). In situ formed reactive oxygen species–responsive scaffold with gemcitabine and checkpoint inhibitor for combination therapy. Sci. Transl. Med. 10, eaan3682.

Wang, D., Wang, T., Yu, H., Feng, B., Zhou, L., Zhou, F., Hou, B., Zhang, H., Luo, M., and Li, Y. (2019). Engineering nanoparticles to locally activate T cells in the tumor microenvironment. Sci. Immunol. 4, eaau5584.

Whiteside, T.L., Demaria, S., Rodriguez-Ruiz, M.E., Zarour, H.M., and Melero, I. (2016). Emerging opportunities and challenges in cancer immunotherapy. Clin. Cancer Res. 22, 1845–1855.

Wolchok, J.D., Kluger, H., Callahan, M.K., Postow, M.A., Ribizl, N.A., Lesokhin, A.M., Segal, N.H., Ariyani, C.E., Gordon, R.-A., and Reed, K. (2013). Nivolumab plus ipilimumab in advanced melanoma. N. Engl. J. Med. 369, 122–133.

Yue, W., Chen, L., Yu, L., Zhou, B., Yin, H., Ren, W., Liu, C., Guo, L., Zhang, Y., and Sun, L. (2019). Checkpoint blockade and nanosonosensitizer-augmented non invasive sonodynamic therapy combination reduces tumour growth and metastases in mice. Nat. Commun. 10, 2025.

Zhang, P., Zhang, L., Qin, Z., Hua, S., Guo, Z., Chu, C., Lin, H., Zhang, Y., Li, W., Zhang, X., et al. (2018a). Genetically engineered liposome-like nanovesicles as active targeted transport platform. Adv. Mater. 30, 1705350.

Zhang, X., Wang, C., Wang, J., Hu, Q., Langworthy, B., Ye, Y., Sun, W., Lin, J., Wang, T., and Fine, J. (2018b). PD-1 blockade cellular vesicles for cancer immunotherapy. Adv. Mater. 30, 1707112.

Zhang, X., Wang, J., Chen, Z., Hu, Q., Yan, J., Dotti, G., Huang, P., and Gu, Z. (2018c). Engineering PD-1-presenting platelets for cancer immunotherapy. Nano Lett. 18, 5716–5725.

Zou, W., Wolchok, J.D., and Chen, L. (2016). PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: mechanisms, response biomarkers, and combinations. Sci. Transl. Med. 8, 329rv324.
Supplemental Information

Converting Immune Cold into Hot
by Biosynthetic Functional Vesicles
to Boost Systematic Antitumor Immunity

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Figure S1. Schematic illustration of the preparation of BFVs/PD1-TRAIL-CAT. Related to Figure 1.

Figure S2. The information of plasmids used in this study. (a) PCDH-CMV-PD1-mCherry-PURO. (b) PCDH-CMV-TRAIL-EGFP-BLASTCIDIN. (c) PCDH-CMV-PDL1-EGFP-PURO. (d) PCDH-CMV-DR5-mCherry-BLASTCIDIN. Related to Figure 1.
**Figure S3.** Full gel scan images. (a, b) Uncropped blot of Figure 1d. (c) Uncropped blot of Figure 1h. Related to Figure 1.

**Figure S4.** (a) The transmission electron microscopy image of BFVs (scale bar: 50 nm). (b) The size distribution of free NVs, BFVs/PD1, BFVs/TRAIL and BFVs/PD1-TRAIL-CAT measured by DLS. (c) The zeta potential of free NVs, BFVs/PD1, BFVs/TRAIL and BFVs/PD1-TRAIL-CAT (n=3). Error bar, mean±s.d. (d) The stability of free NVs, BFVs/PD1, BFVs/TRAIL and BFVs/PD1-TRAIL-CAT which were stored in MEM (without Phenol Red) supplemented with 10% FBS up to 30 days, were measured by DLS (n=3). Error bar, mean±s.d. free NVs: blank vesicles without any functional components; BFVs/PD1: vesicles with surface presenting PD1 proteins; BFVs/TRAIL: vesicles with surface presenting TRAIL proteins; BFVs/PD1-TRAIL-CAT: final vesicles with surface presenting PD1 and TRAIL proteins, and inner core encapsulating CAT. Related to Figure 1.
Figure S5. (a) Confocal images to demonstrate the encapsulation of CAT in BFVs. 3D reconstruction of Z-stack images is resulted from the representative fluorescent vesicle marked by the dashed rectangular frame. Scale bar: 20 µm. (b) Plot profiles along the single merged fluorescence vesicle. (c) TiOSO$_4$ as a probe to monitor the enzyme activity after H$_2$O$_2$ addition to the final products from BFVs and CAT physical mixture or from the co-extrusion procedure. Related to Figure 1.

Figure S6. The specific ratio and content of TRAIL, PD-1 and CAT in BFVs, where the total protein was determined by BCA assay, and the PD1 and TRAIL were determined by ELISA, CAT was determined by fluorescence measurement. Related to Figure 1.
**Figure S7.** The CLSM images to show the co-localization of BFVs/PD1-mCherry with the overexpressed PDL1-EGFP on H22 cells. The H22 cells were transfected with PDL1-EGFP plasmid for 24 h, then incubated with BFVs/PD1-mCherry (50 μg/mL) for 0.5 h at 4 °C. The lower panel is the enlarged images of the white dashed box on the upper panel. Scale bar: 10μm. Related to Figure 2.

**Figure S8.** Representative FACS gating strategy for detecting the cytotoxicity of CTLs in the co-incubation system with CTLs and 4T1 breast cancer cells. Related to Figure 2.
Figure S9. CLSM images to show the co-localization of BFVs/TRAIL-EGFP with the over-expressed DR5-mCherry on H22 cells (scale bar: 10μm, indicated as DR5⁺). The H22 cells were transfected with DR5-mCherry plasmid for 24 h, then incubated with BFVs/TRAIL-EGFP (50 μg/mL) for 0.5 h at 4 °C. The lower panel is the enlarged images of the white dashed box on the upper panel. Related to Figure 3.

Figure S10. Apoptosis of H22 cells with different treatments by flow cytometry analysis using Annexin V-FITC and PI staining. H₂O₂-treated cells were used as the positive control. Related to Figure 3.
Figure S11. Representative FACS gating strategy for detecting the proliferation of CSFE labeled CD8⁺ T cells after incubated with the matured DCs. Related to Figure 3.

Figure S12. The flow cytometry analysis of the apoptotic state of CTLs under hypoxia or normoxia conditions. (a) The flow cytometry analysis of the apoptosis of CTLs under hypoxia condition or normoxia condition by using Annexin V-FITC and PI staining. (b) The statistical analysis data in a are expressed as min to max, show all points (*P<0.05, **P<0.01, ***P<0.001; n=3). (c) The flow cytometry analysis of the exhaustion of CTLs under hypoxia condition or normoxia condition by using TIGIT-PerCP-eFluor 710 staining. (d) The statistical analysis data in c are expressed as min to max, show all points (*P<0.05, **P<0.01, ***P<0.001; n = 3). Related to Figure 4.
Figure S13. Representative FACS gating strategy for detecting the percentage of CD4$^+$ T cells and CD8$^+$ T cells as indicated in Figure 4h. CD4$^+$ T cells and CD8$^+$ T cells are gated on CD3 positive cells. Related to Figure 4.

Figure S14. Representative FACS gating strategy for detecting the percentage of Tregs as indicated in Figure 4i. Treg cells (CD25$^+$ Foxp3$^+$) are gated on CD4$^+$ T cells. Related to Figure 4.

Figure S15. Representative FACS gating strategy for detecting the percentage of MDSCs as indicated in Figure 4j. MDSC are CD11b and Ly-6G/Ly-6C dual positive cells. Related to Figure 4.
Figure S16. In vivo distribution of vesicles in the 4T1 orthotopic murine breast-cancer model. (a) Left panel: In vivo fluorescence imaging of the mouse bearing 4T1 tumor at different time points after i.v. injection. Right panel: Ex vivo fluorescence imaging of major organs and tumor dissected from the mouse after i.v. injection of 48 h. (b) The mean fluorescence intensity (MFI) of tumor and major organs. Related to Figure 6.

Figure S17. Pharmacokinetics of free Cy5, BFVs/PD1 and free NVs. (a) Relative fluorescence intensity of the serum collected from SD rats with i.v. injection of free Cy5, BFVs/PD1(Cy5 labeled, 25 mg/kg based on protein content) or free NVs (Cy5 labeled, 25 mg/kg based on protein content). n=3. Error bar, mean ± s.d. (b) The results of pharmacokinetics were analysis by ExpDec1. The half-life of free Cy5, free NVs and BFVs/PD1 were 3.0 h, 4.0 h and 5.1 h, respectively. The AUC of free Cy5, free NVs and BFVs/PD1 were calculated to be 64.3 ± 30.5 mg/L*h, 85.8 ± 22.6 mg/L*h and 124.0 ± 30.5 mg/L*h respectively. Related to Figure 6.
Figure S18. Growth curves for orthotopic tumor on mice after tail vein intravenous injection with PBS, free NVs, BFVs/PD1, BFVs/TRAIL, BFVs/PD1-TRAIL and BFVs/PD1-TRAIL-CAT. Related to Figure 6.

Figure S19. Original photographs of tumor tissues after various treatments in Figure 6d. Related to Figure 6.
Figure S20. Uncropped images of Figure 6e. Scale bar: 50 μm. Related to Figure 6.

Figure S21. The maturation of DCs (gated on CD11c positive cells) in LNs isolated from the tumor-bearing mice with various treatments by flow cytometry. (a) FACS gating strategy for detecting the maturation of DCs in the LNs. Firstly, we separated the CD11c+ DCs from all cells and then analyzed the CD80 and CD86 in the CD11c+ DCs. (b) The data were acquired by flow cytometry to analyze the maturation of DCs. Related to Figure 6.
Figure S22. Representative FACS gating strategy for detecting the percentage of CD4$^+$ T cells and CD8$^+$ T cells in the blood. CD4$^+$ and CD8$^+$ are gated on CD3$^+$ T cells. Related to Figure 6.

Figure S23. Representative FACS gating strategy for detecting the percentage of CD8$^+$ T$_{EM}$ and CD8$^+$ T$_{CM}$ in the blood. T$_{EM}$ (CD44$^+$ CD62L$^-$) and T$_{CM}$ (CD44$^+$ CD62L$^+$) are gated on CD8$^+$ T cells. Related to Figure 7.
**Figure S24.** Representative FACS gating strategy for detecting the percentage of CD4⁺ TEM and CD4⁺ TCM in the blood. TEM (CD44⁺ CD62L⁻) and TCM (CD44⁺ CD62L⁺) are gated on CD4⁺ T cells. Related to Figure 7.

**Figure S25.** Body weights of the bilateral 4T1 tumor model with indicated treatments. error bar, mean ± s.d (n=8-10). Related to Figure 5.
Figure S26. Body weights of the orthotopic 4T1 tumor model with indicated treatments. error bar, mean ± s.d (n=9-10). Related to Figure 6.

Figure S27. Histological images for H&E staining obtained from the heart, liver, spleen, lung, and kidney of mice with indicated treatments. Tissues were harvested at the day 23 post-injection. Scale bar: 50 μm. Related to Figure 6.
Figure S28. Blood biochemistry analysis of liver and kidney function markers after intravenous injection with PBS and BFVs/PD1-TRAIL-CAT. Data are present as the mean ± s.d (n=3). Related to Figure 6.
Figure S29. (a) The cell viability of 4T1 cells in different concentration of H$_2$O$_2$, or different pH, which was determined by CCK8 assay. (b-d) APCA analysis of the activities of TRAIL and PD1 proteins on BFVs in mimic TME (cell culture medium with 10 μM H$_2$O$_2$ at pH 6.5) in vitro. (b) 4T1 cells incubated in different culture medium; (c) 4T1 cells incubated with BFVs with membrane presenting TRAIL (BFVs/TRAIL) in different culture medium; (d) 4T1 cells incubated with BFVs with membrane presenting PD1 (BFVs/PD1) in different culture medium for 2 h, and then activated CD8$^+$ T cells were added and incubated for 24 h. Related to Figure 6.
Table S1. CD8\(^+\) cell frequency in digested tumor. Related to Figure 4.

| Group             | A: CD8\(^+\) cell frequency in CD3\(^+\) (P2 region) (%) | B: Normalization Factor P2 region frequency in ALL EVENTS (FSC/SSC) (%) | C: CD8\(^+\) cell frequency in ALL EVENTS (%) = A \times B |
|-------------------|--------------------------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------|
| PBS               | 19.8                                                   | 2.36                                                                   | 0.47                                                     |
| CAT               | 21.8                                                   | 2.9                                                                    | 0.63                                                     |
| BFVs/PD1-TRAIL-CAT| 35.3                                                   | 3.76                                                                   | 1.33                                                     |

Note: The data in calculation formula is resulted from Figure S12.

Table S2. CD4\(^+\) cell frequency in digested tumor. Related to Figure 4.

| Group             | A: CD4\(^+\) cell frequency in CD3\(^+\) (P2 region) (%) | B: Normalization Factor P2 region cell frequency in ALL EVENTS (FSC/SSC) (%) | C: CD4\(^+\) cell frequency in ALL EVENTS (%) = A \times B |
|-------------------|--------------------------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------|
| PBS               | 57.4                                                   | 2.36                                                                   | 1.35                                                     |
| CAT               | 52.4                                                   | 2.9                                                                    | 1.52                                                     |
| BFVs/PD1-TRAIL-CAT| 41.9                                                   | 3.76                                                                   | 1.58                                                     |

Note: The data in calculation formula is resulted from Figure S12.

Table S3. Treg cell frequency in digested tumor. Related to Figure 4.

| Group             | A: CD25\(^+\) Foxp3\(^+\) cell frequency in CD4\(^+\) (P2 region) (%) | B: Normalization Factor P2 region cell frequency in ALL EVENTS (FSC/SSC) (%) | C: CD4\(^+\) CD25\(^+\) Foxp3\(^+\) cell Frequency in ALL EVENTS (%) = A \times B |
|-------------------|------------------------------------------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------|
| PBS               | 27.7                                                   | 4.41                                                                   | 1.22                                                     |
| CAT               | 15.8                                                   | 4.54                                                                   | 0.72                                                     |
| BFVs/PD1-TRAIL-CAT| 11.3                                                   | 5.51                                                                   | 0.62                                                     |

Note: The data in calculation formula is resulted from Figure S13.
Table S4. MDSC cell frequency in digested tumor. Related to Figure 4.

| Group                  | A: CD11b⁺ Ly-6G/Ly-6C⁺ cell frequency in P1 region (%) | B: Normalization Factor P1 region cell frequency in ALL EVENTS (FSC/SSC) (%) | C: CD11b⁺ Ly-6G/Ly-6C⁺ cell frequency in ALL EVENTS (%) |
|------------------------|--------------------------------------------------------|--------------------------------------------------------------------------|--------------------------------------------------------|
| PBS                    | 29.0                                                   | 18.5                                                                    | 5.37                                                   |
| CAT                    | 17.6                                                   | 19.7                                                                    | 3.47                                                   |
| BFV/PA1-TRAIL-CAT      | 10.1                                                   | 18.1                                                                    | 1.83                                                   |

Note: The data in calculation formula is resulted from Figure S14.

Transparent Methods

Establishment of genetically engineered stable cell lines

First, we constructed the plasmids which encoded murine PD1 with mCherry-tag fused at the C-terminal region (pcdh-CMV-PD1-mCherry-puro) and murine TRAIL with EGFP-tag fused at the C-terminal region (pcdh-CMV-EGFP-EGFP-blastcidin). Lentivirus were produced by using HEK 293FT cells with Lipofectamine 3000 Transfection Kit (Thermo Fisher Scientific, lot:2098843) and concentrated by ultracentrifugation at 25,000 rpm for 90 min at 4 °C. Then, the HEK 293FT cells were infected with the lentivirus and incubated with 5 μg/mL polybrene (Santa Cruz biotechnology, sc-134220). Two days after infection, the 293FT cells were cultured in DMEM with 10% FBS and screened with puromycin (5 μg/mL) or blastcidin (10 μg/mL) to select cells stably expressing murine PD1 or TRAIL for a week. Finally, the PD1-mCherry stable expressing HEK 293FT cells or TRAIL-EGFP stable expressing HEK 293FT cells were cultured in DMEM medium with 10% FBS.

Preparation of biosynthetic functional vesicles (BFVs)

PD1-mCherry stable expressing HEK 293FT cells and TRAIL-EGFP stable expressing HEK 293FT cells were cultured in DMEM medium with 10% FBS. For
collecting cell membrane, the cells were harvested with trypsin and washed with PBS for 2 times by centrifugation with 300×g for 5 min. Then, the Membrane and Cytosol Protein Extraction Kit (Beyotime, P0033) was used to extract the cell membrane. Afterwards, the cell membrane was washed with PBS containing PMSF and protease inhibitor cocktail for 3 times. To prepare biosynthetic functional vesicles (BFVs), the cell membrane and catalase (CAT) protein in PBS buffer were evenly mixed (mass ratio=1:1, based on protein weight) and then passed through 1 μm filter for 11 times. The entire solution was centrifuged at 13,000×g for 30 min at 4 ℃, and the obtained sediment was washed with PBS buffer for 3 times, followed by successively passed through 0.4 μm,0.2 μm and 0.1 μm pore-sized polycarbonate membrane filters for another 11 times. The vesicles were then purified by Sephadex G-75 column to obtain the final product of BFVs.

To determine the specific ratio and content of TRAIL, PD1 and CAT, Cy5-NHS labeled CAT was used to prepare the final product of BFVs according to above method. After lysis by the Solution B supplied in the Membrane and Cytosol Protein Extraction Kit (Beyotime, P0033), the BFV lysates were divided into four equal parts. One part was quantified by the Easy II Protein Quantitative Kit (BCA, TransGen Biotech, DQ111-01) to determine the total protein content; one part was used to determine the CAT content by fluorescence measurement according to the established concentration-emission correlation curve of Cy5 labeled CAT; the residual two parts were determined by ELISA to respectively analyze the TRIAL and PD1 content (R&D SYSTEMS, DY1121 for TRAIL and DY1021 for PD1).

**Western blot**

Immunoblotting analysis was used to characterize the stable expression of PD1-mCherry and TRAIL-EGFP in HEK 293FT cells. Briefly, the stable expression
cells and the parent HEK 293FT cells were lysed with RIPA buffer for 30 min on ice. Then, the supernatant was collected after centrifugation at 10,000×g for 30 min at 4 ℃. The protein concentration was quantified by the Easy II Protein Quantitative Kit (BCA, TransGen Biotech, DQ111-01), and the loading buffer was added equally into the supernatant before heated to 100℃ for 10 min. Then 10% SDS-PAGE was used to separate the samples that were further analyzed by immunoblotting using the primary antibody of PD1 (1 μg/mL, abcam, ab214421), TRAIL (1 μg/mL, abcam, ab10516) and the corresponding secondary antibodies, and finally revealed by SuperSignal™ West Pico PLUS Chemiluminescent Substrate and recorded by the ChemiDoc™ MP imaging system.

**Immunoprecipitation (IP) assay**

To verify the right outside-out orientation of PD1 on the biosynthetic functional vesicles, the IP assays were performed. Briefly, the vesicles with surface presenting PD1 (termed as BFVs/PD1) were dispersed in 1 mL ice-cold PBS, and then incubated with 100 μL of 50% protein A/G agarose beads for 2 h at 4 ℃ with gentle rotation. Afterwards, the above mixture underwent centrifugation at 10,000 rpm for 15 min at 4 ℃ to precipitate the protein A/G agarose beads that were nonspecifically bond with BFVs/PD1. Then, the supernatant containing BFVs/PD1 was incubated with PD1 primary antibody (10 μg/mL, abcam, ab214421) and protein A/G agarose beads in the rotation condition overnight at 4 ℃. Afterwards, the beads were washed gently with ice-cold PBS for 3 times. Finally, the samples were subjected to 10% SDS-PAGE and analyzed by western blot using the relevant antibodies. Meanwhile, the group that used IgG to substitute PD1 primary antibody was used as the control.

**Cell binding assay**
The 4T1 cells were cultured in the confocal dishes overnight, then BFVs/PD1 or free NVs labeled with FITC (50 μg/mL based on protein content) were added to the medium and incubated for another 4 h at 37 ℃. Meanwhile, the cells that were pretreated with anti-PDL1 antibody (10 μg/mL, abcam, ab213480) for 2 h before the addition of BFVs/PD1 were selected as the competition control group. Afterwards, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min and stained with DAPI for 10 min. Finally, the cells were washed with PBS for 3 times before observed by CLSM (Zeiss LSM780).

Protein co-localization assay

The 4T1 cells or H22 cells were cultured in the confocal dishes overnight, then these cells were transfected with PDL1-EGFP plasmid for 24 h, followed by the incubation with BFVs/PD1 (50 μg/mL based on protein content) for 30 min at 4 ℃. Then, the cells were imaged by CLSM as mentioned above. Similarly, the co-localization assay between TRAIL on vesicles and DR5 on cancer cells was carried out through the same protocol.

Isolation of CD8+ T cells from mouse spleen

CD8+ T cells were isolated from mouse spleen according to the following method. In brief, the spleen was harvested from 8 week old Balb/c mice and kept in RPMI 1640 medium on ice. Then, the cells were flushed out from spleen by RPMI 1640 culture medium through a syringe. The medium containing cells was passed through 40 μm cell strainer to remove the large debris. Afterwards, the cells were collected by centrifugation at 800×g for 5 min, followed by suspended in red blood cell lysis buffer for 10 min at room temperature to lyse red blood cells. Subsequently, the cells were washed with RPMI 1640 medium by centrifuging 5 min at 800×g. Then, the
CD8⁺ T cells were sorted through CD8 (TIL) MicroBeads (Miltenyi Biotec, 130-116-478) according to the manufacturer’s instructions. The CD8⁺ T cells were seeded in a 6-well plate with KBM 581 medium and supplemented with IL-2 (10 ng/mL, R&D systems, MX2918061), anti-CD3ε antibody (5 μg/mL, biolegend, 100331) and anti-CD28 antibody (2 μg/mL, biolegend, 102112) to activate the CD8⁺ T cells. The activated CD8⁺ T cells were cultured for 5 days before seeded in 6-well plate for further use.

**CTL cytotoxicity assay**

The 4T1 cells in 6-well plate were incubated with free NVs (50 μg/mL based on protein content), BFVs/PD1 (50 μg/mL based on protein content) or anti-PDL1 antibody (10 μg/mL) for 2 h, then the activated CTLs (CD8⁺ T cells) were added into 4T1 cells (CTLs:4T1 = 1:1, number ratio). After 24 h of co-incubation, the culture medium was collected for determining Granzyme B by using Mouse Granzyme B ELISA Kit (Boster, EK1115), while the cells were stained with Annexin V-FITC/PI (DOJINDO Laboratories, NX610) and anti-CD3-APC antibody (Thermo Fisher Scientific eBioscience™, 12-0081-82). Finally, the cells were washed with PBS for 3 times and detected by flow cytometry (BD, FACSVerse, USA). PDL1overexpression 4T1 cells with PBS treatment were used as the negative control.

**CRT expression on cancer cells after different treatment**

The 4T1 cells in the confocal dishes were incubated with BFVs/PD1 or BFVs/PD1-TRAIL, and the cells treated with PBS were taken as control. After 24 h of incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and then stained with the primary antibody of CRT (5 μg/mL, abcam, ab2908) for 2 h and the corresponding secondary antibody for 1 h at room temperature. Then,
the cells were washed with PBS and stained with Hoechst for 10 min. Finally, the cells were observed by CLSM (Zeiss LSM780).

**Isolation of DC cells from mouse bone marrow**

DC cells were isolated from bone marrow according to the following method. Firstly, the tibias and femurs were isolated from 8 week old Balb/c mice and kept in RPMI 1640 medium on ice. The end of each bone was cut off with scissors, and then the cells were flushed out from bone by RPMI 1640 culture medium through a syringe. The medium containing cells was passed through 40 μm cell strainer to remove the large debris. Then, the cells were collected by centrifugation at 800×g for 5 min, followed by suspended in red blood cell lysis buffer for 10 min at room temperature to lyse red blood cells. Subsequently, the cells were washed with RPMI 1640 medium through centrifuging 5 min at 800×g, then seeded in a 6-well plate with RPMI 1640 medium and cultured with mouse granulocyte/macrophage colony stimulating factor (mGM-CSF, 20 ng/mL, R&D systems, 415-ML-020/CF) and IL-4 (10 ng/mL, 404-ML-010/CF) for 3-5 day to induce DC differentiation for further usage.

**DCs maturation assay**

The 4T1 cells were treated with BFVs/PD1 or BFVs/PD1-TRAIL for 24 h, and then the supernatant was transferred into a new 24-well plate with DCs for triggering their maturation. The DCs treated with LPS was used as the positive control. After 48 h of incubation, the culture medium was collected for cytokine detection by using Mouse ELISA Kit (TNFa, Boster, EK0527; IFNγ, Boster, EK0375; IL12-P70, Boster, EK0422), while the cells were stained with the antibody of CD11c-APC (eBioscience™, 17-0114-82), CD80-PE (eBioscience™, 12-0801-82) and
CD86-PE-Cy7 (eBioscience™, 25-0862-82). Finally, the cells were washed with PBS for 3 times and detected by flow cytometry.

**Measurement of O₂ production**

The oxygen concentration in aqueous solution was measured using a portable dissolved oxygen meter (Rex, JPBJ-608, China). For measurement of BFVs to generate O₂, the BFVs (10 μg/mL based on protein content) were added into H₂O₂ (0.5 mM) aqueous solution, and then the oxygen electrode probe was inserted into the tube to measure the oxygen concentration of the solution in real time.

**Intracellular H₂O₂ and O₂ detection**

Hydrogen peroxide assay Kit (Cell-based, abcam, ab138874) and [Ru(dpp)₃]Cl₂ probe were used to detected the intracellular H₂O₂ and oxygen levels, respectively. In detail, the 4T1 cells were seeded into a 96-well plate and incubated in hypoxia container. After 24 h of incubation, the BFVs/PD1-TRAIL or BFVs/PD1-TRAIL-CAT were added into 96-well and further incubated for 2 h. After being washed twice with PBS, the intracellular H₂O₂ and O₂ were detected according to the manufacturer’s instructions and observed by a fluorescence microscope (Zeiss Axio Vert.A1, Germany).

**Antitumor evaluation on bilateral 4T1 tumor model**

The Balb/c mice were purchased from China Wushi, Inc. (Shanghai, China). All animal experiments were approved by the Animal Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University and were carried out according to the institutional guidelines. For establishing this model, the left side of each mouse was subcutaneously (s.c.) injected with 1×10⁶ 4T1 cells and the right side was s.c. injected with 5×10⁵ 4T1 cells. When the volume of left side tumor reached about 100
mm$^3$ while the right side tumor volume reached about 50 mm$^3$, the tumor-bearing mice were randomly divided into 7 groups for subsequent experiment. Each tumor-bearing mice was intratumorally (i.t.) injected in the left side of the tumor with PBS, free NVs (5 mg/kg based on protein content), BFVs/PD1 (5 mg/kg based on protein content), BFVs/TRAIL (5 mg/kg based on protein content), anti-PDL1 antibody (0.4 mg/kg, biolegend, 124328), BFVs/PD1-TRAIL (5 mg/kg based on protein content) and BFVs/PD1-TRAIL-CAT (5 mg/kg based on protein content) for a total of 4 times in every 3 days. The tumors were measured by using a vernier calipers and the volume ($V$) was calculated to be $V = axb^2/2$, where $a$ is the longest and $b$ is shortest diameter of the tumor, respectively. To assess potential toxicities, mice were monitored for weight change. Mice were sacrificed when exhibited signs of impaired health or when the tumor volume exceeded 1200 cm$^3$.

**Pharmacokinetics of vesicles**

PD1 presenting vesicles (BFVs/PD1) and blank vesicles (free NVs) labeled with Cy5-NHS were $i.v.$ injected in SD rats via tail-vein in 200 μL of final volume at a dose of 25 mg/kg based on protein content. Peripheral blood was collected at different time points after injection, and the fluorescence of the serum was measured.

**In vivo distribution of BFVs**

The 4T1 orthotopic murine breast-cancer model was established by injection of 4T1 cells ($5 \times 10^5$) into the mammary fat pads of mice on the abdomen to investigate the in vivo distribution of BFVs labeled with ICG-NHS after intravenous ($i.v.$) injection. At different time post-injection, the whole body fluorescence images were acquired by NIR II fluorescence imaging system (UNITED WELL). After 48 h of injection, the
mice were euthanized, and the tumor and other major organs including heart, liver, spleen, lung, kidney, and intestine were dissected and imaged.

In addition to the histopathological observation of major organs, we also evaluated the toxicity of BFVs by blood routine and serum biochemistry analysis. After treatment, the blood of SD rats was collected in EDTA2K spray-coated tubes and analyzed using an automated hematology analyzer in one go.

**Antitumor evaluation on orthotopic 4T1 tumor model**

When the volume of tumor reached about 50 mm$^3$, the mice were randomly divided into 6 groups for the following treatments. PBS, free NVs (25 mg/kg based on protein content), BFVs/PD1 (25 mg/kg based on protein content), BFVs/TRAIL (25 mg/kg based on protein content), BFVs/PD1-TRAIL (25 mg/kg based on protein content) and BFVs/PD1-TRAIL-CAT (25 mg/kg based on protein content) were administered into tumor-bearing mice by tail-vein injection. The tumor size was measured as mentioned above. The tumor and major organs (heart, liver, spleen, lung and kidney) were harvested at the end of antitumor studies, fixed in 4% formalin solution, dehydrated, deparaffinization and subjected to H&E staining, Ki67 immunohistochemical and other immunofluorescence staining analysis.

**Immune memory effect for inhibiting tumor lung metastasis**

The orthotopic 4T1 tumor-bearing mice underwent the aforementioned treatments. At the day 23, the residual tumor was surgically removed from the mice and then the wound was closed. One week later, $5 \times 10^5$ 4T1 cells were injected into the mice through caudal vein. After 2 weeks, the lungs were isolated from treated mice to photograph and count the metastasis nodules, and further fixed in 4% formalin solution for H&E staining.
**Intra-tumoral H$_2$O$_2$ concentration detection**

The tumor bearing Balb/c mice were respectively *i.t.* injected with 100 µL of PBS, CAT (5 mg/kg) or BFVs/PD1-TRAIL-CAT (CAT, 5 mg/kg). After injection for 4 h, the tumors isolated from euthanized mice were weighted and homogenized with 800 µL cold acetone by automatic sample rapid grinding machine. Then, the supernatant was collected by centrifugation at 13,000 rpm for 10 min. Subsequently, 10 µL of TiOSO$_4$ (0.03 M) and 20 µL of NH$_3$·H$_2$O were added into the supernatant and the sediments were immediately collected by centrifugation at 13000 rpm for 10 min. Finally, the sediments were dispersed in 300 µL of H$_2$SO$_4$ (1mM) to measure the absorbance at 405 nm by using the Spectra Max M5 microplate reader (Molecular Devices, USA). The final result of intra-tumoral H$_2$O$_2$ concentration was calculated according to the standard curve.

**Intra-tumoral O$_2$ detection**

To determine whether the BFVs can alleviate hypoxia in tumor, the tumor bearing Balb/c mice were established and treated with PBS, CAT or BFVs/PD1-TRAIL-CAT (5 mg/kg based on protein content) by *i.t.* injection. The photoacoustic images at different time points were acquired to evaluate the dissolved oxygen status of the tumor site by small animal ultrasonic imaging system (VisualSonic Vevo 3100 LAZR system, Canada). We further confirmed this result by immunohistochemistry of tumor slices as follows. After two days of injection, the mice were administrated with a hypoxia probe Hypoxyprobe™-1(1.5 mg/mouse) by intraperitoneal injection, then the tumors were harvested after 1 h. The harvested tumors were sliced and
immune-stained with FITC-MAb antibody (5 μg/mL) for CLSM observation. The hypoxia probe and antibody were from Hypoxyprobe™ Green Kit (Hypoxyprobe, Inc, HP6-100 Kit, USA).

**Immune response analysis in vivo**

To examine DC maturation in vivo, 4T1-tumor bearing mice were treated with various formulation of vesicles as indicated. Then, the draining inguinal lymph nodes were isolated after 3 days of treatment. The cells were harvested from lymph nodes by gently grinding and then examined by flow cytometry after staining with CD11c-APC (eBioscience™, 17-0114-82), CD80-PE (eBioscience™, 12-0801-82) and CD86-PE-Cy7 (eBioscience™, 25-0862-82).

To examine the infiltration of CTLs, MDSCs, Tregs in tumor, the 4T1-tumor bearing mice were treated with indicated conditions. Then, the tumors were isolated after 3 days of treatment. Subsequently, the tumors were digested in RPMI 1640 by collagenase type IV (1 mg/mL), hyaluronidase (0.2 mg/mL), deoxynbonuclease I (0.02 mg/mL) for 1 h at 37 ℃. Then, the medium containing cells were passed through 40 μm cell strainer to remove the large debris, the cells were collected by centrifugation at 800×g for 5 min, and further purified by a density gradient centrifugation by 30% percoll separating solution at 800×g for 30 min at 4 ℃. Afterwards, the cells were harvested and then examined by flow cytometry after stained with anti-CD3-APC (eBioscience™, 17-0032-82), anti-CD4-FITC (eBioscience™, 11-0042-85) and anti-CD8-PE (eBioscience™, 12-0081-82) antibodies for CTLs, anti-CD4-FITC (eBioscience™, 11-0042-85), anti-CD25-PerCP-Cy5.5 (eBioscience™, 46-0251-82) and anti-Foxp3-PE-Cy7 (eBioscience™, 25-5773-80) antibodies for Tregs, anti-CD11b-APC (eBioscience™,
17-0112-82) and anti-Ly-6G/Ly-6C-PE (eBioscience™, 12-5931-82) antibodies for MDSCs.

To examine the memory T cells, the blood collected from the treated mice was diluted with PBS (blood volume:PBS volume = 1:1) and subject to a density gradient centrifugation in Ficoll-Paque™ PREMIUM sterile solution at 800 × g for 30 min at 4 °C. The cells were harvested and then examined by flow cytometry after staining with anti-CD4-FITC (eBioscience™, 11-0042-85), anti-CD44-PE-Cy7 (eBioscience™, 25-0441-82) and anti-CD62L-PerCP-Cy5.5 (eBioscience™, 45-0621-82) antibody or anti-CD8-PE (eBioscience™, 12-0081-82), anti-CD44-PE-Cy7 (eBioscience™, 25-0441-82) and anti-CD62L-PerCP-Cy5.5 (eBioscience™, 45-0621-82) antibody.

Intracellular cytokine assay

To examine the produced cytokines in tumor, the tumors isolated from sacrificed mice were weighted and homogenized with 1 mL PBS (1mL PBS per 50 mg tumor) containing PMSF and protease inhibitor cocktail. Then the supernatants were collected by centrifugation at 10,000 rpm for 10 min. Subsequently, the collected supernatant was used for cytokine detection by using Mouse ELISA Kit (TNFα, Boster, EK0527; IFNγ, Boster, EK0375; IL-12, Boster, EK0422; Granzyme B, Boster, EK0417; IL-10, Boster, EK1115; TGF β, Boster, EK0515).

Statistical analysis

All results are presented as the mean ± s.d. or min to max with show all points as indicated. Significance among different groups was calculated using One-way ANOVA (and nonparametric) with post-hoc tests. Survival rate in different groups
was compared using a Log-Rank test. All statistical analyses were carried out by GraphPad Prism 6.01. The P-value < 0.05 was considered as statistically significant.