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Human Cancer Cell membrane Coated Biomimetic Nanoparticles Reduce Fibroblast-mediated Invasion and Metastasis, and Induce T Cells

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Abstract

Biomimetic nanoparticles (NPs) combine the flexibility and reproducibility of synthetic materials with the functionality of biological materials. Here, we developed and characterized biomimetic poly (lactic-co-glycolic acid) (PLGA) NPs coated with human cancer cell membrane fractions (CCMFs) to form CCMF coated PLGA (CCMF-PLGA) NPs. We evaluated the ability of these CCMF-PLGA NPs to disrupt cancer cell-stromal cell interactions, and to induce an immune response. Western blot analysis verified the plasma membrane purity of CCMFs. Confocal fluorescence microscopy and flow cytometry confirmed the presence of intact membrane-associated proteins including CXCR4 and CD44 following membrane derivation and coating. CCMFs and CCMF-PLGA NPs were capable of inhibiting cancer cell migration towards human mammary fibroblasts. Intravenous injection of CCMF-PLGA NPs significantly reduced experimental metastasis *in vivo*. Following immunization of Balb/c mice, near-infrared fluorescence imaging confirmed the migration of NPs to proximal draining lymph nodes. A higher percentage of CD8⁺ and CD4⁺ cytotoxic T-lymphocyte populations were observed in spleens and lymph nodes of CCMF-PLGA NP-immunized mice. Splenocytes isolated from CCMF-PLGA NP-immunized mice had the highest number of interferon gamma producing L cells as detected by the

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Supporting information

Supplementary experimental details: validation of the membrane fraction components; characterization of MDA-MB-231 MFs, and MDA-MB-231 CCMF-PLGA NPs; right-side-out of MDA-MB-231 MFs and MDA-MB-231 CCMF-PLGA NPs; co-localization of MDA-MB-231 MFs with PLGA-DiD; the integrity of U87-CXCR4 CCMF-PLGA-DiD NPs after being internalized by MDA-MB-231 cells; the number of IFN- γ producing splenocytes obtained from immunized mice.

ELISpot assay. CCMF-PLGA NPs hold promise for disrupting cancer cell-stromal cell interactions, and for priming the immune system in cancer immunotherapy.

Graphical Abstract

Keywords

cancer cell membrane biomimetic nanoparticles; cancer cell-fibroblast interaction; invasion; metastasis; immune response

Introduction:

Biomimetic nanoparticles (NPs) are emerging as exciting nanoplatforms that incorporate biological functionality into synthetic constructs.¹ A combination of the flexibility of synthetic materials and the functionality of biological materials allows effective navigation and interfacing of these NPs in complex biological systems. Cellular functions such as biointerfacing, self-identification, signal transduction, and compartmentalization are regulated by the cell membrane through a collection of biomolecules embedded in the cell membrane lipid bilayer.² Cell membrane coated NPs provide a novel biomimetic platform that can mimic the function of their source cell when interacting with surrounding biological components.³ NPs consisting of red blood cell (RBC) membrane coated poly(lactic-co-glycolic acid) (PLGA), a biocompatible polymer approved by the FDA,⁴ were among the first reported cell membrane coated NPs that exhibited a demonstrably longer circulation time resulting from the coating.⁵ These NPs were further engineered as nanosponges to absorb pore-forming toxins⁶ or as toxoid vaccines to safely deliver non-disrupted pore-forming toxins.⁷ Cell membrane coating technology has been applied to membranes derived from platelets^{8–12} and nucleated cells, such as macrophages,^{13–16} neutrophils,¹⁷ beta cells,¹⁸ and cancer cells.^{19–26}

Stromal cells such as cancer associated fibroblasts (CAFs) mediate many of the aggressive characteristics of cancer and play a crucial role in proliferation, invasiveness, metastasis, and angiogenesis of cancer.²⁷ NPs coated with cancer cell membranes acquire the membrane phospholipid bilayer structure together with the repertoire of surface proteins from cancer cells, making them potentially useful as decoys to interfere with cancer cell-stromal cell interactions. These “artificial cancer cells” have not been previously investigated in the setting of cancer cell-stromal cell interactions.^{19–26} In addition, cancer cell membrane coated NPs may be capable of delivering tumor-specific antigens and activating downstream immune responses. In a recent report, NPs consisting of CpG-loaded PLGA cores coated with B16-F10 mouse melanoma cell membranes were found to reduce tumor growth following immunization of mice.²⁶

Here we coated human cancer cell membrane fractions (CCMFs) on PLGA NPs to form CCMF-PLGA NPs. The design concept of obtaining CCMF-PLGA NPs and the study purpose are outlined in Scheme 1. We characterized NP protein profile, size, purity, cellular internalization, and integrity. We selected high and low CXCR4 expressing U87 glioma cells, and high and low CD44 expressing human breast cancer cells, to establish the intactness of the cell membrane through flow cytometry detection of CXCR4 and CD44 on these NPs, following cell membrane isolation and NP coating procedures. We characterized the pharmacokinetic profile of these cancer cell membrane coated PLGA NPs after intravenous injection. We investigated, for the first time, the ability of these cancer cell coated NPs to reduce fibroblast-mediated invasion and experimental metastasis, and evaluated their ability to induce an immune response in immunocompetent mice.

Results

Protein content, size, zeta-potential and stability characterization:

Western blots of a series of subcellular fractions along with purified membrane are shown in Figure 1a. Whole cell lysate (Lys), and post nuclear supernatant (PNS) predominantly contained endoplasmic reticulum and cytosolic proteins. After centrifugation for 10 min at $7,000 \times g$, the pelleted mitochondrial fraction (Mito) showed a large amount of the mitochondrial marker (ATP5a) and the plasma membrane marker (Na^+/K^+ -ATPase), and an absence of the cytosolic marker (GAPDH). The remaining supernatant was further centrifuged for 30 min at $100,000 \times g$ to pellet out the crude membrane (CM) fraction. The CM fraction contained a significant amount of the endoplasmic reticulum marker (GRP78), but no ATP5a and negligible amounts of GAPDH. Good separation of MFs from lysosome, Golgi and endoplasmic reticulum components was observed following the final sucrose gradient centrifugation. Compared with CM, U87-CXCR4 MFs remained free of ATP5a or GAPDH but, more importantly, had more Na^+/K^+ -ATPase and less GRP78 than CM, indicating successful enrichment of plasma membrane associated proteins and negligible contamination from subcellular organelle proteins.

After checking the plasma membrane purity of U87-CXCR4 MFs, we examined retention of CXCR4 following membrane isolation. As shown in Figure S1a, a higher CXCR4 content was detected in CM and MF components from high CXCR4 expressing U87-CXCR4 cells compared to those from low CXCR4 expressing U87 cells, confirming the preservation of

membrane bound CXCR4 receptors. An increase of Na^+/K^+ -ATPase content from PNS to MF in both U87 and U87-CXCR4 cells further verified the enrichment of plasma membrane proteins that was consistent with the results in Figure 1a. The membrane-to-core ratio in U87 CCMF-PLGA NPs was 0.28 mg of membrane protein per 1 mg of PLGA NPs, and in U87-CXCR4 CCMF-PLGA NPs was 0.25 mg of membrane protein per 1 mg of PLGA NPs. As shown in Figure S1b, the MF component formed a top layer with a discernable stratification from the layers formed by endoplasmic reticulum, lysosomal, and Golgi components. Similar studies were performed with high-CD44 expressing MDA-MB-231 cells and low-CD44 expressing BT474 cells. Subcellular fractions from MDA-MB-231 and BT474 cells examined by western blot analysis (Figure S2a) showed a higher amount of CD44, a cell surface adhesion receptor, in MDA-MB-231 subcellular components but not in BT474 subcellular components. Enrichment of Na^+/K^+ -ATPase and barely detectable levels of GRP78 and GAPDH were observed in both MDA-MB-231 and BT474 MFs.

PLGA NPs examined by transmission electron microscopy (TEM), showed a relatively uniform spherical morphology and an average diameter of 50 nm (Figure 1b, left). U87-CXCR4 MFs formed a coil-like shape with a broad size distribution ranging from 100 nm to 300 nm (Figure 1b, middle). Physical extrusion of NPs with CCMFs allowed the PLGA NPs to be coated with an ~5 nm thick plasma membrane layer (Figure 1b, right), that was in agreement with the thickness of the phospholipid bilayer. The membrane coating looked intact and even. Z-average diameters (Figure 1c) and zeta-potential (Figure 1d) of PLGA NPs, U87-CXCR4 MFs, and U87-CXCR4 CCMF-PLGA NPs were 79.8 nm, 336 nm and 168 nm, and -34.3 mV, -24.9 mV and -25.0 mV, respectively. U87-CXCR4 CCMF-PLGA NPs had a hydrodynamic size between that of PLGA NPs and U87-CXCR4 MFs, with a zeta-potential resembling U87-CXCR4 MFs. These values indicated successful coating of PLGA NPs by the flexible MFs. Comparable hydrodynamic sizes were also observed with PLGA NPs, MDA-MB-231 MFs, and MDA-MB-231 CCMF-PLGA NPs (Figure S2b). The size increase of U87-CXCR4 CCMF-PLGA NPs over PLGA NPs was more than the ~15 nm thickness of the phospholipid bilayer. There are two possible reasons for this increase. CCMFs were harvested following cell homogenization and fractionation. It is possible that the CCMFs had some intracellular debris still attached to the membrane making them thicker than 15 nm. The other reason for the size increase is that occasionally we observed more than one PLGA NP coated by CCMFs. Since U87-CXCR4 MFs and PLGA NPs were mixed and physically extruded through a 400-nm polycarbonate porous membrane to obtain U87-CXCR4 CCMF-PLGA NPs, U87-CXCR4 MFs were sheared during the process of physical extrusion resulting in the smaller size of U87-CXCR4 CCMF-PLGA NPs compared to U87-CXCR4 MFs.

To study the stability of NPs, PLGA NPs, and U87-CXCR4 MFs, and U87-CXCR4 CCMF-PLGA NPs were re-suspended in 0.25 mM of sucrose buffer, and their Z-average diameters were measured by DLS over 9 days. As shown in Figure 1e, the constant hydrodynamic size of CCMF-PLGA NPs confirmed their long-term stability in physiological buffers.

Preservation of membrane-associated proteins in CCMFs and CCMF-PLGA NPs:

We evaluated the integrity of membrane-associated proteins after membrane isolation and PLGA coating by quantifying the G protein-coupled receptor CXCR4 and the glycoprotein CD44, as these membrane proteins are overexpressed on U87-CXCR4 and MDA-MB-231 cells, respectively. Fluorescence images of CCMFs and CCMF-PLGA NPs after staining with PE-conjugated anti-human CXCR4 antibody or APC-conjugated anti-human CD44 antibody are displayed in Figure 2a and Figure S3a. Differences in CXCR4 levels were retained in U87-CXCR4 MFs and U87-CXCR4 CCMF-PLGA NPs. Similarly CD44 was only detected in MDA-MB-231 MFs and MDA-MB-231 CCMF-PLGA NPs. Control experiments with PE or APC-conjugated isotype antibody showed no fluorescence. These results were further confirmed by the data in presented Figure 2b and Figure S3b, of cells, CCMFs, and CCMF-PLGA NPs stained with antibodies/isotypes and analyzed by flow cytometry. U87-CXCR4 or MDA-MB-231 cells, CCMFs and CCMF-PLGA NPs had the highest PE or APC fluorescence intensities. Together with the confocal microscopy data, these results confirmed the stable translocation of membrane-associated proteins from the source cells to CCMFs and CCMF-PLGA NPs.

Purity of CCMF-PLGA NPs:

To check the purity of CCMF-PLGA NPs and verify the CCMF coating on PLGA NP cores, CCMF-PLGA NPs were doubly labeled with a fluorescent antibody on the membrane coating and a DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) dye in the core. CCMFs labeled with fluorescent antibody alone were used as controls. U87-CXCR4 MFs were labeled with PE-conjugated anti-human CXCR4 mouse monoclonal antibody, and MDA-MB-231 MFs were sequentially labeled with anti-CD44 monoclonal antibody followed by Alexa 488 labeled goat anti-mouse secondary antibody. As shown in Figure 3, in U87-CXCR4 CCMF-PLGA NPs there was good overlap between PE and DiD fluorescent signals in the merged images, indicating a well-defined membrane coating on the PLGA NPs. We also found a few green dots in the merged images resulting from free U87-CXCR4 MFs without PLGA cores. Similar to U87-CXCR4 CCMF-PLGA NPs, we also observed good overlap between Alexa 488 and DiD fluorescent signals (Figure S4), confirming that MDA-MB-231 MFs and PLGA NPs co-localized and that MDA-MB-231 MFs had coated the PLGA NPs.

Internalization of CCMF-PLGA NPs in cells:

To check the integrity of CCMF-PLGA NPs after internalization in cells, we labeled U87-CXCR4 CCMF-PLGA NPs with fluorescein in the CCMFs and DiD in the PLGA core. As shown in Figure S5, U87-CXCR4 CCMF-PLGA NPs were internalized by MDA-MB-231 cells and were localized in the perinuclear region. Fluorescein signal overlapped with signal from DiD in the overlaid images, indicating that U87-CXCR4 CCMF-PLGA NPs remained intact up to 1 h after internalization.

Disruption of stromal cell-cancer cell interaction:

When human mammary fibroblasts (HMFs) were seeded at the bottom of transwell chamber plates to act as a cancer cell chemoattractant, the presence of CCMFs or CCMF-PLGA NPs

(at 40 µg of protein on CCMFs) with HMFs reduced the migration of both U87 and U87-CXCR4 cells by approximately 30%. Representative bright-field images of migrated cancer cells stained by crystal violet shown in Figure 4a reflect this reduction in migration of cancer cells in the presence of CCMFs or CCMF-PLGA NPs. Bar graphs shown in Figure 4b demonstrate the significant reduction of migrated cancer cells in the presence of CCMFs and CCMF-PLGA NPs. PLGA NPs alone did not induce any inhibition of cancer cell migration.

Pharmacokinetic and bio-distribution study:

We investigated the pharmacokinetic profile and bio-distribution pattern of CCMF-PLGA NPs in SCID mice. CCMFs and CCMF-PLGA NPs were labeled with IRDye 700DX NHS ester (IR700), and PLGA NPs were loaded with DiR (1,1'-di-octadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) dye. IR700 labeled U87-CXCR4-MFs and U87-CXCR4 CCMF-PLGA NPs (100 µg of protein) were injected into athymic Balb/c nude mice through the tail vein. The dose of PLGA-DiR NPs was determined by a fluorescence phantom study, to obtain the concentration of PLGA-DiR NPs that gave the equivalent fluorescence intensity as IR700 labeled U87-CXCR4-MFs (100 µg of protein). We collected blood from mice before and at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h *p.i.* of NPs. As shown in Figure 5a, U87-CXCR4 CCMF-PLGA NPs and U87-CXCR4 MFs had similar pharmacokinetic profiles. Both showed a rapid decay of concentration within the first 8 h *p.i.* At 8 h *p.i.*, the concentration of U87-CXCR4 CCMF-PLGA NPs and U87-CXCR4 MFs in blood dropped to 0.2% ID/g and 0.1% ID/g, respectively. However, PLGA-DiR NPs stayed in the bloodstream much longer, and the blood concentration of PLGA-DiR NPs at 8 h *p.i.* was 7.1% ID/g, half of peak value at 0.25 h *p.i.* Membrane antigens present on the MFs and the CCMF-PLGA NPs may have triggered recognition by immune cells and accelerated clearance resulting in a significantly shorter circulation time in the bloodstream. Compared to U87-CXCR4 MFs, U87-CXCR4 CCMF-PLGA NPs demonstrated a slightly decreased clearance rate, especially during the first 4 h *p.i.* Representative fluorescence images obtained *in vivo* over 24 h are shown in Figure 5b. Representative fluorescence images of NP bio-distribution in major organs at 24 h *p.i.* are shown in Figure 5c. A significant amount of PLGA-DiR NPs was detected in the liver and spleen at 24 h *p.i.* U87-CXCR4 CCMF-PLGA NPs had a similar bio-distribution pattern as U87-CXCR4 MFs. Both localized primarily in the liver with a peak uptake at approximately 8 h *p.i.*

Metastasis inhibition:

To determine if the reduction in cancer cell migration that we observed in culture resulted in a decrease of metastasis *in vivo*, we intravenously injected MDA-MD-231 cells constitutively expressing luciferase (231-luc) with or without 231-luc CCMF-PLGA NPs, and continued to treat these mice with 231-luc CCMF-PLGA NPs or PBS injected *i.v.* As shown in Figure 6a, bioluminescence from metastatic foci was detected in lungs *in vivo* in the PBS group as early as one week after injecting cancer cells, and continued to remain high, compared to the treated group at all the time-points. These *in vivo* imaging results were consistent with end-point bioluminescence intensity differences in open chest-cavity imaging of euthanized mice as shown in Figure 6b. *Ex vivo* bioluminescence imaging of inflated lungs was performed to detect metastatic lesions (Figure 7a). Quantitative analyses of lung images identified a significant reduction of bioluminescence in the treated group

(Figure 7b). Histological analysis of hematoxylin and eosin (H&E) stained sections confirmed a significant reduction of metastatic burden (Figures 7c–d). These data collectively confirmed that experimental metastasis was significantly reduced in mice treated with 231-luc CCMF-PLGA NPs.

Localization of NPs in draining lymph nodes:

Since CCMFs carry a repertoire of membrane proteins from their source cancer cells, CCMFs and CCMF-PLGA NPs may have the potential of acting as cancer vaccines and inducing an immune response. We subcutaneously injected immunocompetent Balb/c mice in the tarsal region with PLGA-DiR NPs, IR700 labeled U87-CXCR4 MFs or U87-CXCR4 CCMF-PLGA NPs. Near-infrared (NIR) fluorescence *in vivo* imaging (Figure 8a) was capable of tracking the location of NPs after injection. *Ex vivo* images shown in Figure 8b confirmed the presence of PLGA-DiR NPs, U87-CXCR4 MFs and U87-CXCR4 CCMF-PLGA NPs to proximal draining popliteal and inguinal LNs. U87-CXCR4 MFs and U87-CXCR4 CCMF-PLGA NPs demonstrated higher fluorescence intensities than PLGA-DiR NPs in LNs, indicating enhanced antigen capture by the immune system. There was no noticeable uptake of NPs in the spleen.

Cytotoxic T-lymphocytes increase triggered by CCMF-PLGA NP-immunization:

At 7 days after the last injection, spleens, popliteal and inguinal LNs were isolated and teased apart into single cell suspensions. We performed flow cytometry to measure the percentage of cytotoxic T-lymphocytes (CTLs) in the population of cells isolated from the spleens and LNs. Representative examples of the flow cytometry data are shown in Figure 9a for the spleen and 9b for LNs. A summary of data from three mice per group is presented in Figures 7c and d. U87-CXCR4 CCMF-PLGA NP injected mice had significantly higher CD4⁺ cells in the LNs and CD8⁺ CTLs in spleens and LNs compared to the other groups. An ELISpot assay demonstrated that splenocytes isolated from U87-CXCR4 CCMF-PLGA NP injected mice had the highest frequency of interferon gamma (IFN γ) producing T cells (Figure S6).

Discussion

Right-sidedness of cancer cell membrane coating:

In our studies focusing on disrupting cancer cell-stromal cell interactions it was important to demonstrate that the NPs were coated with cell membranes with the right-side exposed. The right-sidedness of RBC membranes after coating on PLGA NPs has been previously confirmed by immunogold staining of CD47 protein²⁸ and quantification of glycoprotein content and sialyl groups on the membrane surface.²⁹ This right-side-out membrane orientation was likely governed by the electrostatic interaction that favored attachment between a negatively charged polymeric core and the less negatively charged intracellular side of RBC membranes. Similar to RBC membranes, the extracellular side of cancer cell membrane is negatively charged.³⁰ The electrostatic repulsion between the negatively charged PLGA NPs and extracellular side of cancer cell membranes resulted in a right-side-out membrane orientation on CCMF-PLGA NPs. We selected fluorescently-labeled antibodies that recognized epitopes located on the extracellular domains of CXCR4 and

CD44 proteins to label CCMFs and CCMF-PLGA NPs. Particles from high CXCR4 or CD44 samples (Figures 2a, S3a) were fluorescing, supporting the presence of the extracellular domain on the outside of the MFs and the CCMF-PLGA NPs. However, since antibodies were not available to target the intracellular domain of CXCR4 and CD44 proteins, we cannot rule out the possibility of a wrong-side-out membrane orientation in some NPs.

Inhibition of HMF-mediated cancer cell migration:

The presence of CCMF-PLGA NPs mixed with HMFs resulted in a significant decrease of cancer cell migration towards HMFs in a transwell chamber assay. This reduction was not dependent on the presence of CXCR4. The CXCR4-CXCL12 axis is a major signaling pathway mediating the interaction between cancer cells and fibroblasts.³¹ CXCR4 is a seven transmembrane spanning G-protein-coupled receptor, overexpressed in more than 23 tumor types as well as metastasis.³² CXCL12 is a ligand of CXCR4 with dissociation constant K_d of 4.5 nM.³³ However the reduction of cancer cell migration did not occur through changes in this axis caused by the NPs.

Based on our calculations, with a K_d of 4.5 nM, 40 μ g of U87-CXCR4 MFs would only absorb approximately 1% of the CXCL12 that was present at a concentration of 10 nM in the medium. This decrease of CXCL12 concentration was insufficient to directly affect cancer cell migration, explaining why there were no differences between U87-CXCR4 MFs and U87 MFs as well as their corresponding NPs.

Our data confirmed that internalization of CCMFs or CCMF-PLGA NPs disrupted the ability of HMFs to attract cancer cells, although the exact mechanism is still under investigation. Fibroblasts have been observed to track to the premetastatic niche prior to the arrival of cancer cells.³⁴ NPs that disrupt the ability of fibroblasts to attract cancer cells may disrupt the metastatic cascade and formation of metastasis. In a previous study, neutrophil cell membrane coated PLGA NPs were found to capture circulating tumor cells *in vivo* to prevent early metastasis.¹⁷ Here, we have shown for the first time, that CCMF-PLGA NPs actively reduced the ability of fibroblasts to attract cancer cells, and confirmed the ability of CCMF-PLGA NPs to significantly reduce metastasis.

Cancer-specific antigen delivery and immune response:

CCMF-PLGA NPs elicited an immune response in immune competent mice. In a recent study, a biomimetic NP formulated to deliver B16-F10 mouse melanoma cell membranes along with the immunological adjuvant CpG, demonstrated effective antigen presentation that resulted in a potent antitumor immune response against B16-F10 melanoma tumors in an immunocompetent mouse model.²⁶ Here we expanded the investigation to human glioblastoma cells selected for high (U87-CXCR4) and low (U87) CXCR4 expression, and human breast cancer cells selected for high (MDA-MB-231) and low (BT-474) CD44 expression. We used NIR fluorescence imaging to track the delivery of NPs during immunization to visualize the distribution of the NPs. From the *in vivo* and *ex vivo* fluorescence imaging, we confirmed the localization of U87-CXCR4 CCMF-PLGA NPs in proximal draining LNs. We detected an increase of CTLs and IFN γ triggered by U87-

CXCR4 CCMF-PLGA NPs in these mice indicating that cell surface antigens present on the NPs were recognized by the mouse immune system. Since U87-CXCR4 cells are of human origin and the NPs were injected into immunocompetent mice, these studies do not demonstrate a cancer-cell specific immune response, but do open the possibility of using such formulations together with immunogenic adjuvants in combination with checkpoint inhibitors for cancer treatment. U87-CXCR4 CCMF-PLGA NPs tended to be more immunogenic than U87-CXCR4 MFs in terms of increased CTLs and IFN γ that may have occurred from the MFs being fully extended on the NPs compared to the curled formations of MFs alone. This extended membrane coating may have increased the exposure of antigens to the immune system.

Conclusion

In summary, we successfully synthesized human CCMF-coated biomimetic NPs and characterized their protein profile, size, morphology, purity, integrity and sidedness. CCMF-PLGA NPs disrupted the migration of cancer cells towards fibroblasts, and significantly reduced metastatic burden. U87-CXCR4 CCMF-PLGA NPs demonstrated the ability to increase the population of CTLs in immune competent mice highlighting the potential of these cancer cell-membrane cloaked NPs as promising personalized cancer vaccines to induce a cancer-specific immune response. Our data support investigating the ability of these CCMF-coated NPs in inducing cancer-cell specific immune response. If successful, each patient's cancer cells, obtained post-operatively or through biopsy, can be used to synthesize these 'immunosomes' for immunotherapy applications in combination with checkpoint inhibitors. In the future, iron-oxide NPs can be coated with CCMFs to detect these NPs in deep-seated tissues with MRI and pave the way for translational applications. Embedding these NPs with granulocyte macrophage colony-stimulating factor (GM-CSF) may stimulate dendritic cells and further enhance the adaptive immune response. The availability of such NPs has the likelihood of significantly improving the outcome of treatment with checkpoint inhibitors. Since shape plays an important role in NP internalization, these studies may lead to the future development of NPs of different shapes using inactivated viruses cloaked with CCMFs to activate the adaptive immune response in cancer immunotherapy.

Methods

Cell culture:

The human glioblastoma U87 and U87-CXCR4 (a U87 cell line stably transfected with human CXCR4) cell lines were provided by Dr. Sridhar Nimmagadda.³⁵ HMFs were kindly provided by Dr. Gary Luker. Two wild-type human breast cancer cell lines, MDA-MB-231, and BT-474 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HMFs and U87, MDA-MB-231, and BT-474 human cancer cells were cultured in 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) supplemented MEM (Mediatech, Manassas, VA, USA), DMEM (Mediatech), RPMI 1640 (Sigma), and ATCC 46-X (ATCC) media, respectively. U87-CXCR4 human cancer cells were maintained in DMEM medium supplemented with 15% FBS, 1 μ g/ml puromycin (Sigma-Aldrich), and

300 µg/ml G418 (Mediatech). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Preparation of CCMFs:

CCMFs were harvested from source cancer cells according to a previously reported method with modification.^{36–37} Briefly, cells were grown in 150-mm petri dishes to full confluency (four petri dishes for each cell line), and detached with 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) in 1 × phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich). After washing, the cell pellet was suspended in 4 ml of homogenizing buffer containing 225 mM mannitol (Sigma-Aldrich), 75 mM sucrose (Sigma-Aldrich), 0.1 mM EDTA, 30 mM Tris-HCl (pH 7.4, Fisher Scientific, Waltham, MA USA), and EDTA-free mini protease inhibitor (one cocktail tablet for 10 ml of buffer solution, Sigma). Cells were disrupted with a motor-driven homogenizer consisting of an overhead stirrer (VWR, Radnor, PA, USA) running at 2,000 rpm and a Wheaton® Potter-Elvehjem tissue grinder set (VWR) with a smooth PTFE pestle. The homogenized cell mixture was centrifuged twice for 5 min at $1,000 \times g$ to remove intact cells and debris. The post-nuclear supernatant solution was centrifuged for 10 min at $7,000 \times g$ to pellet the crude mitochondria. The remaining supernatant was further centrifuged for 30 min at $100,000 \times g$ to pellet the crude membrane, after which the cytosolic supernatant was discarded. The crude membrane pellet was suspended in 4 ml of 1.4 M of sucrose made in 10 mM HEPES (pH 7.4, Sigma-Aldrich) and 1 mM EDTA. The sucrose gradients (1 ml of 2 M sucrose, 2 ml of 1.6 M sucrose, 4 ml of crude membrane suspension, 3 ml of 1.2 M sucrose, and 2 ml of 0.8 M sucrose) made in 10 mM HEPES (pH 7.4) and 1 mM EDTA were loaded sequentially into a 13.2 ml Ultra-Clear™ thinwall ultracentrifuge tube (Beckman Coulter, Brea, CA, USA), and centrifuged with a Beckman SW 41 Ti rotor (Beckman Coulter) at 28,500 rpm for 2.5 h. The plasma membrane fractions were identified at the 1 M density regions. The plasma membrane fractions were diluted with buffer (25 mM imidazole (Sigma-Aldrich), 1 mM EDTA, pH 7.4), pelleted by centrifugation at $100,000 \times g$ for 30 min, and re-suspended in 1 ml of sucrose buffer (0.25 mM sucrose, 10 mM HEPES (pH = 7.4), 1 mM EDTA and protease inhibitor).

Preparation of CCMF-PLGA NPs:

CCMFs were extruded through a 400-nm polycarbonate porous membrane for eleven passes with a mini extruder (Avanti Polar Lipids, Inc, Alabaster, AL, USA) to harvest cancer cell membrane vesicles. PLGA NPs were prepared using a nanoprecipitation method.⁵ Briefly, the carboxy-terminated 50:50 PLGA (0.67 dL/g, LACTEL Absorbable Polymers) was dissolved in acetone at 1 mg/ml concentration, and 1 ml of the acetone solution was added dropwise to 3 ml of deionized water under vigorous stirring. After stirring in open air for 2 h, acetone was removed under reduced pressure with a rotary evaporator operating at room temperature (RT). The resulting PLGA NP solution was purified, concentrated with 10 K molecular weight cutoff (MWCO) Amicon Ultra-15 Centrifugal Filters (Millipore, Burlington, MA, USA), and re-suspended in deionized water. For the synthesis of fluorescent dye-loaded PLGA NPs (PLGA-DiD NPs and PLGA-DiR NPs), 2 µg of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) dye (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) or 1,1'-dioctadecyl-3,3,3',3'-

tetramethylindotricarbocyanine iodide (DiR, Invitrogen) was added to the 1 ml of PLGA acetone solution prior to PLGA NP synthesis. Cancer cell membrane vesicles and PLGA NPs were mixed at a certain ratio and physically extruded through a 400-nm polycarbonate porous membrane for eleven passes to obtain CCMF-PLGA NPs.

Immunoblot assay:

Various subcellular fractions were lysed in radioimmune precipitation (RIPA, Sigma-Aldrich) buffer and measured by a BCA assay (Pierce, Thermo Fisher Scientific, for proteins. Samples with the same amount of protein loading were fractionated by SDS-PAGE, and transferred to a nitrocellulose membrane. A membrane fraction antibody cocktail (abl40365, Abeam, Cambridge, MA, USA), consisting of antibodies targeting anti-sodium potassium ATPase for plasma membrane, GRP78 for endoplasmic reticulum, ATP5A for mitochondria, and GAPDH for cytosol, was used at 1:250 dilution for membrane immunoblotting. Horseradish peroxidase-conjugated secondary antibody cocktail (abl40365, Abeam) was used at 1:2500 dilution, and the signal was developed using ECL Plus reagents (Thermo Scientific). Membranes were stripped and re-probed with anti-CXCR4 antibody (Prosci, Poway, CA, USA) or anti-CD44 antibody (clone 8E2, Cell Signaling, Danvers, MA, USA).

TEM and dynamic laser scattering (DLS) measurements:

Carbon-coated 400 square mesh copper grids (CF400-Cu, Electron Microscopy Sciences, Hatfield, PA, USA) were first glow discharged, and floated onto a drop of sample solution for 2 min. Subsequently, grids were consecutively negatively stained with two drops of 1% phosphotungstic acid (PTA, Sigma-Aldrich) at pH 7.0 for 30 s. Excess solution was wicked away by filter paper between each staining process. TEM imaging was carried out on a Philips/FEI BioTwin CM120 microscope at 80 kV. A Malvern Zetasizer Nano ZS90 was used to detect the information of particle size and zeta-potential of NPs.

Confocal microscopy:

CCMFs or CCMF-PLGA NPs with 100 µg of protein were suspended in 100 µl of 1 × PBS supplemented with 1% BSA following which 20 µl of phycoerythrin (PE)-conjugated anti-human CXCR4 mouse monoclonal antibody (clone 12G5, R&D Systems, Minneapolis, MN, USA) or 20 µl of APC-conjugated anti-human CD44 mouse monoclonal antibody (clone G44-26, BD Pharmingen™, Franklin Lakes, NJ, USA) were added. PE-conjugated mouse IgG2A isotype (clone 20102, R&D Systems) or APC-conjugated mouse IgG2b K isotype (clone 27-25, BD Pharmingen™) were used as controls. The mixture was kept at RT for 1 h under occasionally stirring, washed with 1 × PBS twice, and pelleted by centrifugation at 20,000 × g for 30 min. The resulting pellet was re-suspended in 100 µl of 1 × PBS. A drop of sample suspension was placed onto a cover glass (22 mm × 60 mm, Fisher Scientific), and imaged by a laser scanning confocal microscope (Zeiss LSM 510-Meta, Carl Zeiss Microscopy GmbH, Jena, Germany). The laser wavelength was set at 561 nm or 633 nm, and the receiving PMT channel was set at 572~625 nm or 650~700 nm for imaging CXCR4 or CD44 proteins, respectively. Images in each group were obtained under identical microscope settings.

Flow cytometry:

Cells were detached using $1\times$ non-enzymatic cell dissociation solution (Sigma), washed and suspended in $1\times$ PBS supplemented with 1% BSA. To examine the expression levels of CXCR4 on U87 and U87-CXCR4 cells, 1×10^6 live cells were stained with 20 μ l of phycoerythrin (PE)-conjugated anti-human CXCR4 mouse monoclonal antibody at 4 °C for 1 h. To detect CD44 levels on MDA-MB-231 and BT-474 cells, 20 μ l of APC-conjugated anti-human CD44 mouse monoclonal antibody was used. For the sample preparation of CCMFs and CCMF-PLGA NPs, the procedure was the same as described in the confocal microscopy section. Flow cytometry measurements were conducted on a FACS Calibur (BD Bioscience); ten thousand events were collected for each measurement and analyzed by FlowJo software (BD Bioscience).

Integrity of CCMF-PLGA NPs:

CCMF-PLGA NPs were doubly labeled with a fluorescent antibody on the membrane coating and a DiD dye in the core. Briefly, U87-CXCR4 CCMF-PLGA-DiD NPs were stained by PE-conjugated anti-human CXCR4 mouse monoclonal antibody according to the procedure described for confocal microscopy. U87-CXCR4 MFs without a PLGA core were used as controls. MDA-MB-231 CCMF-PLGA-DiD NPs were sequentially stained with 2 μ g/ml of anti-CD44 monoclonal antibody (clone MEM-263, Sigma) at RT for 1 h, and secondarily stained by 2 μ g/ml of Alexa 488 labeled goat anti-mouse secondary antibody (Life Technologies, Thermo Fisher Scientific) at RT for 30 min. MDA-MB-231 MFs stained with the identical procedure were used as controls. PE fluorescence was recorded as described in the confocal microscopy section. Alexa 488 fluorescence from the NP shell was acquired by excitation at 488 nm and emission at 525 nm, and DiD signals from the NP core were obtained with excitation at 633 nm and emission at 665 nm. Images in each comparison group were acquired under the identical experimental settings.

Transwell migration assay:

HMF cells in 0.75 ml of cell suspension at a density of 2×10^4 cells/ml were plated into each well of a 24-well companion plate (Corning, Coming, NY, USA). After overnight incubation, medium was replenished with serum-free medium with or without 40 μ g of CCMFs or CCMF-PLGA NPs. A Falcon™ cell culture insert (8 μ m, transparent PET membrane, Falcon, Coming) was placed into each well and plated with 5×10^4 of cancer cells suspended in 0.5 ml of serum-free medium. The plate was incubated further for one day. Cells inside the inserts were scraped off by cotton swabs, and cells migrated to the bottom of the insert membrane were stained with 0.2% crystal violet (Sigma-Aldrich) in 20% methanol solution for cell counting under a microscope. The percent migration value was obtained by normalizing to the number of cells that migrated to HMF cells alone.

Animals:

All the animal care and *in vivo* procedures were conducted in accordance with the regulations of the Institutional Animal Care and Use Committee of The Johns Hopkins University. Six to eight-week-old female athymic Balb/c (nu/nu) mice and female immunocompetent Balb/c mice were purchased from Charles River (Wilmington, MA).

Pharmacokinetic and bio-distribution study:

CCMFs and CCMF-PLGA NPs were labeled with IRDye 700DX NHS ester (IR700, LI-COR Biosciences, Lincoln, NE, USA) at a ratio of 10 nmol of IR700 to 0.5 mg of protein. PLGA-DiR NPs, IR700 labeled U87-CXCR4-MFs and U87-CXCR4 CCMF-PLGA NPs (100 µg of protein or the equivalent amount of PLGA-DiR) were injected into athymic Balb/c mice through the tail vein (n = 5 per group). The dose of PLGA-DiR NPs was determined from a phantom study to determine the amount of PLGA-DiR NPs that provided equivalent fluorescence intensity as IR700 labeled U87-CXCR4-MFs (100 µg of protein). Blood was collected retro-orbitally in anesthetized mice before and at various time points (15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h) post injection (*p.i.*). A 20 µl blood droplet was placed on a glass slide for fluorescence imaging. The concentration of NPs in blood was quantified in terms of the fluorescence intensity of the blood droplet in units of %ID/g. *In vivo* fluorescence images of mice were acquired on a Li-Cor Pearl® Impulse imager (LI-COR Biosciences) before and after injection at different time points (1 h, 8 h, 24 h). At 24 h of *p.i.*, mice were sacrificed, main organs were harvested, and *ex vivo* fluorescence images were acquired.

Lung metastasis study:

MDA-MD-231 cells constitutively expressing luciferase (231-luc, Sibtech Inc, Brookfield, CT, USA) were intravenously injected at a density of 1×10^6 cells per 50 µl Hank's balanced salt solution of through the tail vein of nude mice. The treated group (n = 5) was initially injected with 231-luc cells mixed with 231-luc CCMF-PLGA NPs (10 µg of protein) and intravenously injected weekly with 231-luc CCMF-PLGA NPs (10 µg of protein) for two subsequent doses. The control group (n = 5) was intravenously injected with $1 \times$ PBS.

VivoGlo™ Luciferin (Promega, Madison, WI, USA) was injected weekly through intraperitoneal (*i.p.*) injection at a dose of 3 mg per mouse. *In vivo* bioluminescence imaging (BLI) was performed at 15 min *p.i.* of luciferin with a Xenogen IVIS Spectrum scanner (Perkin-Elmer, Waltham, MA, USA). Three weeks after cell injection, mice were sacrificed at 15 min *p.i.* of luciferin, the chest cavity was opened, and lungs were isolated and inflated for *ex vivo* BLI. The inflated lungs were fixed overnight in 4% buffered formaldehyde solution. Approximately 10–15 fixed lung pieces were placed in a plastic cassette, embedded in paraffin, and sectioned at 5 µm thickness. Lung sections were stained with H&E, and scanned at 20× magnification on an Aperio ScanScope (Leica Biosystems Inc., Buffalo Grove, IL, USA). Metastatic burden was quantified by determining the percentage of area occupied by metastatic nodules to the total lung area using ImageScope software (Leica Biosystems, Richmond, IL, USA).

Immunization:

Immunocompetent Balb/c mice were injected with three doses of either PBS or PLGA or U87-CXCR4-MFs or U87-CXCR4 CCMF-PLGA NPs through a subcutaneous injection in the lateral tarsal region (n = 3 per group). Mice were injected every three days with 50 µg of protein on CCMFs or the equivalent amount of PLGA, or the equivalent volume of PBS.

Fluorescence tracking:

To track the NPs in injected mice, U87-CXCR4-MFs and U87-CXCR4 CCMF-PLGA NPs were labeled with IR700, and PLGA NPs were replaced by PLGA-DiR NPs. The dose of PLGA-DiR NPs was determined from a phantom study to determine the equivalent fluorescence intensity from PLGA-DiR NPs as IR700 labeled U87-CXCR4-MFs (50 µg of protein). *In vivo* fluorescence images of Balb/c mice (n = 3 per group) before and after injection were acquired on a Li-Cor Pearl® Impulse imager. Spleens, popliteal, inguinal LNs and muscle tissue were isolated at 3 days after the last injection and placed on a glass slide for fluorescence imaging.

Immune response assay:

At 7 days after the last injection, spleens, popliteal and inguinal LNs were isolated and teased apart into single cell suspensions. Splenocytes and lymphocytes (1.5 million suspended in 100 µl of FACS buffer) were stained with 2 µl of Alexa Fluor® 488 Rat Anti-Mouse CD8a (BD Pharmingen™) and 2 µl of PE Rat Anti-Mouse CD4 antibodies (BD Pharmingen™) for 30 min at 4 °C. Flow cytometry measurements were conducted on a FACS Calibur (BD Bioscience), with ten thousand events collected for each measurement, and analyzed by FlowJo software (BD Bioscience). ELISpot assay was used to quantify the frequency of IFN-γ producing T cells from spleens and draining LNs. Splenocytes and lymphocytes were collected at 7 days after the last injection. A 100 µL cell suspension (3×10^6 cell/ml) was incubated with NPs (30 µg of protein on CCMFs, or the equivalent amount of PLGA, or the equivalent volume of PBS) for 24 h in a 96-well ELISpot plate of mouse IFN-γ kit (R&D Systems). A well with 100 µL of medium was used as a negative control, while recombinant mouse IFN-γ was used as a positive control. The assay was performed according to the manufacturer's protocol.

Statistical analysis:

Statistical analysis was performed with an unpaired one-sided student t-test (Microsoft Excel), assuming unequal variance. Values of $P < 0.05$ were considered significant, unless otherwise stated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of PLGA NPs, U87-CXCR4 MFs, and U87-CXCR4 MF-PLGA NPs. (a) Western blots of U87XCR4 MFs by probing plasma membrane-specific marker (Na⁺/K⁺-ATPase), endoplasmic reticulum marker (GRP78), mitochondrial maker (ATP5a), and cytosol marker (GAPDH). Notations: Lys (cell lysate), PNS (post nuclear supernatant), Mito (mitochondria fraction), CM (crude membrane), and CCMF (cancer cell membrane fraction). (b) Representative TEM images of PLGA NPs, U87-CXCR4 MFs, and U87-CXCR4 CCMF-PLGA NPs with insets showing high magnification images. Scale bars in the insets are 100 nm, 500 nm, and 20 nm, respectively. Number distribution curves (c) and zeta-potential values (d) of PLGA NPs, and U87-CXCR4 MFs, and U87-CXCR4 CCMF-PLGA NPs measured by DLS. (e) Stability of PLGA NPs, and U87-CXCR4 MFs, and U87-

CXCR4 CCMF-PLGA NPs suspended in 0.25 mM sucrose buffer over time measured by DLS.

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Figure 2.

Surface proteins are right-side-out in U87-CXCR4 MFs and U87-CXCR4 CCMF-PLGA NPs. (a) Confocal microscopy images of CCMFs and CCMF-PLGA NPs stained with PE-conjugated antihuman CXCR4 antibody (upper panel, only recognizing extracellular CXCR4 epitope) and PE-conjugated isotype IgG2a control. Scale bar = 50 μm . (b) Flow cytometry analysis of U87-CXCR4 cells, U87-CXCR4 MFs and U87-CXCR4 CCMF-PLGA NPs after staining with PE-conjugated anti-human CXCR4 antibody. U87 compartments and PE-conjugated isotype IgG2a were used as controls.

Figure 3.

Co-localization of U87-CXCR4 MFs with PLGA-DiD. Overlap of fluorescence signals from PE stained CXCR4 receptors (left panel, green pseudo color) located on the shell, and DiD (middle left panel, red pseudo color) located in the core, of U87-CXCR4 CCMF-PLGA-DiD NPs. Middle right and right panel show bright field (BF) and merged images, respectively. U87-CXCR4 MFs without PLGA-DiD encapsulation were used for comparison. Scale bar = 50 μm .

Figure 4.

Migration assays. (a) Representative bright-field images of migrated cancer cells. (b) Percent cancer cells migrating towards HMFs in the presence or absence of pre-incubation of CCMFs or CCMFs-PLGA NPs. Values are normalized to number of cancer cells migrating towards HMFs and represent Mean $\pm$ SEM from three chamber wells per group. * $P < 0.05$ for CCMFs and CCMFs-PLGA NPs groups compared to the HMF groups. Scale bar = 50 μ m.

Figure 5.

Pharmacokinetic and bio-distribution study on PLGA-DiR NPs, U87-CXCR4 MFs, and U87-CXCR4 CCMF-PLGA NPs. (a) Retention curves of PLGA-DiR NPs, U87-CXCR4 MFs and U87-CXCR4 CCMF-PLGA NPs in mouse plasma over a period of 24 h *p.i.* of NPs (100 μ g for each) through tail vein. Values represent Mean $\pm$ SEM from five mice per group. (b) Representative NIR fluorescence *in vivo* images of nude mice over a period of 24 h *p.i.* of NPs (100 μ g for each). (c) Representative fluorescence images of major organs harvested at 24 h *p.i.* of NPs (100 μ g for each). H, heart; Li, liver; Sp, spleen; M, muscle; Lu, lung; K, kidney; I, intestine; St, stomach; B, blood.

Figure 6.

In vivo bioluminescence imaging of metastasis. (a) *In vivo* bioluminescence imaging over the course of three weeks. Experimental lung metastasis was established by intravenously injecting MDA-MD-231 cells constitutively expressing luciferase (231-luc) through the tail vein of Balb/c nude mice. The treated group was intravenously injected with the same number of 231-luc cells mixed with 231-luc CCMFs-PLGA NPs and injected with two additional doses of 231-luc CCMF-PLGA NPs once a week over the 3 weeks. Control mice were injected with PBS. (b) Bioluminescence imaging of the chest cavity at the end of the 3

week treatment. Two upper left panels have separate scale bars, and the other panels share the same scale bar.

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Figure 7.

Evaluation of metastatic tumor burden in lungs. (A) *Ex vivo* bioluminescence imaging of metastatic nodules in lungs. At the end of the 3 week treatment, lungs from the PBS group (upper) and treated group (lower) were isolated, inflated, and imaged for bioluminescence. Two upper left panels have individual scale bars, and the other panels share the same scale bar. (B) Average radiance from bioluminescence in the PBS group (upper) and treated group (lower). * $P < 0.05$ for 231-luc CCMF-PLGA NPs treated group compared with PBS group using Student's *t* test. (c) Representative H&E stained images of lung sections from the PBS group (upper) and the 231-luc CCMF-PLGA NPs treated group (lower). Magnified images (5 $\times$) of outlined areas are shown on the right. Arrowheads point to metastatic nodules. (d) Metastatic burden in lungs determined from the percentage of metastatic nodule area to the total lung area. * $P < 0.05$ for 231-luc CCMF-PLGA NPs group compared with PBS group using Student's *t* test. Values represent Mean $\pm$ SEM from five mice per group ($n = 5$).

Figure 8.

NIR fluorescence tracking of NPs following immunization. (a) Representative NIR *in vivo* images of Balb/c mice over a period of 3 days following the first injection. Arrow points to the fluorescent inguinal LN from U87-CXCR4 CCMF-PLGA NPs-injected mouse. (b) Representative NIR images of resected lymph nodes (LNs) and spleens 3 days after the last injection. Balb/c mice were immunized with subcutaneous injections of PLGA-DiR, U87-CXCR4 MFs, or U87-CXCR4 CCMF-PLGA NPs into the lateral tarsal region above the ankle. Injections were given three days apart, and 50 μ g of protein on CCMFs or the equivalent amount of PLGA-DiR was given in each dose. Mu (muscle), Pop (popliteal LNs), In (inguinal LNs), Sp (spleen).

Figure 9.

Representative populations of CD8⁺ and CD4⁺ cytotoxic T-lymphocytes isolated from (a) spleens and (b) draining LNs at 7 days after the last injection. Balb/c mice were injected with three doses of PBS or PLGA or U87-CXCR4 MFs or U87-CXCR4 CCMF-PLGA NPs through subcutaneous injection into the lateral tarsal region. Injections were given three days apart, and 50 μ g of protein on CCMFs or the equivalent amount of PLGA, equivalent volume of PBS was given in each dose. Cells were stained with FITC-labeled anti-CD8 and PE-labeled anti-CD4 antibodies for 30 min at 4 $^{\circ}$ C, and analyzed using a flow cytometer. Percentage of CD4⁺ (c) and (d) CD8⁺ cytotoxic T cells in spleens and LNs. *P < 0.05 for U87-CXCR4 CCMF-PLGA versus PBS group. Values represent Mean $\pm$ SEM from three mice per group.

Scheme 1.

(a) Schematic illustration of the preparation of cancer cell plasma membrane fraction coated PLGA NPs (CCMF-PLGA NPs). Cancer cell derived plasma membrane fractions (CCMFs) were derived from their source cells through a series of homogenization, differential centrifugation, and sucrose density gradient centrifugation treatments. CCMFs together with the associated proteins were translocated to PLGA NPs through extrusion processes. (b) The purpose was to determine the ability of these cancer cell-mimicking NPs to disrupt cancer cell-stromal cell interactions, reduce metastasis, and prime the immune system for cancer immunotherapy.