UNIVERSITY OF CALIFORNIA SAN DIEGO

Natural and Engineered Cancer Cell Membrane-Coated Nanoparticles for Antitumor Immunotherapy

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by

Yao Jiang

Committee in charge:

Professor Liangfang Zhang, Chair
Professor Yi Chen
Professor Jesse Jokerst
Professor Wei Wang
Professor Dong-Er Zhang

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The dissertation of Yao Jiang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

This dissertation is dedicated to my husband, Charlie Xu, who has been incredibly supportive, patient, and loving during my journey throughout graduate school.

This dissertation is also dedicated to my parents: Rongrong Shao and Xinghua Jiang, who chose to raise me in the “gardener” way, supported every decision I have made, and nurtured me into a confident and curious young woman.

I am truly blessed and forever grateful to have them in my life. All my work would never happen without their support.

EPIGRAPH

Even youths grow tired and weary,
and young men stumble and fall;
but those who hope in the Lord
will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint.

Isaiah 40:30-31

TABLE OF CONTENTS

LIST OF FIGURES

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VITA

2015 B.S. in Bioscience, Huazhong University of Science and Technology, China

2016 M.S. in Nanoengineering, University of California San Diego, USA

2021 Ph.D. in Nanoengineering, University of California San Diego, USA

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Natural and Engineered Cancer Cell Membrane-Coated Nanoparticles for Antitumor Immunotherapy

by

Yao Jiang

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Professor Liangfang Zhang, Chair

Advancement in the understanding of tumor immunology has led to the development of novel therapies that can augment endogenous immunity and elicit potent antitumor immune responses. Concurrently, nanomedicine has revolutionized the way we fight diseases with unique physical properties and distinct mechanisms of action. In particular, cell membrane-coated

nanoparticles, which are biomimetic nanotherapeutics derived from natural plasma cell membrane, have demonstrated tremendous benefits in cancer immunotherapy.

Herein, the first chapter of this dissertation will be dedicated to the design principles and current status of cancer cell membrane-coated nanoparticles for anticancer vaccination. The second chapter of the dissertation will focus on the design, fabrication, and biological functions of a biomimetic anticancer vaccine that can co-deliver tumor antigens and immunostimulatory adjuvants. This nanovaccine utilizes natural cancer cell membrane to provide multivalent-antigen specificity, drains efficiently to lymphatic system to engage immune cells, and elicits a strong immune response to control tumor growth in both prophylactic and therapeutic settings. The third chapter of the dissertation will focus on the design, fabrication, and biological functions of a biomimetic nanoparticle platform that can be used to mobilize specific T cell subsets without the need for professional antigen-presenting cells. This nanoparticle utilizes engineered cancer cell membrane that express a T cell co-stimulatory marker and present peptide epitopes, the two signals necessary to promote tumor antigen-specific T cell immune responses. These two platforms both represent powerful tools that can be used to develop personalized cancer immunotherapies down the road.

This dissertation will serve as a paradigm to rationally design both natural and engineered cancer cell membrane coated nanoparticles for antitumor immunotherapy. By harnessing the amply available engineering tools, researchers could enhance the functionalities of cell membrane beyond the natural properties of parent cells, and significantly expand the application of cell membrane-coated nanoparticles beyond antitumor immunotherapy.

Chapter 1

Introduction

1.1 Introduction

Our immune system is a complex network of cells, proteins, and physical barriers that work together to keep the human body free from disease. When mobilized correctly, it has the ability to seek out and eliminate foreign invaders with exquisite specificity. Malfunctioning or underperforming immunity is often the root cause of many disease states. For example, an overactive immune system can result in autoimmunity, which is characterized by proinflammatory states and leads to the destruction of healthy tissue [1, 2]. On the other hand, an underactive immune system can lead to enhanced susceptibility to infection, which is becoming increasingly dangerous given the rise of antibiotic resistance [3]. With regards to tumorigenesis, it has been shown that the immune system is integral in helping to prevent the proliferation of malignant cells [4]. It is now known that for tumors to successfully grow, cancerous cells must generally go through a prolonged evolutionary process in order to develop mechanisms for immune evasion [5]. Tumors can manipulate the surrounding microenvironment to support growth and suppress host immune responses using cytokine and growth factor secretion [6], extracellular matrix restructuring [7], and cellular signaling [8, 9]. It is for this reason that an intense amount of research has been focused on leveraging the immune system to fight off cancer [10]. In general, cancer immunotherapies seek to train, augment, or supplement the body's own ability to eliminate malignant growths. There are numerous classes of immunotherapy, and they can act on different stages of immunity, ranging from initial antigen presentation up to the final effector stages [11, 12]. Depending on the specific type of cancer being treated, early returns have thus far been promising, and a number of immunotherapies have proven to be highly potent in scenarios where the previous clinical standard of care had little effect [13-15].

Anticancer vaccination is a class of cancer immunotherapy that focuses largely on training the immune system to recognize and mount a response against tumors in an antigen-specific manner [12, 16]. Over the course of recent human history, vaccines have represented an attractive means of managing the spread of disease, as most are easy to administer and can promote the development of sterilizing immunity [17]. Particularly in the case infectious diseases, vaccination has proven to be highly effective, having likely helped to prevent millions of deaths as a result of large-scale prophylaxis campaigns [18]. Despite the favorable history of antibacterial and antiviral vaccines, anticancer vaccination unfortunately has not achieved the same level of success [19, 20]. Unlike with those against pathogens, there are additional hurdles that must be overcome in order for vaccines against tumors to be effective. One of the main challenges comes from the fact that most tumors are lowly immunogenic and originate from one's own healthy cells. As such, it is incredibly difficult for the immune system to correctly identify malignant tissue. Additionally, vaccines against established tumors must be administered therapeutically, requiring the need for formulations that are highly potent in addition to being tumor-specific. This has oftentimes necessitated the use of complex strategies for immune system manipulation [19-21], many of which are lowly viable in a clinical setting given poor cost-to-benefit ratios. In 2010, the United States Food and Drug Administration approved the first and only therapeutic anticancer vaccine, sipuleucel-T [22]. This autologous cell-based therapy trains patient-derived immune cells against a common prostate cancer antigen before reinfusion of the cells back into the patient. The treatment has been shown to marginally increase patient survival time, but the complex logistics and high cost of manufacturing a personalized cell-based vaccine have limited its commercial viability.

To address the hurdles faced by traditional vaccination schemes against cancer, many researchers have turned toward nanotechnology to help guide the design of nanovaccines capable

of producing potent, specific, and durable antitumor responses [23, 24]. Compared with traditional vaccines, those manufactured at the nanoscale have unique physical and material properties that make them better suited for immune manipulation. Through purposeful engineering, nanovaccines can be formulated with antigen and adjuvant payloads in a manner that maximizes immune responses through efficient delivery to specific cellular subsets. Ultimately, the goal is to leverage such platforms for the controlled programing of endogenous immunity to reverse tumor burden. In this review, we start by covering some basic background information regarding anticancer vaccines and the current state of traditional platforms. We then discuss developments in the field of anticancer nanovaccines, focusing on platforms for both nonspecific and antigen-specific immune modulation. Finally, we introduce an emerging class of biomimetic nanoparticles based on cell membrane coating nanotechnology. This top-down strategy directly leverages nature's own design principles as a means of fabricating multifunctional and multiantigenic nanosystems, which have the potential to play an important role in the future of anticancer vaccination.

1.2 Background on Anticancer Vaccination

1.2.1 Cancer Immunology and Immunotherapy

Cancer is generally characterized by an accumulation of mutations that allows for uncontrolled cell proliferation. As tumors grow, they are in a constant battle with the immune system and must evolve mechanisms for escape over time [5]. Due to the random nature of the mutations that lead to malignancy, phenotypes can vary greatly among different cancers, as well as among cells within the same tumor. This heterogeneity not only serves as a challenge for

traditional cancer therapeutics, but also acts as an immune evasion mechanism, increasing the likelihood of some mutant cell populations remaining undetected [20, 25]. Another immune escape mechanism occurs through antigen shedding [26]. As part of their normal growth, cells generate a large amount of waste products, and these unwanted products are commonly secreted through membrane vesicles. When released in large abundance, this process can also deplete the parent cell of tumor-specific antigens, thus enabling the altered cancer cells to avoid destruction by cytotoxic T cells. Furthermore, shed antigens released into the bloodstream can act as decoys for neutralizing cancer-specific antibodies. Solid tumors can employ additional means of escape, whereby their local microenvironments are remodeled to promote immune tolerance [27].

A better understanding of how cancer interacts with the immune system has allowed for the development of new and effective therapeutics. The goal of cancer immunotherapies is to leverage a patient's own immune system to eradicate tumors in a highly specific and relatively safe manner [28]. One example is through an overall activation of the immune system by administering proinflammatory cytokines, which are immunomodulatory molecules released by activated immune cells [29, 30]. Although the immune stimulation caused by these molecules is nonspecific, an overall boost in immunity can sometimes strengthen immune cells enough to overcome tumor suppression. More specific, tumor-targeted approaches can be achieved using genetically engineered chimeric antigen receptors (CAR) on T cells [31, 32]. In CAR T cell therapy, T lymphocytes are isolated from a patient or a donor through leukapheresis [33]. The cells are then genetically modified to express a receptor that can recognize tumor-associated antigens, leading to elimination of the corresponding cells. Altered T cells are purified, expanded *ex vivo*, and finally infused back into patients for treatment. For some cancer types, this CAR approach has displayed striking efficacy in the clinic.

Antibodies have also been widely used to elicit antitumor immunity. For example, tumor-targeted monoclonal antibodies that recognize tumor antigens can opsonize cancer cells and trigger antibody-dependent, cell-mediated cytotoxicity [34]. Furthermore, by conjugating antibodies with chemotherapeutics, these cytotoxic cargos can be more accurately targeted to the tumor site and induce immunogenic cell death [35]. More recently, antibody-based checkpoint inhibitors have been used to directly modulate the function of specific immune cell subsets [36]. Immune checkpoints involve inhibitory receptors such as programed cell death protein 1 (PD1) and cytotoxic T lymphocyte–associated protein 4 (CTLA4) that regulate T cells. By presenting the corresponding ligands, the cytotoxic activity of T cells can be inhibited by tumor cells and regulatory immune cells. In checkpoint blockade therapy, antibodies target and block these receptor binding sites, thus removing the inhibitory signals on the T lymphocytes and unleashing their full potential for eliminating cancer cells. Despite their ability to elicit strong antitumor responses, efficacy of checkpoint blockades can vary greatly by patient [37]. This discrepancy may be explained by the fact that the therapy generally relies on the presence of preexisting tumor-targeted T cells [38]. For this reason, checkpoint blockades are being actively explored for use in combination with other therapies such as anticancer vaccination, which can help to generate new T cell populations [39, 40].

1.2.2 Current State of Cancer Vaccines

Cancer vaccines introduce tumor-relevant antigenic material in a manner that leads to downstream mobilization of the immune system [28]. As the most immunogenic mutations have likely already been selected out by the time cancer is detected [5], the presence of tumor antigens

alone is usually not sufficient to drive proper immune stimulation. As such, tumor antigens are almost always combined with an adjuvant in order to enhance the immune response [41]. In the basic process, delivered antigens are taken up by professional antigen-presenting cells (APCs), such as dendritic cells, which process and break down the antigens, followed by presentation of the peptide fragments via major histocompatibility complexes (MHCs) [42]. With the help of the adjuvant, the APCs mature, enabling engagement and activation of cancer-relevant T cells. Finally, the activated T cells can help to promote tumor elimination, either by further propagating immune activation or by directly seeking out and destroying the cancer cells.

Antigenic delivery to the immune system can be achieved in multiple ways. The most straightforward is the direct administration of tumor antigens. In single-antigen approaches, a tumor antigen overexpressed on cancer cells is administered parenterally [43]. This has been shown to elicit a robust immune response against the target antigen, especially in combination with an adjuvant; however, this approach may ultimately be thwarted by tumor heterogeneity. Whole cell preparations are another source of antigenic material that can theoretically be used to vaccinate against the full breadth of tumor antigens [44]. However, this strategy often suffers from inadequate antitumor immune responses due to the interference from irrelevant proteins. In response to the often weak immunity generated by the above approaches, dendritic cells can be pulsed with an antigen and stimulated *ex vivo* [21]. Once this process is completed, the cells are then injected back into the patient in a process similar to CAR T cell therapy. The manipulated dendritic cells can subsequently migrate to the body's immune centers, where they train endogenous T cells. In a final method, antigenic uptake can happen *in situ* at the tumor site, taking advantage of processes such as immunogenic cell death, which provide autologous tumor antigens

under an immunostimulatory context [45]. *In situ* vaccinations can also be achieved with oncolytic viruses that selectively infect and destroy cancer cells [46].

In April of 2010, the United States Food and Drug Administration gave its first approval to a therapeutic anticancer vaccine, sipuleucel-T, for the treatment of prostate cancer [47]. In this therapy, patient-derived dendritic cells are pulsed with prostatic acid phosphatase, which is expressed in a significant number of patients with prostate cancer [48]. After exposure to the antigen, along with granulocyte-macrophage colony-stimulating factor, the activated dendritic cells are introduced back into the patient. It was demonstrated in a clinical trial that sipuleucel-T was able to extend median survival by 4.1 months, which paved the way for its eventual approval [47]. The successful translation of this treatment has motivated the further clinical exploration of anticancer vaccine formulations, and a search on ClinicalTrials.gov yields over 200 results for active trials. Examples of current clinical studies include dendritic cell therapies for glioblastoma (NCT01808820), oncolytic viruses for ovarian cancer (NCT00408590), peptide vaccines for recurrent glioblastoma (NCT02754362), and whole cell vaccines for breast cancer (NCT00317603).

Although cancer vaccines have had some success in the clinic, their limited ability to produce strong antitumor responses has hindered their widespread adoption. Despite its regulatory approval, the long-term financial viability of sipuleucel-T has come into question. The labor-intensive processes involved in its manufacture necessitate its high cost, which may be hard to justify given that the treatment only modestly prolongs median survival. Single-antigen peptide vaccines are able to elicit potent immune responses against the tumor cells that display the relevant antigenic epitopes; however, due to the heterogeneity of cancers, antigen-negative cells can eventually escape detection and proliferate without competition [20]. This approach is also not

universal, and personalized identification and manufacture of vaccines based on tumor-specific neoantigens may not yet be viable on a large scale [49, 50]. Whole cell vaccination with tumor lysates has the potential to elicit multiantigenic immunity, but the final immune response is often dampened by the presence of extraneous proteins [44]. This underscores the fact that, even when delivering the correct antigenic material, current vaccination strategies may not have sufficient immunostimulatory capacity to overcome the tolerogenic tumor microenvironment.

1.2.3 Advantages of Nanovaccines

Nanotechnology offers many opportunities for improving the treatment efficacy of cancer vaccine formulations compared to traditional strategies (Figure 1.1). A major advantage is the ability to formulate the antigen and adjuvant components together in a manner that maximizes immune stimulation [51]. Flexibility in nanoparticle synthesis methods and material choice allows for the incorporation of different classes of molecules, such as proteins, polysaccharides, nucleic acids, lipids, proteins, and polymers. For example, electrostatic interactions can be used to bind nanoparticles and payloads with opposite charges together [52], or lipid-based cargoes can be incorporated into the bilayer of liposomes through an insertion technique [53, 54]. Cargoes can also be encapsulated through chemical conjugation [55], or they can be decorated onto the nanoparticle surface [56]. Oftentimes, the nanocarriers themselves can also be fabricated using biologically active vaccine components. For example, it has been demonstrated that both calcium phosphate [57], a mineral-based adjuvant, and certain antigen proteins [58] can be made into nanoparticulate form.

Loading of antigen and adjuvant into nanoparticles can serve a variety of purposes. Encapsulation of vaccine components has been shown to increase immunogenicity by protecting the integrity of the molecules from enzymes in the body, such as nucleases, proteases, and phosphatases [59]. Nanoparticulate delivery not only protects the adjuvant from degradation, but can also protect the body from the systemic toxicity of the adjuvants, which can cause side effects such as fever, lethargy, diarrhea, and nausea [60]. Nanoencapsulation can also be used to enhance immune responses by providing extended release properties. Certain gel-like or polymeric nanoparticle platforms can act as depots, slowly releasing adjuvants and antigens over a long period of time [61]. Finally, there are a wide range of techniques available for loading both antigens and adjuvants into the same nanocarrier, which has been shown to dramatically increase antigen-specific immune responses by unifying the pharmacokinetics of the coencapsulated payloads [51].

In terms of payload delivery, nanoparticles can be designed to better target immune cells and immune-rich organs. At their size range, nanoparticulate vaccine formulations more easily drain into the lymphatic system after administration, enabling efficient delivery to the lymph nodes [62, 63], which contain high densities of immune cells. The localization of the nanoparticles can be further improved by modifying their outer layer to display ligands specific to immune cell surface receptors [64, 65]. Nanoformulations can also be designed to promote intracellular localization in a manner that maximizes the biological activity of the payloads. For example, nucleotide-binding oligomerization domain–like agonists and small interfering RNA (siRNA) can be delivered directly to the cytosol using nanoparticles designed to penetrate through cell membranes [66], and toll-like receptors (TLRs) can be engaged by various agonists when delivered into cells via an endosomal pathway [67]. Overall, careful choices in the use of

materials, loading methods, and synthesis techniques for nanoparticle-based formulations can all lead to improved vaccine efficacy.

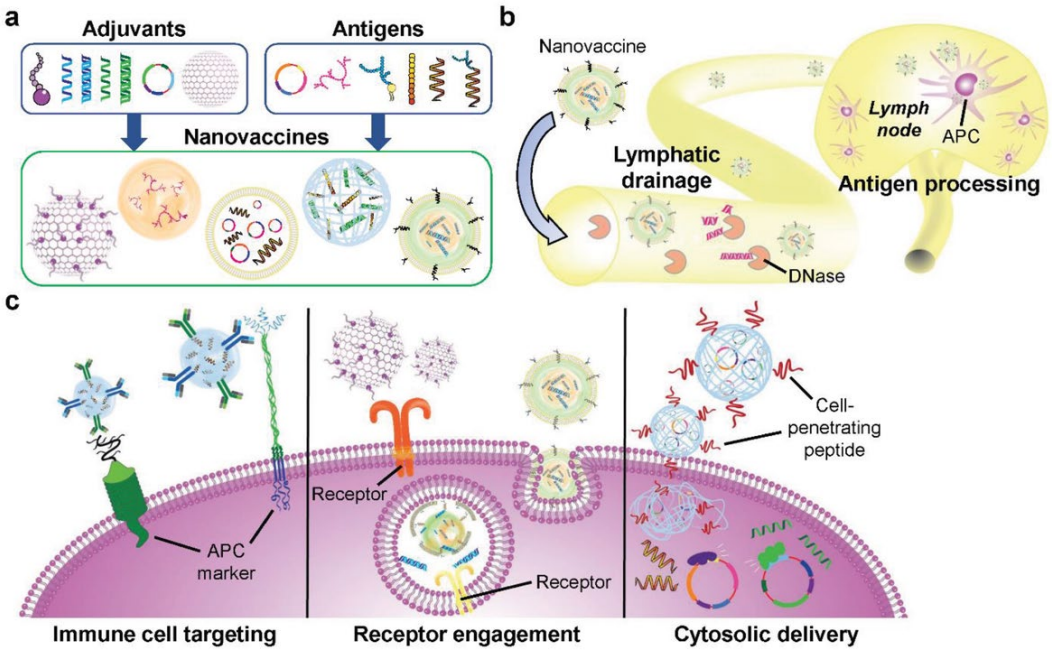

Figure 1.1: Advantages of nanoparticles for vaccine design. a) Various combinations of adjuvants and antigens can be formulated using nanoparticle platforms such as liposomes, emulsions, nanogels, and many others. b) Nanovaccines can access the lymphatic drainage system for lymph node delivery while protecting cargoes from environmental degradation. Once at the lymph nodes, the nanocarriers can deliver their cargoes to antigen-presenting cells (APCs) for immune processing. c) Nanovaccine properties can be tuned to efficiently deliver their cargoes for maximum immune activation. For example, nanoparticles can be modified to target specific subsets of immune cells. They can also be delivered to specific intracellular compartments, where receptors for immune pathways can be triggered.

1.3 Nanoparticle-Based Cancer Vaccines

1.3.1 Nonspecific Modulation

Some immunomodulatory nanoparticle platforms work to nonspecifically boost immune system function. While not strictly considered vaccines, these systems do rely on a patient's own tumor as the source of antigenic material and work by augmenting immune processes such as

antigen processing and antigen presentation. This is generally achieved by manipulating the immune system in a way that reduces immunosuppression or activates specific immune cell subsets to potentiate a response against cancer cells. In some cases, these formulations can also be combined with tumor cell killing mechanisms to increase exposure to tumor-associated antigens.

1.3.1.1 Enhancing Physical Proximity of Immune Cells

An intuitive method for boosting antitumor immune activity is to bring the principal immune cells responsible for tumor elimination closer to their target. To achieve this, nanoparticles can be decorated with two different antibodies, one to target and/or activate immune cells, and another to target the tumor cells. By using these bifunctional nanoparticles, nearby immune cells can be targeted to tumors, increasing the chance of exposure to released tumor antigens or apoptotic cancer cells while enhancing immune stimulation. In a first example, biodegradable poly(lactic acid) nanoparticles were decorated with antibodies against the dendritic cell costimulatory marker CD40, as well as an antibody against human epidermal growth factor receptor 2 (HER2)/neu, a common tumor antigen overexpressed in human breast cancer [68]. The anti-CD40 antibody was found to both bind and activate dendritic cells, inducing a strong proinflammatory immune response that could be directed toward neu^+ tumors. Intratumoral injection of the nanoparticles yielded 100% rejection, while systemic injections resulted in 70% of mice rejecting neu^+ tumors. Importantly, rechallenge of mice that rejected the primary tumor did not lead to any subsequent tumor growth. In another example, polystyrene nanoparticles were conjugated with antibodies against HER2/neu and calreticulin, a protein that facilitates phagocytosis in APCs [69]. Macrophages treated with these multivalent bispecific nano-bioconjugate engagers were able to better take up $HER2^+$ cancer cells and presented tumor-

associated antigens via MHC surface complexes. Intratumoral and intravenous injections of the nanoparticles led to higher infiltration of $CD8^+$ T cells and inhibited the growth of HER2-expressing tumors. Upon rechallenge, treated mice rejected $HER2^+$ cancer cells but not $HER2^-$ cells, demonstrating the specificity of the treatment and the durability of the response. Instead of binding APCs to tumor cells, it has also been demonstrated that antigen-specific T cells can be linked to cancer cells in a similar manner [70]. Conjugation of nanoparticles with SIY–MHC complexes effectively enabled binding to 2C T cells, while the inclusion of anti-CD19 allowed for crosslinking with $CD19^+$ Raji cancer cells. Shortly after intratumoral injection of the nanoparticles, mice were infused with adoptively transferred 2C T cells, which led to significant retardation of tumor growth.

1.3.1.2 Reduction of Immunosuppression

The immunosuppressive tumor microenvironment is a hurdle for most anticancer immunotherapy treatments, as effector cells can be rendered ineffective by inhibitory proteins or anti-inflammatory cytokines. For example, a melanoma-specific peptide vaccine was found to be effective for early stage melanoma, but it failed to demonstrate efficacy at later disease stages due to increased levels of immunosuppressive cytokines like tumor growth factor β (TGFβ) in the tumor microenvironment [71]. To address this, a liposome–protamine–hyaluronic acid nanoparticle was designed to deliver siRNA against TGFβ into tumor cells [72]. Injection of the nanoparticles halved the levels of TGFβ in the tumor microenvironment while doubling the efficacy of the vaccine. This improvement was discovered to be caused by an increase in $CD8^+$ T cells in the late stage tumor tissue along with a marked decrease in regulatory T cell levels. Other immunosuppressive efforts focus on the expression of signaling proteins on tumor tissue that

interact with immune cells. Well-known pathways such as PD1 can be intercepted using checkpoint blockades, but systemic administration can have toxic side effects, potentially leading to the development of autoimmune diseases and pathological inflammation [73]. In one recent work, platelet-derived microparticles were used as a carrier for antibodies against programed death-ligand 1 (PDL1) [74]. After tumor resection, residual cancer cells can oftentimes start to regrow the tumor or be released into circulation. These remaining cells can express PDL1 in response to inflammation, making it highly difficult for the immune system to destroy them and prevent tumor recurrence. Due to the abundance of exposed collagen in wound sites, platelet microparticles were chosen as the delivery vehicle for anti-PDL1 given their inherent targeting ability. Intravenous injection of the microparticles immediately after incomplete tumor resections was shown to greatly reduce tumor regrowth and metastasis formation in both B16-F10 melanoma and triple-negative 4T1 breast cancer mouse models. Similarly, immunotherapy mediated by low dose doxorubicin has been shown to have partial efficacy against B-Raf proto-oncogene mutant melanoma, but it failed at long-term efficacy likely due to the emergence of the Wnt family member 5a (Wnt5a) protein on cancer cells. Wnt5a can induce dendritic cell tolerance and cause fibrosis of tumor tissue, as well as prevent T cell infiltration. A lipid–protamine–DNA nanoparticle loaded with plasmid DNA encoding for a Wnt5a trap was able to transiently reduce Wnt5a levels in the tumor microenvironment and significantly boost treatment efficacy using doxorubicin [75].

1.3.1.3 Immune System Activation

The immune system can be boosted through the introduction of immunostimulatory payloads, including pathogen-associated molecular patterns (PAMPs), costimulatory markers, cytokines, and other signaling proteins. Adjuvant administration has been found to be a powerful

nonspecific modulator to aid in cancer immunotherapy. PAMPs such as single-stranded DNA, double-stranded RNA, and lipopolysaccharides are recognized by the TLRs found on immune cells and help to promote downstream inflammatory responses. Many of these PAMPs, such as CpG oligonucleotides (ODNs) recognized by endosomal TLR9, have been extensively used as adjuvants in conjunction with a coinjection of proteins or peptides to promote specific immune responses [76-80]. Other TLR-targeted PAMPs such as monophosphoryl lipid A (MPLA) [81, 82] and imidazoquinoline [83] have been used in nanoparticle formulations as adjuvants, and some PAMPs have even been coloaded together to simultaneously engage multiple different TLRs [84].

Cyclic dinucleotides (CDNs), small nucleic acids characteristic of invading microbes, are a family of type I interferon (IFN)–producing PAMPs. These CDNs are in phase I clinical trials, but they require very high dosages to ensure that adequate amounts can get into the cytosol to interact with their stimulator of interferon genes (STING) receptor. Encapsulation of CDNs into nanoparticles can improve cytosolic delivery and enhance immune responses at lower concentrations. In one work, cyclic diguanylate was encapsulated into polyethylene glycol-functionalized lipid nanoparticles and used to adjuvant soluble ovalbumin (OVA) protein [85]. After vaccination, a significant increase in both $CD8^+$ and $CD4^+$ T cells was observed, and T cells restimulated with OVA produced fivefold increases in IFNγ and tumor necrosis factor α (TNFα). Further, a CDN-adjuvanted B16-F10 vaccine formulation induced a sevenfold higher frequency of gp100-specific $CD8^+$ T cells and significantly delayed B16-F10 tumor growth. CDNs have also been incorporated into nanoparticles consisting of cationic poly(β-amino ester) (PBAE), a polymer widely used for cytosolic delivery of DNA [86]. Delivery of cyclic diguanylate to THP-1 cells using a PBAE carrier yielded an equivalent amount of IFN regulatory factor 3 activation as free CDN, but at a 100-fold lower dose of adjuvant. When the nanoparticles were given as an

intratumoral injection along with anti-PD1 antibodies, complete remission of B16-F10 tumors was seen at an order of magnitude lower CDN dosage than the soluble form.

The repetitive protein structure of viral capsids self-assembled into nanoparticles can also serve as a PAMP. For example, cowpea mosaic virus is a noninfectious agent that self-assembles into hollow, icosahedral 30 nm virus-like particles, which can have strong antitumor immunotherapeutic activity (Figure 1.2) [87, 88]. Inhalation of the virus-like particles by B16-F10 tumor–bearing mice increased tumor-infiltrating neutrophils, activated neutrophils in the lung microenvironment, and elevated levels of neutrophil-secreted cytokines. Significantly delayed tumor growth was seen after injections of the nanoparticles via various routes in multiple different tumor models. In particular, the virus-like particles were able to eliminate primary B16-F10 tumors in half of mice upon intratumoral injection, as well as provide long-term antitumor immunity as shown by rejection of a contralateral B16-F10 rechallenge. Other virus-like particles such as the papaya mosaic virus [89], influenza virus [90], and tomato yellow leaf curl virus [91] have also shown strong adjuvanting properties that can be taken advantage of for immune modulation.

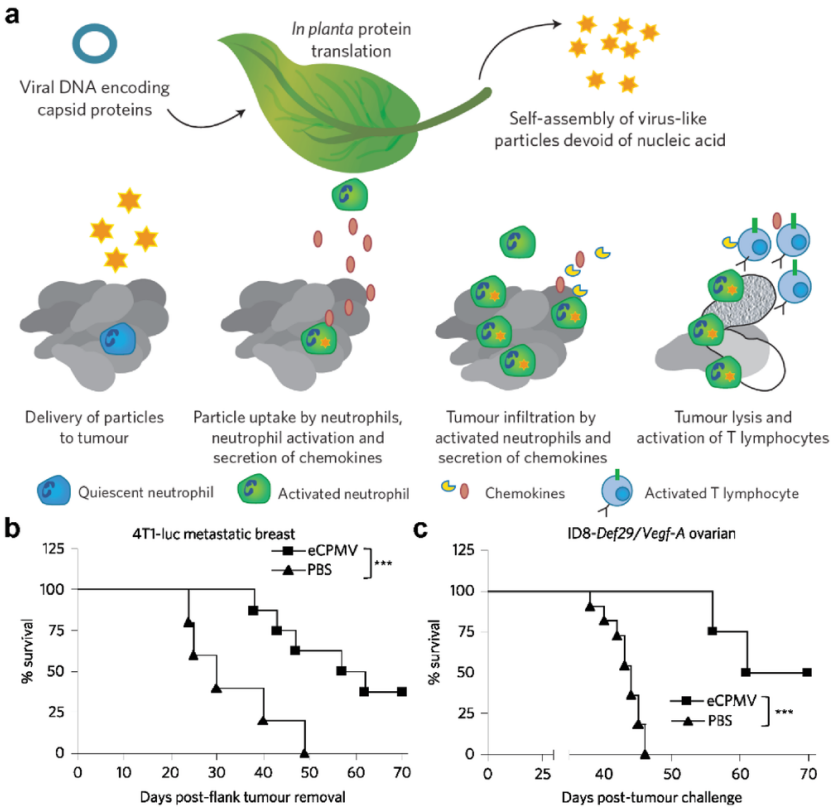

Figure 1.2: Virus-like nanoparticles for *in situ* anticancer vaccination. a) Schematic depicting the synthesis of empty cowpea mosaic virus (eCPMV) nanoparticles and their expected mechanism of action for tumor treatment. b,c) When used to treat tumor-bearing mice, virus-like nanoparticles significantly enhanced survival in both a 4T1-luc metastatic breast cancer model (b) and an ID8-Def29/Vegf-A ovarian cancer model (c). Reproduced with permission Copyright 2016, Springer Nature.

Cytokines serve a very important role in the adaptive immune system and can also be used for potent immune activation. For instance, mast cells can influence dendritic cell migration to the lymph nodes and upregulate inflammatory responses through the release of granules full of immune mediators like TNF. To mimic this natural boosting of the immune system, synthetic mast cell granules were synthesized by trapping TNF into a nanoparticle matrix of chitosan–heparin [92]. Like real mast cell granules, the particles drained to lymph nodes and promoted germinal center formation. Due to the modular nature of the nanoparticles, TNF could be replaced with interleukin-12 (IL12) to promote polarization of immune cells toward proinflammatory phenotypes, such as IFNγ-secreting T cells. Delivery of IL2, a crucial cytokine for T cell survival

and proliferation, has also been explored as a method to enhance T cell–mediated immunotherapy. Hydroxyethyl starch nanocapsules were coupled with IL2 using copper-free click chemistry, and incubation with T cells resulted in a high level of uptake and a fourfold increase in division index compared to unmodified nanocapsules. It has been shown previously that nanoparticles delivering a combination of different classes of immune-activating adjuvants can promote increased therapeutic efficacy [93]. Combinations of cytokines with other molecules, such as PAMPs [94] and costimulatory ligands [95], have also been shown to synergistically activate immune cells.

1.3.1.4 Immune Activation and Immunosuppressive Intervention Combination

Beyond combining different methods of activating immune cells, simultaneous use of immunosuppressive intervention and immune activation can also yield impressive results. For example, combining IL10 siRNA and CpG ODN into a pathogen-mimicking nanoparticle resulted in a balanced Th1/Th2 cytokine response that improved antitumor efficacy [96]. Immune activating R848 has also been delivered to T cells by encapsulation in nanoparticles that were targeted to T cells expressing PD1 [97]. To enhance costimulation while reducing immunosuppression, dual-targeted nanoparticles have been developed with both agonistic and antagonistic antibodies conjugated onto the same surface. In one case, anti-4-1BB was attached onto particles to activate the 4-1BB costimulation pathway on $CD8^+$ T cells, while the conjugation of anti-PDL1 served to block PDL1 expressed on the surface of cancer cells [98]. Alternatively, nanoparticles decorated with anti-OX40 and anti-PD1 were able to target T cells expressing both receptors, simultaneously activating them and preventing their anergy [99]. In both the cases

above, T cells were less inhibited by the immunosuppressive tumor microenvironment, leading to enhanced antitumor efficacy in a variety of mouse cancer models.

1.3.1.5 Combination with Traditional Anticancer Therapies

In the examples discussed thus far, it can be understood that the immunostimulatory nanoparticle platforms relied on the natural immune processing of tumor cells as the source of antigenic material. To facilitate the generation of tumor antigens and downstream immune activation, another strategy is to actively promote the release of material from tumors while concurrently introducing nonspecific immune modulators. For example, administration of the immunotherapeutic potato virus X alone caused a modest decrease in the growth rate of B16-F10 cancer cells, similar to monotherapy with doxorubicin. However, coadministration of both the components led to a significant improvement in antitumor efficacy [100]. In another work, cytotoxic cationic silica nanoparticles were used to induce necrotic cell death while delivering a STING agonist to the immune cells in the tumor microenvironment [101]. Finally, "sticky" nanoparticles were designed to capture antigens *in situ* before being phagocytosed by immune cells [102]. After administration of anti-PD1 antibodies, primary tumors were irradiated and then injected with the antigen-capturing nanoparticles. Taking advantage of the abscopal effect, protein-loaded nanoparticles could then travel to the lymph nodes to facilitate an adaptive immune response, which led to the eventual destruction of a secondary tumor in 20% of mice.

1.3.2 Specific Modulation

The ultimate goal of vaccination is to stimulate the immune system while simultaneously guiding a specific response against the desired target. For cancer immunotherapy, this target is often a lowly immunogenic antigen that is differentially expressed by tumor cells. As a result, an ideal cancer vaccine requires delivery of the relevant antigens along with a potent immunological adjuvant, which can be used to force the immune system to mount an antitumor response. In recent research, nanotechnology has been employed to further improve the efficacy of cancer vaccines using several strategies, including inherent nanoparticle adjuvancy, codelivery of antigen and adjuvant, targeted delivery to immune cells, enhanced immune cell uptake and cross-presentation, and cytosolic delivery.

1.3.2.1 Inherent Nanoparticle Adjuvancy

There is a wide variety of materials and structures that can be made into nanoparticles, and one strategy for the formulation of nanovaccines is to carefully choose a material that is naturally immunostimulatory. This can help to streamline nanoparticle fabrication by reducing the complexity of the final formulation. As an example, nanoparticles made of viral capsids naturally activate the immune system, largely due to the conservation of repetitive protein structures or the retention of nucleic acid-based PAMPs. These virus-like particles can engage TLRs in immune cells while delivering an antigenic payload. Even very lowly immunogenic tumor–associated antigens like idiotypic immunoglobulin from B cell lymphomas can elicit a strong humoral response when delivered by nanoparticles made of potato virus X coat proteins [103]. Other gel-like nanoparticles can be made by crosslinking materials that mimic the structure of PAMPs, such

as hydrophobic polymers [104], peptides [105, 106], or DNA [107], while also encapsulating antigens. d-tetra-peptide hydrogels in particular show promise as a vaccine adjuvant. Nanoformulations made by mixing irradiated tumor cells with a self-assembling hydrogel made of the d configuration of naphthylacetic acid-modified GFFY peptide were able to significantly protect mice from both E.G7 and 4T1 tumor challenges [108].

Immune responses to antigens can also be naturally boosted by carefully tuning their release over time. Nanogels are especially adept at this, as protein-to-polymer ratios can be precisely varied to change matrix spacing and cargo release rates [109, 110]. Some formulations have shown impressive sustained protein release, such as a PBAE layer-by-layer microparticle that extended release half-life from 4.9 to 143.9 h [111], or a hyaluronic acid-based nanogel that released proteins for over one week in rats [112]. Antigen delivery can be further improved by modifying nanogels to be retained at the immunization site, promoting sustained release of the payload in the presence of immune cells [113]. Polymeric nanoparticles can also provide sustained protein release profiles, as in the case of a poly(lactic-*co*-glycolic acid) (PLGA)-based formulation that was shown to release OVA protein for over a week [114]. When modified to carry gp100 or B16-F10 lysate, the same particles could produce approximately threefold greater T cell activation compared to equivalent doses of protein in soluble form, and this resulted in superior B16-F10 tumor suppression.

1.3.2.2 Codelivery of Antigen and Adjuvant

In general, delivery of antigens alone is not enough to trigger a strong immune response, requiring the use of an adjuvant to boost immune activation. For example, OVA antigen conjugated to poly(propylene sulfide) nanoparticles showed no anti-OVA immune response in

mice, but high levels of dendritic cell maturation and OVA-specific T cell generation were observed when the same particles were delivered along with an administration of CpG, resulting in protection against influenza–OVA challenge [115]. Furthermore, vaccines generally work the best when the antigen and adjuvant are delivered concurrently to the same APC, which can be readily accomplished using nanoparticle-based systems. This idea was shown systematically with a model cancer vaccine consisting of a polymeric nanoparticle loaded with an OVA peptide and the TLR7/8 agonist R848 [116]. Administration of a nanoparticle encapsulating both the payloads resulted in higher anti-OVA IgG production compared to either component in free form, one component in free form and the other encapsulated, or both the components encapsulated separately. In addition, codelivery of both the components together enhanced downstream T cell–mediated lysis of OVA-expressing cells and elicited increased local cytokine production. Many platforms have been designed for the codelivery of antigen and adjuvant together, including inter-bilayer-crosslinked multilamellar vesicles loaded with OVA antigen inside and MPLA interspersed throughout their lipid bilayers [117]. Immunization with this formulation led to an impressive 28% of $CD8^+$ T cells exhibiting OVA specificity, which was 14 times greater than observed when using soluble OVA and MPLA. These specific T cells also retained their functionality, as shown by high IFNγ production upon restimulation with OVA *ex vivo*.

When vaccinating against a heterogenous target like cancer cells, multiepitope vaccine formulations can be employed to prevent immune escape and tumor recurrence [20]. Modular vaccine designs, exemplified by recent work describing designer nanodisks [118], can help overcome this barrier. Synthetic high-density lipoprotein nanodisks were mixed with cholesterol-modified CpG ODN for immunogenicity and further functionalized with cysteine-modified, tumor-specific neoantigens for specificity. Mice immunized with nanodisks harboring a

combination of three antigens experienced an expansion in their pool of antigen-specific $CD8^+$ and $CD4^+$ T cells when compared to those receiving soluble formulations. The multiantigen formulation also showed significantly better control of B16-F10 tumor growth compared to single-antigen or dual-antigen formulations. Impressively, when mice were vaccinated in combination with anti-PD1 and anti-CTLA4, 90% were cured of their tumor burden.

1.3.2.3 Immune Cell Targeting

Due to the easy surface functionalization properties of nanoparticles, the efficacy and efficiency of nanovaccines can be improved by including an immune cell targeting moiety. Vaccine processing mainly takes place in APCs, and thus the most common immune cells targeted are dendritic cells and macrophages. A variety of surface markers can be targeted, such as the C-type lectin mannose receptor (CD206) by the inclusion of mannose on the nanovaccine surface [71, 119, 120]. In one example, the targeting ability of mannose was examined, and it was observed that functionalization could increase particle uptake into bone marrow–derived dendritic cells [121]. Strong localized signal of a fluorescently labeled targeted nanovaccine was seen in the draining lymph nodes at 24 h, while particles without mannose started to lose signal as early as 12 h after injection. Other surface markers such as CD11c [122], scavenger receptor class B type 1 [123], DEC205 [124, 125], and macrophage galactose-specific C-type lectin [55] have also been commonly targeted.

1.3.2.4 Efficient Cytosolic Entry

Traditional cancer vaccines suffer from difficulty in entering the cytosol of immune cells. Cytosolic entry can help to facilitate the presentation of antigens by MHC-I and subsequent

mobilization of $CD8^+$ effector T cells. In addition, there are several maturation pathways and pathogen recognition receptors located in the cytosol that can be leveraged to boost the potency of vaccine formulations. As most nanoparticles are taken up endosomally, there exist many strategies for facilitating endosomal escape. Due to the characteristic acidic environment of the endosomal compartment, redox-responsive nanovaccines can be used to achieve this goal. For example, some polymeric nanoparticles can act as proton sponges and induce lysosome swelling and rupture when encountering low pH environments [126]. Lysosomal rupture–triggered reactive oxygen species have also been shown to enhance proteasome activation, which can help to trigger MHC-I antigen presentation [55]. In one case, the common transfection agent, polyethylenimine, was coated onto the surface of antigen-loaded polymeric nanoparticles, and this helped to facilitate cross-presentation of the loaded antigen after uptake [127]. Similar reducible polymeric systems like poly(γ-glutamic acid) nanoparticles [128] and cationic dextran nanogels [129] have also shown a similar ability for facilitating MHC-I restriction. Besides endosomal escape, there are other ways to enter the cytosol from the endosomal compartment. OVA-loaded α-alumina nanoparticles can engage noncanonical autophagy, where antigens are diverted into autophagosomes and the delayed antigen degradation allows for increased cross-presentation [130]. By taking advantage of this process, significant levels of OVA-specific T cells could be induced, enabling mice to completely reject established B16-OVA tumors *in vivo*. In another strategy, nanoparticles can be designed to directly cross cell membranes by incorporating cell penetrating peptides onto their surfaces [131-133]. Macropinocytosis of lipid-coated nanovaccines has also been reported [134].

Cytosolic localization gives delivered antigens access to MHC-I presentation, but it can also be leveraged to enhance immune stimulation. Recent work has shown that retinoic acid-inducible protein 1 ligands and STING ligands may be stronger activators of the immune system

than traditional TLR-based adjuvants like CpG and MPLA [85, 135]. PC7A synthetic nanoparticles have been used to deliver antigen while simultaneously activating the STING pathway (Figure 1.3) [136, 137]. When loaded with OVA, the nanoformulation induced a threefold increase in antigen cross-presentation due to endosomal disruption by the redox-responsive PC7A. Once in the cytosol, the PC7A also engaged the STING receptor, resulting in higher immune activation compared to poly(I:C) or other polymeric nanoparticle groups. The combination of potent STING activation and efficient antigen cross-presentation led to significant antitumor efficacy against loaded antigens in B16-OVA, B16-F10, MC38, and TC-1 mouse tumor models.

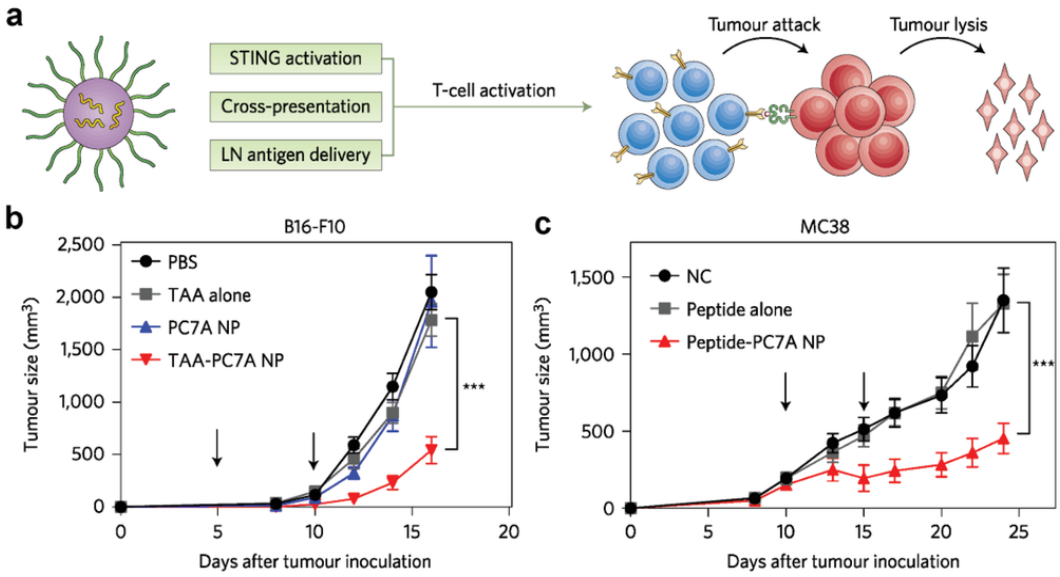

Figure 1.3: Synthetic nanoparticles activating the STING pathway for antitumor vaccination. a) Schematic depicting an antigen-loaded synthetic nanocarrier (PC7A) and its proposed mechanism of action. b,c) When used to treat tumor-bearing mice, antigen-loaded PC7A nanoparticles significantly enhanced survival in both a B16-F10 melanoma model (b) and an MC38 colon cancer model (c). Reproduced with permission. Copyright 2017, Springer Nature.

Instead of delivering antigens directly to the cytosol, some recent work has also focused on delivery of antigen-encoding RNA for *in situ* transcription and antigen production [120, 134, 138]. Acid-dissolvable calcium phosphate nanoparticles carrying messenger RNA (mRNA)

encoding the tumor-associated tyrosinase-related protein 2 (TRP2) could elicit stronger antigen-specific T cell responses and humoral responses against B16-F10 melanoma compared to peptide delivery [120]. In addition, PDL1 siRNA could be delivered to directly downregulate PDL1 in dendritic cells to reduce immunosuppression. Cytosolic delivery of both the mRNAs was shown to have a potent antitumor effect, significantly better than cytosolic delivery of either component alone. This strategy of RNA antigen sourcing has also been implemented using a highly modular RNA–lipoplex platform [134]. RNA-containing lipoplexes were optimized to target the spleen by modifying the charge ratios of the components, and the resulting formulation was shown to be taken up into the cytoplasm of dendritic cells and macrophages via macropinocytosis. The nanoparticles also induced TLR7-triggered IFNα production and IFN-α/β receptor–dependent activation of APCs. Introduction of antigen-encoding RNA induced generation of functional antigen-specific T cells and memory cells, which resulted in potent antitumor efficacy in several tumor models. Moving toward clinical translation, three human patients with advanced malignant melanoma received five doses of the nanovaccine encoding for four tumor antigens. All three patients showed systemic IFNα production, along with de novo priming and amplification of T cells against the vaccine antigens.

1.3.2.5 Artificial Antigen Presentation

Most cancer vaccines work by manipulating APCs, which can then further stimulate antigen-specific T cells and B cells. Recently, there has been significant interest in developing artificial APCs (aAPCs) that are capable of directly stimulating effector cells [139]. This strategy was originally developed in order to effectively expand T cells *ex vivo* for adoptive cell therapies such as CAR T cell therapy [140]. These aAPCs, which include both live cell–based and synthetic

micro-/nanoparticle-based platforms, mimic professional APCs and can strongly activate T cells while avoiding the intensive labor, high cost, and difficulty in quality control when using autologous APCs. Similar to their natural counterparts, aAPCs require at least two signals to induce T cell activation. The first signal, a peptide–MHC complex, binds to its cognate T cell receptor (TCR) and establishes antigen specificity. To become fully activated, T cells require a second signal in the form of costimulatory molecules such as CD80 and CD86, which engage their corresponding receptor on the T cell surface [139]. With these two signals, aAPCs have the potential to act as a vaccine-like platform that can expand antigen-specific T cell populations, but without the use of immunological adjuvants. In addition to the minimum two signal requirement, at times a third signal, in the form of soluble cytokines, can further enhance the survivability of the activated T cells [141].

To generate nanoscale aAPCs capable of engaging and activating T cells, MHC–Ig along with a costimulatory signal, in the form of CD80 or anti-CD28, has been decorated onto the surface of nanoparticles (Figure 1.4) [142]. When administered subcutaneously, nanoscale aAPCs exhibited greater lymphatic drainage compared with microscale aAPCs, which largely remained at the injection site. When administered into tumor-bearing mice that received adoptively transferred antigen-specific T cells, the nanoparticles were able to help significantly control tumor growth. It has also been demonstrated that aAPCs can be fashioned using magnetic nanomaterials [143]. After incubation with their cognate T cells, these magnetic aAPCs helped to induce significant proliferation and could also guide the T cells to tumors with the use of a magnetic field. In the future, such a platform may be directly used *in vivo* to promote antitumor activity. Interestingly, it has been found that the shape of nanoscale aAPCs can have a significant impact on their biological activity [144]. Ellipsoid nanoparticles were fabricated by stretching spherical

PLGA nanoparticles, followed by conjugation with anti-CD28 and MHC–Ig loaded with a gp100 tumor antigen epitope. After intravenous injection, it was observed that the ellipsoid particles could induce more antigen-specific T cells in circulation compared with their spherical counterparts. Although there are currently limited examples of nanoparticulate aAPCs being used *in vivo*, this nanovaccine-like platform holds significant potential given its ability to help bypass the complicated processes of antigen processing and presentation.

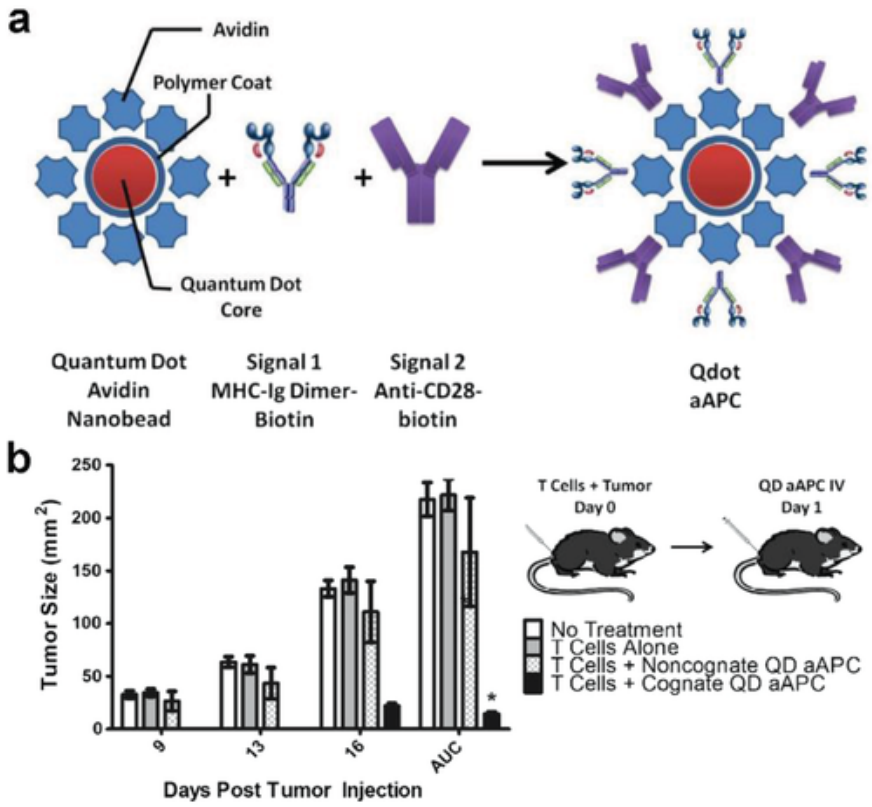

Figure 1.4: Quantum dot (QD) nanoparticles for artificial antigen presentation. a) Schematic depicting the artificial antigen presenting cell (aAPC) structure, where both the signals are attached to the nanoparticle surface using biotin–avidin interactions. b) When injected intravenously into B16 tumor–bearing mice that were also adoptively transferred with antigen-specific T cells, the aAPCs were able to significantly control tumor growth. Reproduced with permission. Copyright 2014, Elsevier Inc.

1.4 Cell Membrane-Coated Nanovaccines

1.4.1 Background

As discussed thus far, nanoparticle technology has the potential to significantly alter the landscape of anticancer vaccination, enabling the design of new nanovaccines with improved efficacy compared with traditional formulations. More recently, there has been a noticeable paradigm shift within the field of nanomedicine in which a greater emphasis has been placed on biomimetic design principles [145-148]. Along these lines, a new cell membrane coating approach has emerged in which nanoparticles are cloaked with a layer of cell-derived membrane [149-151]. In contrast to traditional bottom-up synthetic strategies, top-down membrane coating directly leverages naturally occurring biological material for the fabrication of multifunctional nanoparticles. Using red blood cells (RBCs) as the source of membrane material, it was demonstrated that RBC membrane–coated nanoparticles gained the ability to avoid immune clearance and circulated for extended periods of time (Figure 1.5) [152]. The cell-mimicking properties of these biomimetic nanoparticles result from the transference of the originating cell's membrane proteins onto the surface of the nanoparticle substrate [153]. This approach for functionalization has proven to be highly generalizable, allowing for the delivery of a wide range of cargoes using different types of materials for the inner core [154, 155]. The outer membrane layer can also be modified with further functionality by facile means, affording additional flexibility to membrane-coated platforms [54, 156].

Since the first work on RBC membrane–coated nanoparticles was reported, research on cell membrane coatings has expanded in multiple directions. In addition to modulating the material

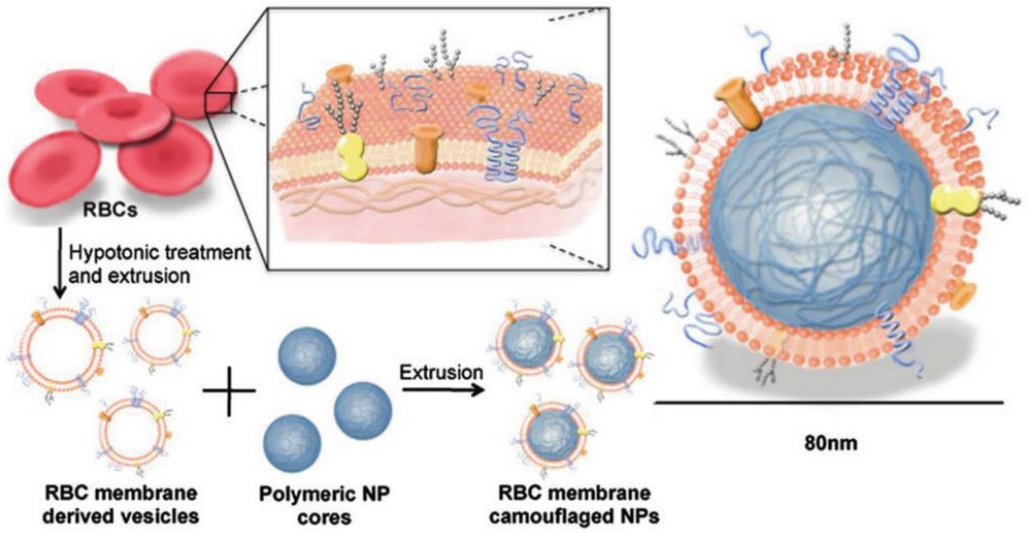

Figure 1.5: Functionalization of nanoparticles with a cell membrane coating. Schematic depicting the fabrication of red blood cell (RBC) membrane–coated nanoparticles. RBC vesicles are obtained by hypotonic treatment, followed by coating onto polymeric nanoparticle cores using extrusion. The resulting membrane-coated nanoparticle exhibits a characteristic core–shell structure. Reproduced with permission. Copyright 2011, National Academy of Sciences.

composition of the inner core, the membrane can be sourced from a plethora of cell types, each resulting in unique formulations with novel properties. For example, platelet membrane–coated nanoparticles exhibit the ability to target bacteria and damaged vasculature [157, 158], while cancer cell membrane–coated nanoparticles can homotypically target cancer cells [159]. White blood cell membrane, with its various toxin and cytokine receptors, has utility for treating sepsis [160]. Other membrane-coated formulations have also been reported using stem cell membrane [161], endothelial cell membrane [162], and even hybrid membranes generated from multiple cell types [163]. As a result of all the complex functionalities that can be incorporated, this approach has enabled the resulting biomimetic nanoparticles to excel in nontraditional areas of nanomedicine. A major example is detoxification, where membrane-coated particles can act as nanosponges to neutralize toxins by taking advantage of their interactions with cell membranes [164-166]. By neutralizing these toxins and preventing them from attacking healthy cells, these nanoscale decoys have utility for the treatment of bacterial infections, animal envenoming, and

even exposure to chemical warfare agents. The ability of cell membrane–coated nanoparticles to bind and present multiple antigens, combined with the flexibility of choosing various core materials, has also made them suitable for vaccine design [23, 24].

1.4.2 Cell Membrane-Coated Nanoparticles for Antibacterial Vaccination

Overall, vaccines represent one of the most efficient methods of reducing the global health burden posed by bacterial infections [167]. Toxoid vaccination represents an effective means of disarming bacteria of their virulent proteins, making it harder for the pathogens to colonize their host. This strategy is currently used in the clinic to vaccinate against tetanus and diphtheria [168]. In order to make bacterial toxins safe for administration, they are generally inactivated with harsh chemical or heat treatments that can damage antigenicity and reduce vaccination efficacy. By contrast, RBC nanosponges have demonstrated the ability to naturally detain and neutralize bacterial toxins when the two are mixed together, forming what are referred to as nanotoxoids [167, 169]. Using methicillin-resistant *Staphylococcus aureus* (MRSA) and its major virulence factor α-hemolysin as a model system, the corresponding nanotoxoid was able to generate significant antitoxin titers, improving overall antibacterial immunity compared to a heat-denatured toxoid formulation [170]. While the control toxoid required 60 min of high heat exposure to achieve an acceptable safety profile, the nanotoxoid demonstrated excellent safety on a number of cell types at the outset. In animal models of both systemic and skin toxin burden, nanotoxoid vaccination on a prime with two boosts schedule resulted in almost complete protection. A later study also demonstrated the efficacy of this approach against live MRSA infection [171].

As the mechanism of toxin binding to membrane-coated nanoparticles relies on function rather than the specific structure of the toxin, the nanotoxoid platform can be easily generalized. To generate a multiantigenic nanotoxoid, RBC nanosponges were mixed with a crude hemolytic protein fraction isolated from MRSA culture (Figure 1.6) [172]. It was confirmed that the nanotoxoids contained several toxins on their surface, including α-hemolysin, γ-hemolysin, and Panton–Valentine leukocidin. Further, the nanotoxoids were found to be completely safe, whereas intense heat treatment of the hemolytic protein fraction could not completely abrogate its toxicity. When used as a vaccine, the multivalent nanotoxoids were capable of generating antibody titers against all of the aforementioned toxins, which helped to reduce bacterial burden upon live MRSA challenge. In addition to the nanotoxoid approach, another method of generating multiantigenic vaccines is to directly employ bacteria-derived membrane. Outer membrane vesicles (OMVs) are secreted from bacteria and are important in pathogenesis as well as cell-to-cell signaling [173]. Some vaccines employing OMVs as the antigenic material have already been used in the clinic, as is the case with a formulation against meningococcal infection [174]. OMVs are attractive for use as antibacterial vaccines because they often share a similar biochemical membrane profile with their parent cell [175]. The utility of OMVs can be further improved by coating the material around a nanoparticulate core. In one instance, *Escherichia coli* OMVs were coated onto small gold nanoparticles, which provided increased stability and size control compared with free OMVs [176]. Due to the ability to finely control their size, the membrane-coated particles efficiently localized to the lymph nodes, leading to strong and durable immune activation.

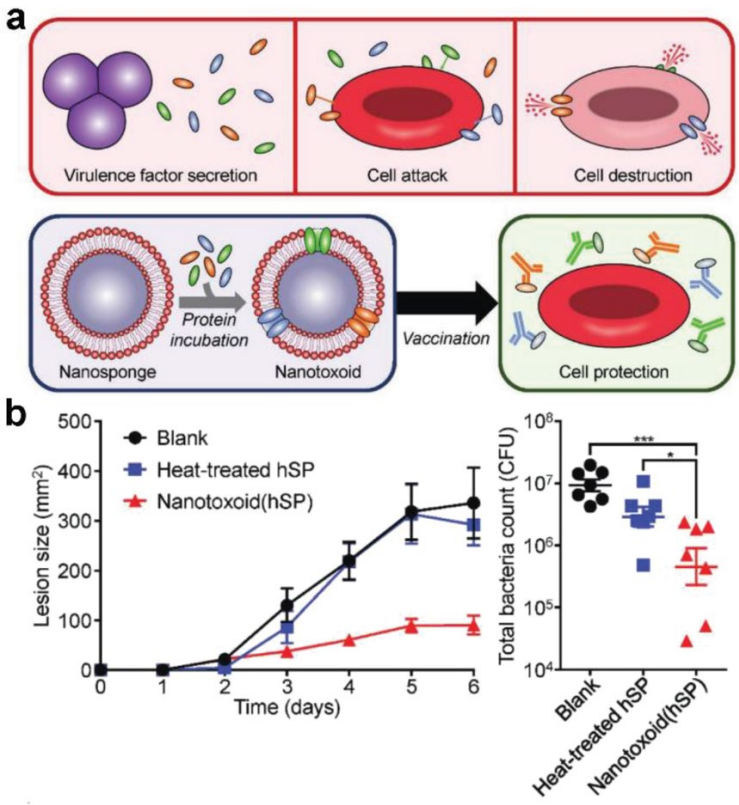

Figure 1.6: Membrane-coated nanoparticles for antibacterial vaccination. a) Schematic depicting the nanotoxoid concept, which can be used to develop vaccines against bacteria-secreted toxins. b) Vaccination using multiantigenic nanotoxoids fabricated with a hemolytic secreted protein (hSP) fraction from methicillin-resistant *Staphylococcus aureus* (MRSA) significantly inhibited lesion formation caused by subcutaneous MRSA challenge, leading to decreased bacteria counts. Reproduced with permission. Copyright 2017, Wiley-VCH.

1.4.3 Cell Membrane-Coated Nanoparticles for Anticancer Vaccination

As a whole, antibacterial vaccines have been extremely successful in reducing mortality rates related to infection. Unfortunately, the same level of clinical success has not been achieved for formulations against cancer. Recently, the extension of cell membrane–coated nanoparticles to anticancer vaccination has become an active area of research. In one example, an RBC membrane–based nanocarrier was designed to deliver a hgp100 tumor antigen peptide and the adjuvant MPLA [177]. The platform was further modified with mannose on the surface to better target dendritic

cells, and this led to enhanced localization to the draining lymph nodes. Both prophylactic and therapeutic efficacy were demonstrated in a B16-F10 subcutaneous tumor model, resulting in a slowing of tumor growth and a reduction in metastasis.

Since cancer cell membranes contain a plethora of autologous tumor antigens, utilizing the purified membrane of cancer cells as the antigenic material can be an effective approach in the design of nanoparticulate anticancer vaccines. This was initially demonstrated using B16-F10 melanoma membrane–coated nanoparticles incorporated with MPLA [159]. The formulation significantly increased the maturation of bone marrow–derived dendritic cells and enhanced the stimulation of antigen-specific T cells. More recently, an in-depth set of studies was conducted using a platform in which cancer cell membrane was coated around CpG ODN–loaded polymer cores (Figure 1.7) [178]. CpG ODN 1826, a potent TLR9 agonist in mice, was encapsulated into PLGA cores through a double emulsion process, and B16-F10 membrane was coated onto the adjuvant-loaded cores by bath sonication. When the formulation was administered subcutaneously into mice, increased maturation of dendritic cells in the draining lymph nodes was observed, as indicated by the upregulation of protein markers such as CD40, CD80, CD86, and MHC-II, when compared to various controls. Notably, CpG encapsulated in nanoparticulate form was able to activate the immune system significantly better than free CpG, likely due to the preferential cellular uptake of the nanoparticles [179-182]. Additionally, it should be noted that TLR9 is located within the endosomal compartment, which highlights the power of leveraging the inherent properties of nanoparticles to purposefully manipulate immune responses. Mice vaccinated with the nanovaccine were able to generate antigen-specific $CD8^+$ T cells against gp100 and TRP2, both of which are melanoma-associated antigens [183]. When immunized mice were challenged with B16-F10 cancer cells, 86% of the mice exhibited no tumor growth, even after 150 days. In a

therapeutic setting, it was demonstrated that the nanoformulation, along with a cocktail of anti-PD1 and anti-CTLA4 checkpoint inhibitors, was able to extend the survival of the tumor-bearing mice compared to either treatment alone.

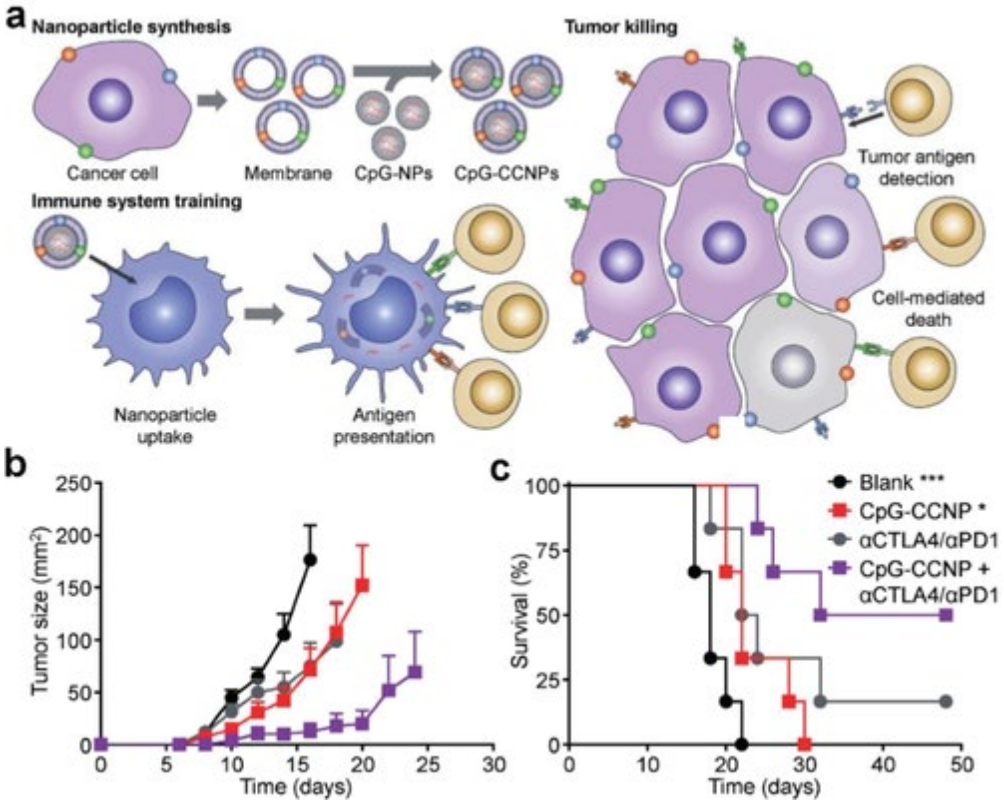

Figure 1.7: Membrane-coated nanoparticles for anticancer vaccination. a) Schematic depicting the fabrication of adjuvant-loaded cancer cell membrane–coated nanoparticles (CpG–CCNPs) and their proposed mechanism of action. b,c) When combined with a cocktail of checkpoint blockades (anti-CTLA4 and anti-PD1), treatment of established B16-F10 melanoma with the CpG–CCNP nanovaccine resulted in significantly slowed tumor growth (b) and improved survival (c). Reproduced with permission. Copyright 2017, Wiley-VCH.

Building upon the concept of using cancer cell membrane–coated nanoparticles for antitumor vaccination, various strategies have been employed to augment immune responses. For example, mannose was introduced to bestow immune cell–targeting properties, helping to enhance uptake by dendritic cells and subsequently promoting their maturation [184]. As a result of this additional functionality, the targeted nanovaccine was able to offer better protection for vaccinated mice. It was claimed that this triple combination of an adjuvant, cancer cell membrane antigens,

and a targeting ligand could work together to generate a robust anticancer immune response similar to levels generated against bacterial infections. In another example, immune stimulation was enhanced via the concurrent delivery of multiple adjuvants in an artificial cancer cell membrane–coated nanoparticle [185]. CpG-encapsulated calcium phosphate cores were fabricated by a water-in-oil microemulsion process and then coated with a membrane-mimicking liposome layer. Then, OVA-expressing B16-F10 cancer cell membrane proteins were purified by dialyzing the membrane against a detergent solution. The membrane proteins, along with the danger-associated molecular pattern Hsp70, were incorporated onto the nanoparticle surface to create the final formulation. This dual-adjuvant formulation was able to significantly upregulate maturation markers such as CD80, CD86, and MHC-II, and treated mice had fewer lung metastasis compared to formulations with just the CpG adjuvant. In all, the works described in this section demonstrate that cell membrane–coated nanoparticles have significant potential to be used as nanovaccines. Armed with the versatility to easily modulate both the adjuvant and the cancer membrane material, which can eventually be derived from a patient's own tumor, this platform may ultimately pave the way for potent, personalized anticancer vaccine therapies.

1.5 Conclusions

In this review, we have discussed the progress of using nanotechnology toward the design of cancer vaccines. In theory, vaccination represents an attractive option for cancer therapy, but in practice there are many challenges that need to be overcome in order for such platforms to achieve widespread clinical adoption. Generally, it is highly difficult for the immune system to generate a potent response against established tumors, which can employ various means to lower their

immunogenicity over time. With the help of nanoscale delivery vehicles, researchers are exploring the design of novel vaccine formulations that can elicit immune responses capable of overcoming tumor immunosuppression. Nanocarriers offer many advantages, including the effective localization of payloads to the desired immune cell populations, loading of multiple cargoes into a single nanoparticle, and prolonged release characteristics.

More recently, a novel type of biomimetic platform, the cell membrane–coated nanoparticle, has emerged as a strong candidate to drive the further improvement of nanovaccine platforms. Membrane coating presents a facile means of introducing multiple functionalities onto the same nanoparticle without the need for complicated synthetic techniques. Regarding anticancer vaccination, the use of cancer cell membrane as the coating material offers an approach for creating vaccine formulations rich in tumor antigens. Combined with a nanoparticulate core carrying potent immune stimulators and the ability to easily target the resulting nanoparticles to antigen presenting cells, cancer cell membrane–coated nanoparticles can achieve strong inhibition of tumor growth. These nanoformulations may be further improved through the continued optimization of adjuvant and membrane antigen combinations. Methods can also be developed for obtaining membrane material from the resected tumors of patients, enabling the facile fabrication of personalized vaccines.

Looking toward clinical translation, a main challenge will be scaling up nanoparticle production in an efficient and cost-effective manner. Nanoformulations will avoid many expenses required for live-cell vaccines, but there will likely need to be a substantial investment of time and resources to adapt current lab-scale manufacturing procedures to high-throughput workflows capable of production at the scale necessary for human patients. These workflows will also need to align with good manufacturing practices to meet quality requirements for regulatory approval.

Finally, significant work will also need to be done on evaluating the synergy between vaccines and other types of cancer therapies. By simultaneously tackling the challenge of cancer treatment on multiple fronts, it may one day be possible to eliminate tumors altogether, regardless of their underlying characteristics.

Chapter 1, in full, is a reprint of the material as it appears in *Advanced Biosystems*, 2019, Ashley Kroll, Yao Jiang, Jiarong Zhou, Maya Holay, Ronnie Fang and Liangfang Zhang. The dissertation author was the primary author of this paper.

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Chapter 2

Cancer Cell Membrane-Coated Nanoparticles for Anticancer Vaccination

2.1 Introduction

Recent successes in the field of immunotherapy have provided convincing evidence that, if properly stimulated, the immune system is capable of successfully battling a variety of cancer types [1-3]. Despite this fact, an effective anticancer vaccine that is widely applicable and facile to administer, while highly sought after, has continued to remain elusive [4, 5]. Fundamentally, the challenge lies in the need to generate potent and specific immune responses that enable the body to successfully distinguish between healthy and diseased tissue [6]. By the time a neoplastic growth reaches the malignant stage, the most immunogenic tumor-specific antigens have generally been eliminated via negative selection [7, 8]. Some promising strategies under clinical investigation have focused on common tumor-associated antigens, which are dysregulated wild-type proteins [9, 10]. However, the applicability of such single-antigen approaches is dependent on tumor phenotype, and they may also be subject to some of the same limitations facing targeted monotherapies as tumors evolve mechanisms of escape [11, 12]. On the other end of the spectrum, whole cell vaccine preparations are capable of delivering a wide range of autologous antigens [13, 14], but they have traditionally been ineffective. This may be a result of significant interference from a surplus of nontumor-related antigenic material [15] or difficulties in direct administration, which have necessitated more complex cell-based strategies [14, 16, 17]. Additionally, the immunosuppressive microenvironment of established tumors is often hard to break [18, 19], leading to suboptimal efficacy despite effective training of the immune system.

Advances in genomics have enabled the elucidation of individual cancer mutanomes, which can be leveraged to identify multiple vaccine epitopes on a personalized level [20, 21]. Other studies have demonstrated that high mutational burden can lead to neoantigen targets that

are recognized by the immune system, and this correlates with clinical response to checkpoint blockade therapies [22, 23]. While personalized epitope identification and vaccine manufacture may currently not be practical at large-scale, these findings confirm that, despite the challenges facing whole cell formulations, there is a wealth of relevant antigens to be found in autologous tumor material. Applying the principles of biomimetic nanotechnology [24-26], we explored the presentation of cancer-derived membrane material in a context that could enable potent, multiantigenic immune responses for anticancer vaccine design (Figure 2.1). It was demonstrated that nanoparticulate delivery of the membrane, along with an immunostimulatory adjuvant, could facilitate enhanced antigen presentation, leading to the activation of tumor-specific cellular responses. Further, when used in conjunction with checkpoint blockade therapy to help break tumor immunosuppression [27-29], the nanovaccine formulation was able to achieve significant control of tumor growth in a therapeutic setting.

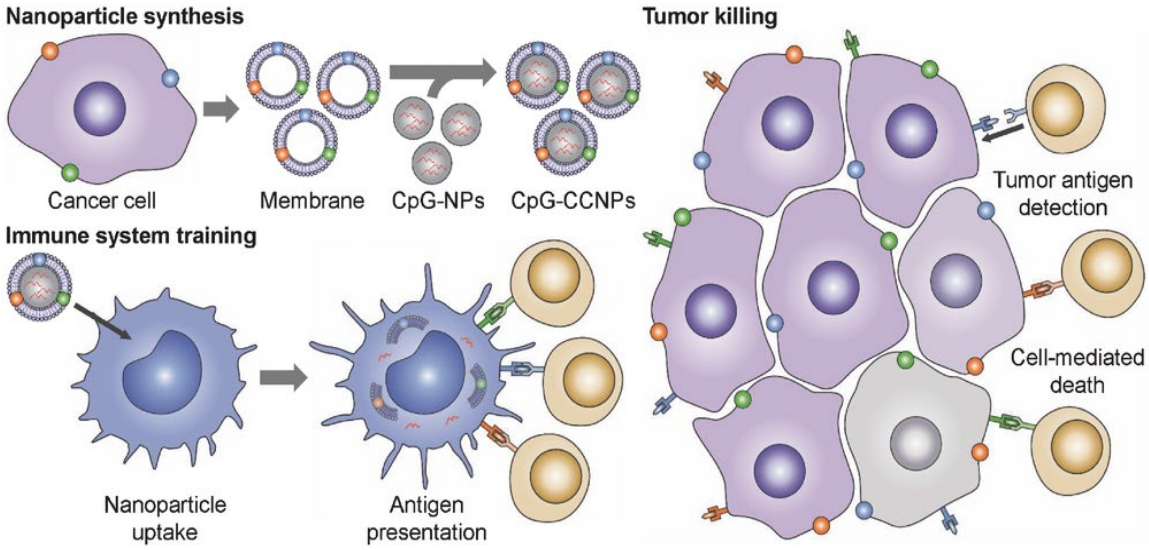

Figure 2.1: Schematic of CpG-CCNPs for anticancer vaccination. Membrane derived from cancer cells (purple), along with the associated tumor antigens (small colored spheres), is coated onto adjuvant-loaded nanoparticle cores (CpG-NPs) to yield a nanoparticulate anticancer vaccine (CpG-CCNPs). Upon delivery to antigen presenting cells (blue), the vaccine formulation enables activation of T cells (tan) with multiple specificities. After detecting the antigens present on the tumor, the T cells are capable of initiating cancer cell death (gray).

2.2 Experimental Methods

2.2.1 B16-F10 Murine Melanoma Cell Culture and Membrane Derivation

B16-F10 mouse melanoma cells (CRL-6475; American Type Culture Collection) were cultured at 37 °C with 5% CO_2 in T175 tissue culture flasks (Becton Dickinson) with Dulbecco's Modified Eagle Medium (DMEM; Mediatech) supplemented with 10% bovine growth serum (Hyclone) and 1% penicillin-streptomycin (Gibco). At 80–90% confluency, ≈16–18 million cells per flask were collected in phosphate buffered saline (PBS; Mediatech) by scraping, pelleted at 700 × *g* for 7 min in a Sorvall Legend Micro21R centrifuge, then resuspended in a 50:50 solution of cryopreservation medium (Hyclone) and complete DMEM. Cell aliquots were stored at −20 °C before use. To derive membrane, cells were first washed in a starting buffer containing 30 × 10^{-3} m Tris-HCl pH 7.0 (Quality Biological) with 0.0759 m sucrose (Sigma-Aldrich) and 0.225 m D-mannitol (Sigma-Aldrich), then mechanically disrupted in the presence of phosphatase inhibitor and protease inhibitor cocktails (Sigma-Aldrich) using a Kinematica Polytron PT 10/35 probe homogenizer at 70% power for 15 passes. Membrane was separated from the resulting homogenate by differential centrifugation using a Beckman Coulter Optima L-90K Ultracentrifuge. Homogenate was pelleted at 10 000 × *g* for 25 min, and the supernatant was then pelleted at 150 000 × *g* for 35 min. The resulting pellet of cell membrane was washed in 0.2 × 10^{-3} m ethylenediaminetetraacetic acid (EDTA; USB Corporation) in DNase free/RNase free water (Invitrogen) and stored in the same solution at −20 °C until use. Total membrane protein content was quantified by a BCA protein assay kit (Pierce).

2.2.2 Cancer Cell Membrane-Coated Nanoparticle Preparation and Characterization

Polymeric cores were prepared using 0.18 dL g^{-1} carboxyl-terminated 50:50 poly(lactic-*co*-glycolic) acid (PLGA; LACTEL Absorbable Polymers) using a double emulsion process. PLGA was dissolved in dichloromethane at a concentration of 50 mg mL^{-1}. 500 μL of polymer was added to 100 μL of 200 × 10^{-3} m Tris-HCl pH 8 and sonicated using a Fisher Scientific 150E Sonic Dismembrator at 70% power pulsed (2 s on/1 s off) for 1 min. An outer aqueous phase consisting of 5 mL of 10 × 10^{-3} m Tris-HCl pH 8 was added to the polymer solution and sonicated at the same setting for 2 min. The emulsion was then added to 10 mL of 10 × 10^{-3} m Tris-HCl pH 8 and magnetically stirred at 700 × *g* for 2.5 h. After stirring, the particles were pelleted at 21 100 × *g* for 8 min, and washed twice in 10 × 10^{-3} m Tris-HCl pH 8. Adjuvant-loaded polymeric cores (CpG-NPs) were made by including CpG oligodeoxynucleotide 1826 (CpG), synthesized using the sequence 5′-TCCATGACGTTCCTGACGTT-3 with all phosphorothioate bonds (Integrated DNA Technologies), at 500 × 10^{-6} m to the inner phase of the double emulsion during nanoparticle synthesis. To optimize the loading, CpG-NPs were made with CpG inputs of 250, 500, 1000, and 2000 pmol per 1 mg of PLGA. Each formulation was lyophilized overnight, then resuspended in 1 mL of acetone. PLGA was precipitated and pelleted with the addition of 1 mL water followed by centrifugation at 21 100 × *g* for 20 min. CpG concentration of the supernatants were measured using a Quant-iT Oligreen ssDNA quantification kit (Invitrogen) according to manufacturer's instructions. Further studies employed an initial input of 1000 pmol CpG per 1 mg of PLGA.

B16-F10 cancer cell membrane-coated CpG-NPs (CpG-CCNPs) were made by pelleting the CpG-NP cores and resuspending them in solution containing B16-F10 cell membrane. The

mixture was sonicated in a 1.5 mL disposable sizing cuvette (Brandtech) using a Fisher Scientific FS30D bath sonicator at a frequency of 42 kHz and a power of 100 W for 2 min. The nanoparticles were washed twice in 10×10^{-3} m Tris-HCl pH 8, and resuspended to a concentration of 25 mg polymer per 1 mL of solution in 5×10^{-3} m Tris-HCl pH 7.5 and 0.2×10^{-3} m EDTA in DNase free/RNase free water for *in vitro* studies or in 10% sucrose with the same buffer concentrations for *in vivo* studies. If not used immediately, particles were stored at −20 °C. In the study, CpG-CCNPs were fabricated with 100 μg of membrane protein per 1 mg of PLGA. Size and surface zeta potential of CCNPs were determined through DLS measurements using a Malvern ZEN 3600 Zetasizer. To test the stability of CCNPs in 10% sucrose solution, particles were stored at 4 °C for 2 weeks with size measured by DLS every other day. The morphology of CCNPs was examined by transmission electron microscopy using a Zeiss Libra 120 PLUS EF-TEM. Samples were resuspended in 10×10^{-3} m Tris-HCl pH 8, deposited onto a glow discharged carbon-coated 400 square mesh copper grid (Electron Microscopy Sciences), and negatively stained with 1 wt% uranyl acetate (Electron Microscopy Sciences).

2.2.3 Membrane Antigen Retention

Identification of characteristic B16-F10 tumor antigens was completed via western blotting. B16-F10 whole cells were collected from culture by scraping, lysed using 0.2% Triton X-100 (Sigma-Aldrich) in water, and sonicated. B16-F10 lysed cells, B16-F10 membrane, and CpG-CCNPs were analyzed for protein content using a BCA assay, then each diluted to 0.2 mg mL^{-1} in water. Each sample was then mixed with NuPAGE 4 × lithium dodecyl sulfate sample loading buffer (Novex) and heated for 10 min at 70 °C. 25 μL of each sample was loaded into 12-well Bolt

4–12% Bis-Tris gels (Invitrogen) and run at 165 V for 45 min in 3-(*N*-morpholino)propanesulfonic acid running buffer (Novex). Proteins were transferred to 0.45 μm nitrocellulose membrane (Pierce) in Bolt transfer buffer (Novex) at 10 V for 60 min. After blocking with 5% milk (Genesee Scientific) in PBS with 0.05% Tween 20 (National Scientific), blots were immunostained with mouse antimouse gp100 (EP4863(2); Abcam), rabbit antimouse TRP2 (E-10; Santa Cruz Biotechnology), or mouse antimouse MART1 (A103; Santa Cruz Biotechnology). The appropriate horseradish peroxidase-conjugated secondary (Biolegend) was used for secondary staining. Membranes were developed with ECL western blotting substrate (Pierce) in an ImageWorks Mini-Medical/90 Developer.

2.2.4 *In Vitro* Uptake and Activity

All animal studies were designed and proceeded in compliance to the University of California, San Diego Institutional Animal Care and Use Committee. Female C57BL/6NHsd mice were obtained at 6–10 weeks old from Envigo Harlan. BMDC culture was adapted from a previously published protocol [25]. Healthy mice were euthanized using carbon dioxide asphyxiation followed by cervical dislocation. Both femurs were dissected, cleaned in 70% ethanol, and cut on both ends. Bone marrow was then flushed out of the bone with a 1 mL sterile syringe using warm BMDC basal media consisting of 500 mL Isocove's Modification of DMEM with 2×10^{-3} m L-Glutamine and 25×10^{-3} m 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Mediatech) supplemented with 50 mL USDA certified fetal bovine serum (Omega Scientific), 500 μL 55×10^{-3} m β-mercaptoethanol (Gibco), 5 mL 200×10^{-3} m L-Glutamine (Gibco), and 5 mL penicillin-streptomycin. Cells were then pelleted at $700 \times g$ for 5 min,

resuspended in BMDC growth media, consisting of the basal media further supplemented with 10 ng mL^{-1} granulocyte/macrophage-colony stimulating factor (GM-CSF; Biolegend), to a concentration of 1×10^6 cells mL^{-1}, and plated into petri plates at 2×10^6 cells per plate. On the third day of culture, 10 mL of BMDC growth media was added to each plate.

To make CpG-CCNPs with fluorescently labeled polymeric cores, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD, ex/em = 644/663 nm; Biotium) was added to the PLGA solution at 0.1 wt% of the polymer during nanoparticle synthesis. For the nanoparticle uptake study, BMDCs were collected on day 5 using 1×10^{-3} mEDTA in PBS. Cells were washed once in PBS, resuspended in BMDC basal media, and plated into 24-well suspension plates. DiD-labeled CpG-CCNPs were added at a final concentration of 1.4 mg mL^{-1}. At each timepoint (0, 15, 30 min, 1, 2, 6, 12, 24 h), media was removed, and the cells were detached with trypsin-EDTA (Gibco). Cells were collected, washed once in trypsin-EDTA, washed twice in PBS, and resuspended in 200 μL of 10% phosphate buffered formalin (Fisher). The adjuvant uptake study was conducted similarly, instead employing CpG-CCNPs synthesized with CpG containing a 5′ 6-FAM modification (Integrated DNA Technologies). Free dye-labeled CpG was used at an equivalent concentration for comparison. For all experiments, after each time point was collected and processed, 1 drop of NucBlue Live ReadyProbe Reagent UV stain (Molecular Probes) was added and data were collected using a Becton Dickinson FacsCanto-II flow cytometer. All data were analyzed using FlowJo software.

The activity of delivered CpG was examined using a BMDC cytokine release assay. BMDCs were plated on day 6 into 96-well plates at a concentration of 8×10^4 cells mL^{-1} in BMDC growth media. Dilutions of CpG-CCNP or free CpG were added to the cells. After 2 h of incubation, the cells were washed three times with fresh BMDC growth media and cultured for

another 2 d. Supernatant was then collected and measured for the presence of proinflammatory cytokines using mouse IL-6 and IL-12p40 ELISA kits (Biolegend) according to manufacturer's instructions.

Antigen and adjuvant colocalization was visualized by imaging BMDCs incubated with dual-labeled CpG-CCNPs. B16-F10 membrane was labeled using CF647 succinimidyl ester dye (Biotium) and used to coat CpG-CCNPs fabricated with FAM-modified CpG. BMDCs were seeded into 8-well chamber slides at 7.5×10^4 cells mL^{-1} and incubated with the nanoparticles for 15 min at 0.7 mg mL^{-1}. Cells were then washed three times with PBS, fixed with 10% formalin for 30 min, then washed again three times with PBS and mounted onto coverslips using VECTASHIELD mounting media with DAPI (Vector Laboratories). Samples were imaged on a Deltavision RT Deconvolution Microscope at 60 × magnification.

2.2.5 *In Vivo* Cellular Localization and Dendritic Cell Activation

To assess *in vivo* localization, DiD-labeled CpG-CCNPs were injected subcutaneously into each hock of female C57BL/6NHsd mice. After 24 h, the popliteal lymph nodes were collected into 500 µL of dissociation buffer consisting of 1 mg mL^{-1} collagenase D from *Clostridium histolyticum* (Roche) and 1 mg mL^{-1}DNase I grade II, from bovine pancreas (Roche) in Dulbecco's PBS with calcium and magnesium (Gibco). Lymph nodes were dissociated manually by pipetting and then were stained with fluorescein isothiocyanate (FITC)-labeled antibodies for dendritic cells (antimouse CD11c, N418; Biolegend), macrophages (antimouse F4/80, BM8; Biolegend), T cells (antimouse CD3, 17A2; Biolegend), B cells (antimouse CD19, 6D5; Biolegend), and granulocytes (antimouse Ly-6G/Ly-6C, RB6-8C5; Biolegend) for 30 min. Appropriate dye-labeled antibody

isotypes (Biolegend) were used for gating purposes with cells from an untreated lymph node. After washing, dead cells were labeled with propidium iodide (Biolegend). Data were collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo software.

Dendritic cell activation following immunization with CpG-CCNPs, CpG-NPs, CCNPs, or additional controls was determined by testing dendritic cell maturation and lymph node cytokine secretion. To test vaccines with antigens and adjuvants delivered as separate components, additional controls of CCNP with free CpG and B16-F10 whole lysate with free CpG were also administered. The CCNPs with free CpG formulation was made by mixing the two components such that the final ratio was 25 mg of PLGA per 3.5 nmol of CpG. Whole cell lysate was prepared by three freeze-thaw cycles at −80 °C for 10 min followed by 10 min at 37 °C. The amount of protein used for the formulation was normalized by the amount of Na^+K^+-ATPase, a characteristic membrane protein, compared with CCNPs as determined by immunoblotting. To examine dendritic cell maturation *in vivo*, 50 μL of each formulation at 25 mg mL^{-1} of nanoparticle, or equivalent, was injected into the hock. After 24 h, the popliteal lymph nodes of all treated mice were collected into 500 μL dissociation buffer and manually dissociated. Cells were stained using FITC antimouse CD11c with either Alexa647-conjugated antimouse CD40 (HM40-3; Biolegend), CD80 (12-10A1; Biolegend), CD86 (GL-1; Biolegend), or MHC-II (M5/114.15.2; Biolegend). Appropriate dye-labeled antibody isotypes (Biolegend) were used for gating purposes with cells from an untreated lymph node. After 30 min of incubation at 4 °C, the cells were washed and stained with CellTrace Calcein Violet, AM (Molecular Probes) in PBS according to manufacturer's instructions. Data were collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo software. To analyze cytokine production, lymph node-derived single cell suspensions were plated with 500 μL of BMDC growth media in 24-well tissue culture plates.

After 48 h, supernatant was collected and analyzed for cytokine content using IL-6 and IL-12p40 ELISA kits according to the manufacturer's instructions.

2.2.6 Adoptive T Cell Proliferation and Native T Cell Generation

B6.Cg-Thy1^{a}/Cy Tg(TcraTcrb)8Rest/J (pmel-1) transgenic mice were obtained from the Jackson Laboratory at 4–6 weeks old. The spleen, popliteal lymph node, and inguinal lymph nodes of one pmel-1 mouse were collected for dissociation into single cell suspensions. The red blood cells in the spleen were removed using lysis buffer (Biolegend), and all remaining cells were pooled together. CD8+ T cells were separated out using CD8a (Ly-2) microbeads (Miltenyi Biotec) on Miltenyi Biotec MACS LS separation columns per manufacturer's instructions. After separation, cells were washed in PBS and stained with carboxyfluorescein succinimidyl ester (CFSE; eBiosciences). Cells were then diluted to 2.5×10^{6} cells mL^{-1} and 200 μL was transferred to naïve C57BL/6NHsd recipients. 2 h postinjection, each mouse was injected with 50 μL of various vaccine formulations in both hocks. 4 d after treatment, the spleens were collected and dissociated into single cell suspensions. Adoptively transferred T cells were stained for using allophycocyanin (APC)-conjugated antimouse CD8a (53-6.7; Biolegend) and Pacific Blue-conjugated antimouse CD90.1 (OX-7; Biolegend). Data were collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo software. CFSE dilution was used to assess the degree of T cell proliferation.

To assess the native generation of antigen-specific T cells, C57BL/6NHsd mice were vaccinated subcutaneously with 50 μL of the different formulations in each hock on days 0, 2, and 4. On day 10, spleens were collected and processed into single cell suspensions using mechanical

dissociation. After lysing the red blood cells, 5×10^6 splenocytes were plated into 6-well suspension plates and pulsed with either 1 μg mL^{-1} of mouse gp100 peptide with sequence EGSRNQDWL (Anaspec) or 1 μg mL^{-1} of TRP2 peptide with sequence SVYDFFVWL (Anaspec) in BMDC growth media. After 7 d, cells were collected, washed in PBS, and stained with APC-conjugated antimouse CD8a and either phycoerythrin (PE)-labeled H-2Db gp100 tetramer (MBL International) or H-2Kb TRP2 tetramer (MBL International). Data were collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo software.

2.2.7 *In Vivo* Immunity and Therapeutic Efficacy

To study the protection conferred by vaccination, C57BL/6NHsd mice were vaccinated with 50 μL of the different formulations at 25 mg mL^{-1} of PLGA, or equivalent, on days 0, 7, and 14. On day 20, the right flank of each mouse was shaved and, on day 21, mice were challenged with 2×10^5 B16-F10 cells subcutaneously on the right flank. Tumors were measured every other day and the experimental endpoint was defined as either death or tumor size greater than 200 mm^2.

To study the antitumor therapeutic effect, C57BL/6NHsd mice were first challenged on the right flank with 5×10^4 B16-F10 cells on day 0. On days 1, 2, 4, and 7, mice were vaccinated subcutaneously in the same flank with 200 μL of the nanoparticulate formulations. The subcutaneous route was chosen in this case to accommodate the larger dosage that was employed. The checkpoint blockade cocktail, consisting of 100 μg anti-CTLA4 (9H10; BioXCell) and 200 μg anti-PD1 (RMP1-14; BioXCell) was administered intraperitoneally on the same days. Tumors were measured every other day and the experimental endpoint was defined as either death or tumor size greater than 200 mm^2.

2.3 Results and Discussion

CpG oligodeoxynucleotide 1826 (CpG), a nucleic acid-based immunological adjuvant known to trigger the maturation of antigen presenting cells, was encapsulated into biodegradable poly(lactic-*co*-glycolic acid) (PLGA) nanoparticle cores via a double emulsion process (Figure 2.2a). The amount of CpG that could be loaded started saturating at an initial input of 1 nmol per 1 mg of PLGA, and ≈100 pmol of the adjuvant could be loaded at this ratio. To introduce tumor antigen material, the membrane derived from B16-F10 mouse melanoma cells was coated onto CpG-loaded PLGA cores (CpG-NPs). The process used for coating did not significantly alter the amount of adjuvant within the polymeric cores. Dynamic light scattering (DLS) measurements showed an increase in nanoparticle size after coating, and the zeta potential of the adjuvant-loaded, cancer cell membrane-coated nanoparticles (CpG-CCNPs) increased to approximately that of pure membrane (Figure 2.2b,c). Successful coating was confirmed by transmission electron microscopy (TEM), which revealed a characteristic core–shell structure (Figure 2.2d). Over time, the CpG-CCNPs stayed stable in solution (Figure 2.2e). Importantly, the presence of known membrane-bound tumor-associated antigens [30], including MART1, TRP2, and gp100, was confirmed by western blotting (Figure 2.2f). When normalized by total protein amount, significant antigen enrichment was observed on the derived membrane and CpG-CCNPs when compared with whole cell lysate.

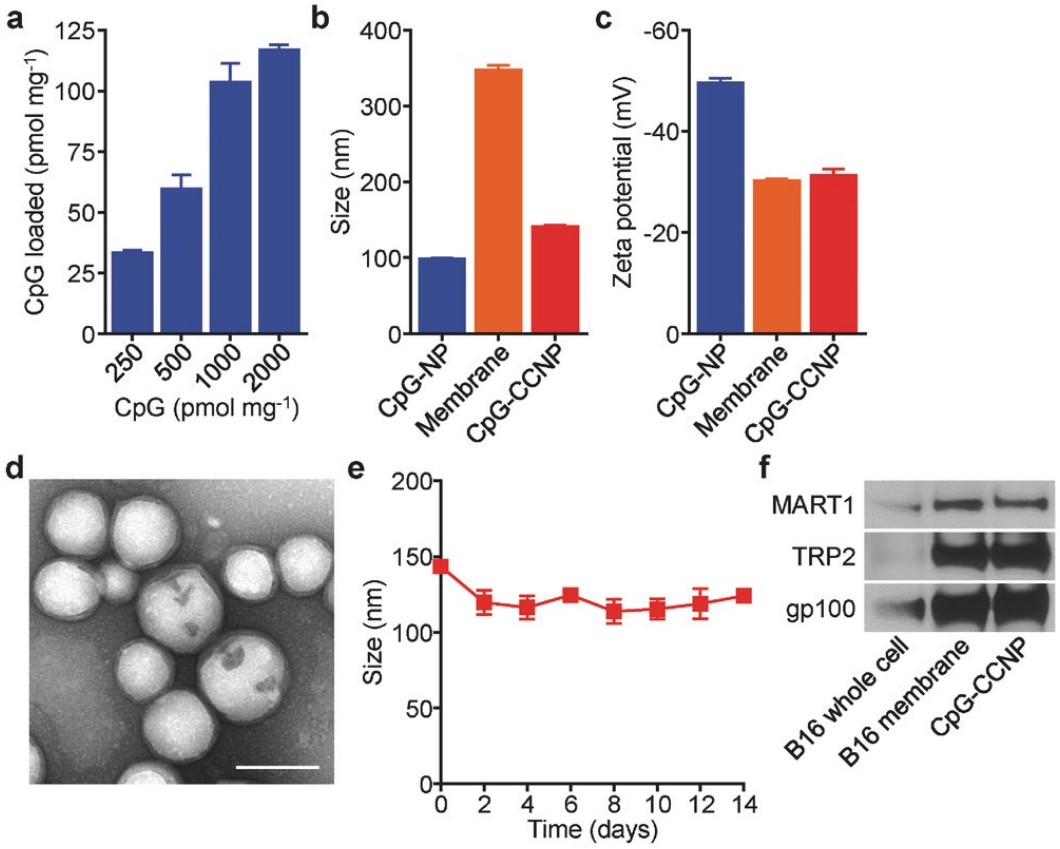

Figure 2.2: Preparation and characterization of CpG-CCNPs. a) CpG encapsulation into PLGA cores with increasing inputs, normalized by polymer weight (n = 3, mean ± SD). b) Size of CpG-NPs, B16-F10 membrane vesicles, and CpG-CCNPs (n = 3; mean ± SD). c) Surface zeta potential of CpG-NPs, B16-F10 membrane vesicles, and CpG-CCNPs (n = 3; mean ± SD). d) TEM image of CpG-CCNPs negatively stained with uranyl acetate. Scale bar = 100 nm. e) Size stability over time of CpG-CCNPs stored in 10% sucrose (n = 3; mean ± SD). f) Western blots for known melanoma-associated antigens MART1, TRP2, and gp100 on B16-F10 cells, B16-F10 membrane, and CpG-CCNPs.

To study the interaction of the nanoformulation with antigen presenting cells, bone marrow-derived dendritic cells (BMDCs) were employed. When incubated with dye-labeled CpG-CCNPs, quick uptake was observed until saturation was achieved at ≈6 h (Figure 2.3a). CpG is known to activate proinflammatory responses in antigen presenting cells [31], which is necessary for generating potent antitumor immunity. Using a fluorescently tagged CpG, the adjuvant was shown to much more readily be internalized by BMDCs when encapsulated within the membrane-coated nanoparticles, which are in the ideal size range for endocytosis [32, 33] (Figure 2.3b). To test the implications of this enhanced internalization and confirm the integrity of CpG after

encapsulation, the biological activity of CpG in free form versus nanoparticulate form was assessed (Figure 2.3c,d). Secretion of two representative proinflammatory cytokines, interleukin-6 (IL-6) and IL-12, was significantly enhanced for the CpG-CCNP formulation, which was approximately an order of magnitude more immunostimulatory than free CpG. This effect is likely due to the fact that nanoparticulate CpG more readily localizes to the endosomal compartment during uptake, where it can engage its endosomal recognition site on toll-like receptor 9 (TLR-9) [34]. It should be noted that CCNPs without adjuvant induced significantly less cytokine secretion when incubated with BMDCs at equivalent nanoparticle concentrations. While the CpG employed in the studies here was murine-specific, other variants could easily be substituted to promote immunity in humans [35]. Further, the integrity of the nanoparticle structure was assessed by fluorescent imaging using dye-labeled CpG and membrane protein, and significant colocalization of the two signals confirmed the ability of the CpG-CCNPs to co-deliver both adjuvant and antigen to the same BMDC (Figure 2.3e). Upon *in vivo* administration subcutaneously via the hock, the nanoformulation could easily be detected at the draining lymph node after 1 h, with some appearing at an adjacent node after 24 h. Little signal was observed at the spleen given its considerable distance from the injection site. Within the draining lymph node, antigen presenting cells such as dendritic cells and macrophages exhibited the highest percentage of nanoparticle uptake; B cells and granulocytes also displayed some uptake, while the limited amount of signal observed for T cells was likely the result of nonspecific interactions with the nanoformulation (Figure 2.3f).

The effect of the nanoformulation on BMDC maturation *in vitro* was studied by looking at the upregulated expression of costimulatory markers CD40, CD80, and CD86, as well as MHC-II. Consistent with the fact that the dendritic cell maturation process is largely driven by the detection

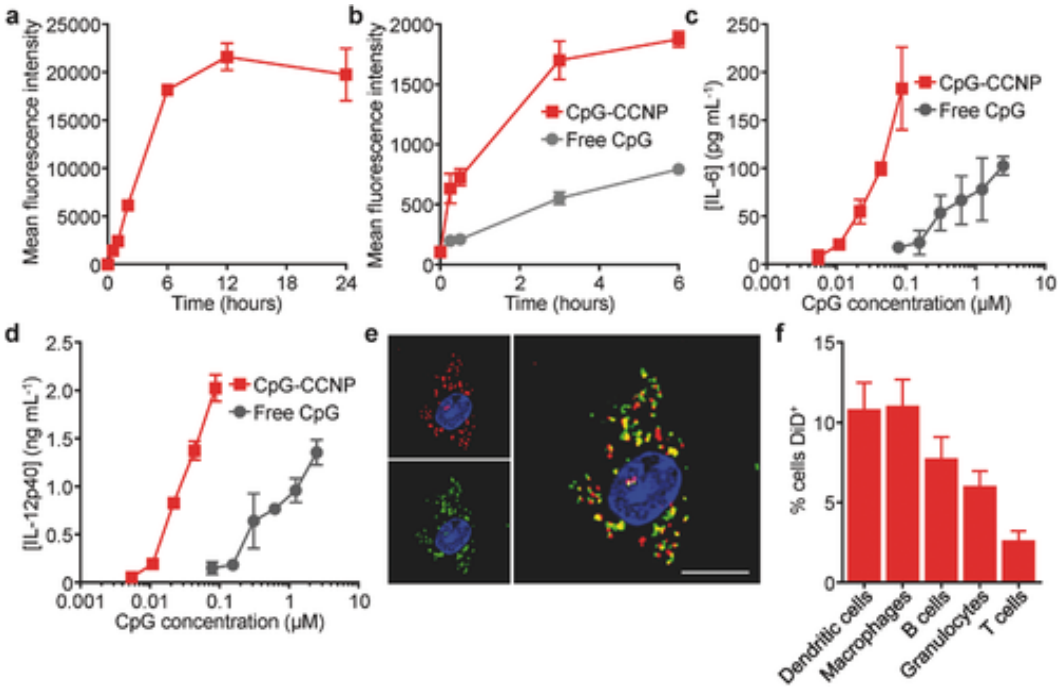

Figure 2.3: Delivery of antigen and adjuvant to immune cells. a) Uptake kinetics of dye-labeled CpG-CCNPs by BMDCs (n = 3; mean ± SD). b) Uptake kinetics of dye-conjugated CpG in free form or within CpG-CCNPs by BMDCs (n = 3; mean ± SD). c,d) Secretion of the proinflammatory cytokines IL-6 (c) and IL-12p40 (d) by BMDCs when incubated with either free CpG or CpG-CCNPs (n = 3; mean ± SD). e) Confocal microscopy colocalization of CpG and membrane proteins upon uptake of dual-labeled CpG-CCNPs by a BMDC. Green = CpG, red = membrane, blue = cell nucleus; scale bar = 10 μm. f) Uptake of dye-labeled CpG-CCNPs by different immune cell subsets in the draining lymph node after *in vivo* administration (n = 6; mean ± SD).

of pathogen-associated molecular patterns such as CpG, it was observed that both CpG-CCNPs and CpG-NPs without any antigen were equally potent. Without CpG, the antigen-only CCNP formulation exhibited significantly decreased activity. A similar pattern was seen when assessing the secretion of IL-6 and IL-12 by the BMDCs. When administered *in vivo*, the CpG-CCNP and CpG-NP formulations were likewise able to induce significant dendritic cell maturation at the draining lymph node after 24 h (Figure 2.4a–d). They also outperformed additional controls, including CCNPs with free CpG and whole cell lysate with free CpG, highlighting the advantage of nanoparticulate formulations. The level of cytokine secretion at the draining lymph node was shown to be mostly dependent on the presence of CpG, with all adjuvanted formulations

performing similarly (Figure 2.4e,f). This effect was localized, as analysis of cytokine levels in the serum did not yield anything significantly above baseline.

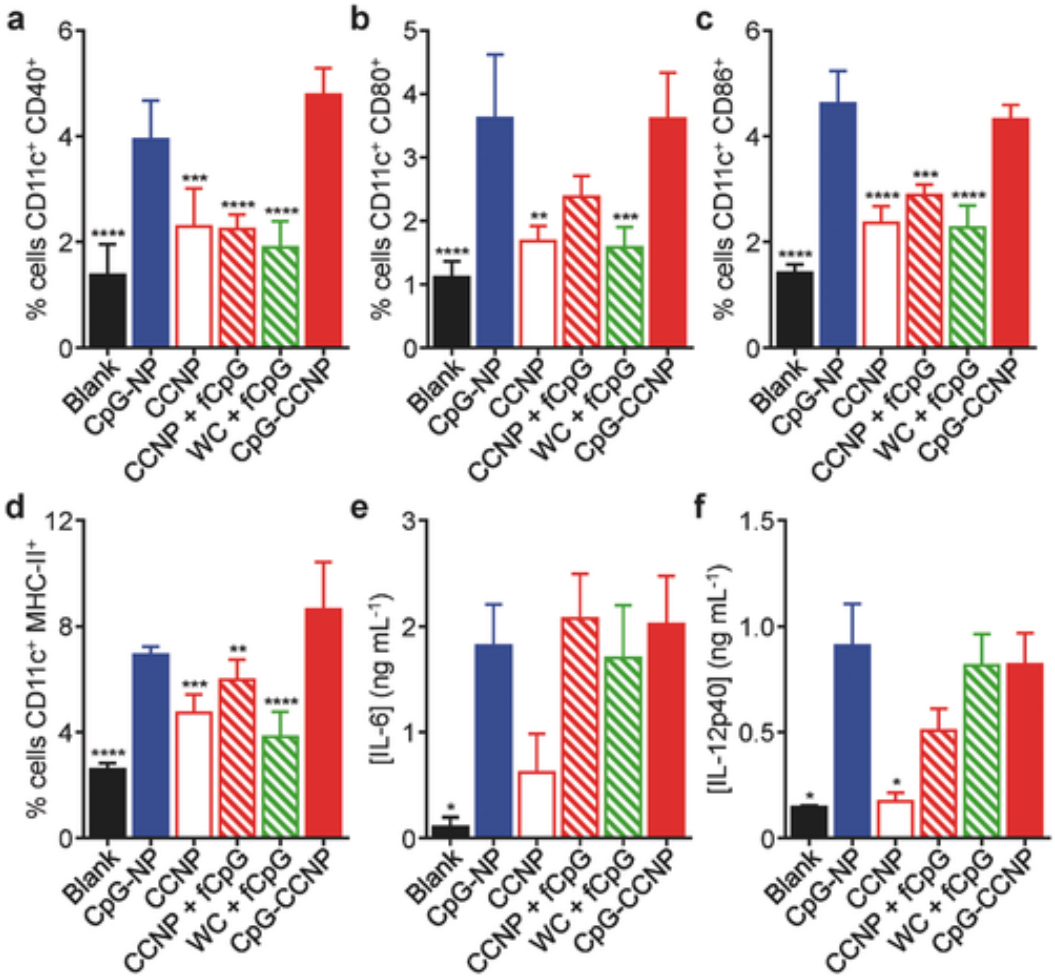

Figure 2.4: Characterization of *in vivo* dendritic cell maturation. Analysis of dendritic cell maturation markers a) CD40, b) CD80, c) CD86, and d) MHC-II in the draining lymph nodes after administration with CpG-CCNPs and various control formulations, including whole cell lysate with free CpG (WC + fCpG), CCNPs with free CpG (CCNP + fCpG), CCNPs, CpG-NPs, and blank solution ($n = 4$; mean ± SD). e,f) Concentration of proinflammatory cytokines e) IL-6 and f) IL-12p40 secreted by immune cells isolated from the draining lymph nodes after vaccination with CpG-CCNPs or various control formulations ($n = 4$; mean ± SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (compared to CpG-CCNP); one-way ANOVA.

To confirm the utility of the CpG-CCNP formulation for antitumor vaccination, its ability to elicit antigen-specific immune responses was verified using T cell-based assays. First, pmel-1 CD8+ T cells, which specifically recognize a gp100 epitope, were adoptively transferred to recipient mice, which were subsequently vaccinated with the various formulations (Figure 2.5a).

Treatment with CpG-CCNPs resulted in the highest degree of pmel-1 T cell proliferation, indicating that the formulation was able to effectively deliver the gp100 antigen for presentation under an immunostimulatory context. Additionally, after a set of vaccinations in naïve mice, the CpG-CCNPs were able to promote the native generation of T cells with multiple tumor antigen specificities (Figure 2.5b,c). T cells specific for both gp100 and TRP2 could be isolated and expanded from mice vaccinated with the CpG-CCNPs. Further, when cultured *ex vivo*, immune cell preparations from mice vaccinated with the formulation showed significantly enhanced production of IFNγ and IL-2 when stimulated with a gp100 peptide, a TRP2 peptide, or whole cell lysate, suggesting robust effector-level response against those targets. While these studies were generally limited to probing for immunity against well characterized epitopes, it could be reasonably inferred that the CpG-CCNP formulation was concurrently generating additional responses against other tumor-relevant antigens.

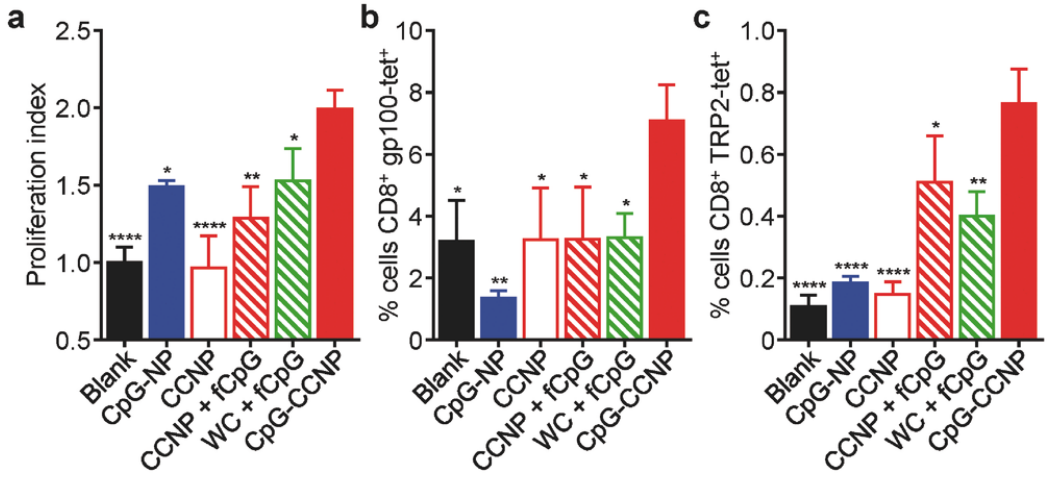

Figure 2.5: Characterization of *in vivo* T cell responses. a) Proliferation index of adoptively transferred pmel-1 CD8+ T cells after *in vivo* stimulation by CpG-CCNPs or various control formulations, including whole cell lysate with free CpG (WC + fCpG), CCNPs with free CpG (CCNP + fCpG), CCNPs, CpG-NPs, and blank solution (n = 3; mean ± SD). b,c) Tetramer staining analysis of T cells specific for gp100 (b) and TRP2 (c) after *ex vivo* restimulation of splenocytes from mice vaccinated with CpG-CCNPs or various control formulations (n = 3; mean ± SD). *p < 0.05, **p < 0.01, ****p < 0.0001 (compared to CpG-CCNP); one-way ANOVA.

To assess if the enhanced cellular immunity afforded by the CpG-CCNP formulation could translate into functional rejection of tumor cells, a prophylactic study using the wild-type B16-F10 model, which is poorly immunogenic [21, 36, 37], was carried out (Figure 2.6a–c). In mice vaccinated with CpG-CCNPs, there was significant activity, and tumor occurrence was prevented in 86% of mice 150 d after challenge with the tumor cells. Formulations consisting of either whole cell lysate with free CpG or CCNPs with free CpG both showed modest control of tumor growth, extending median survival from 20 d for the untreated group to 34 and 40 d, respectively. All but one of the mice in these groups reached the experimental endpoint by day 48 after challenge. CCNPs without adjuvant had minimal protective benefit, with the mice in these groups achieving a median survival of 28 days. Finally, mice vaccinated with CpG-NPs that had no antigenic material exhibited tumor growth kinetics identical to the blank control and displayed a median survival of 22 d. The results suggest that codelivery of both tumor antigen material and the CpG adjuvant together in the same vehicle is necessary for eliciting maximal antitumor immunity. The fact that CpG-NPs alone had no effect is encouraging and demonstrates that the inclusion of cancer

membrane material helped to provide appropriate cues for the specific detection and elimination of malignant cells by the immune system.

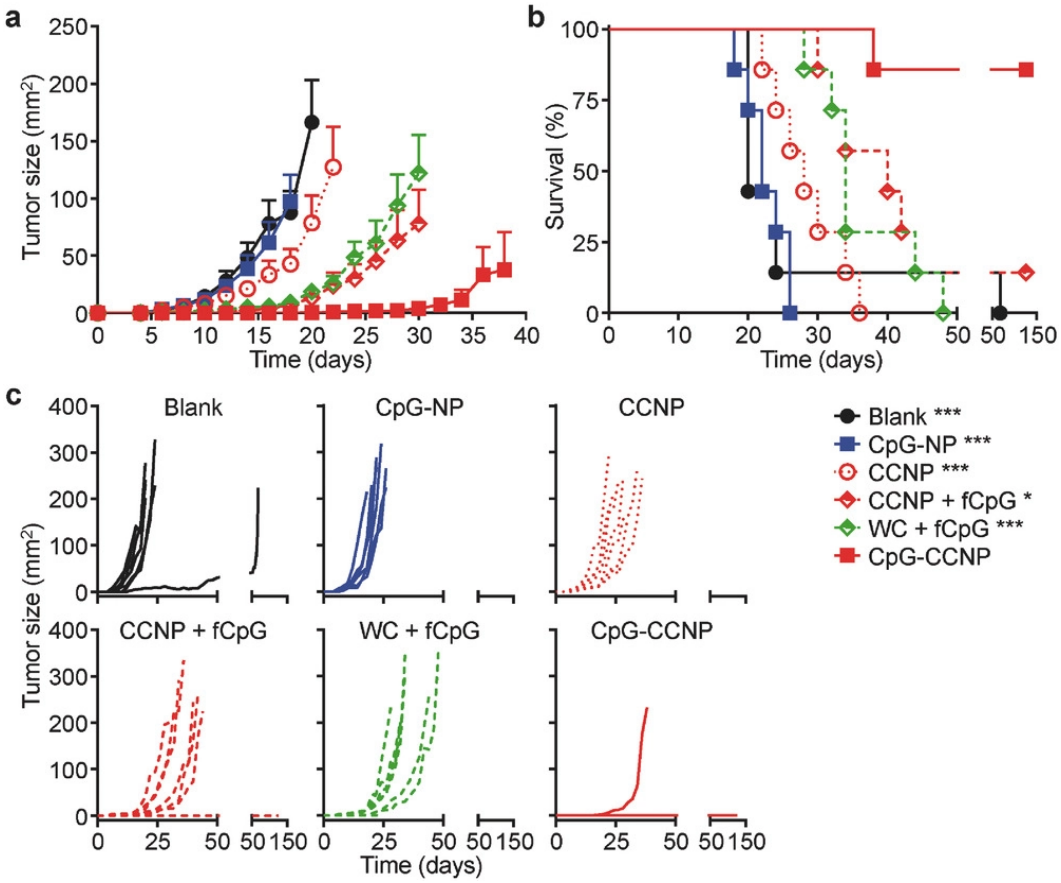

Figure 2.6: Prophylactic efficacy. a–c) Mice immunized with CpG-CCNPs and various control formulations, including whole cell lysate with free CpG (WC + fCpG), CCNPs with free CpG (CCNP + fCpG), CCNPs, CpG-NPs, and blank solution, on days 0, 7, and 14 were challenged with B16-F10 cells on day 21. Average tumor sizes (a), survival (b), and individual tumor growth kinetics (c) were plotted over time ($n = 7$; mean ± SEM). Reporting of average tumor sizes was halted after the first mouse died in each respective group. $*p < 0.05$, $***p < 0.001$ (compared to CpG-CCNP in survival plot); log-rank test.

The utility of the nanoparticulate vaccine formulation was further tested in a more clinically relevant therapeutic setting (Figure 2.7a–c). In this study, mice were challenged with B16-F10 cells and subsequently treated with the nanoformulation. Using this design, CpG-CCNPs alone displayed a modest ability to control tumor growth and extend survival. Given the aggressive nature of the B16-F10 tumor model, the results were not unexpected, especially given that vaccination largely focuses on the training phase of adaptive immunity. Despite adequately

enabling the immune system to recognize the appropriate targets, vaccine formulations for boosting cellular immunity may not be particularly well-suited for potentiating effector functionality in the presence of strong immunosuppression [38]. As such, the CpG-CCNPs were combined with a checkpoint blockade cocktail consisting of anti-CTLA4 and anti-PD1, and treatment with the combination enabled significantly enhanced control of tumor growth. Median survival was extended from 18 d for the blank control to 32 d for the treated group, and 50% of tumors were still below the experimental endpoint threshold on day 48 post challenge. In contrast, the checkpoint blockades, which have not shown significant efficacy in a related B16 model [39], was about as effective as CpG-CCNPs. The results confirm that the nanoparticulate vaccine formulation can act synergistically with other immunotherapies, modulating different aspects of immunity to promote the strongest antitumor responses.

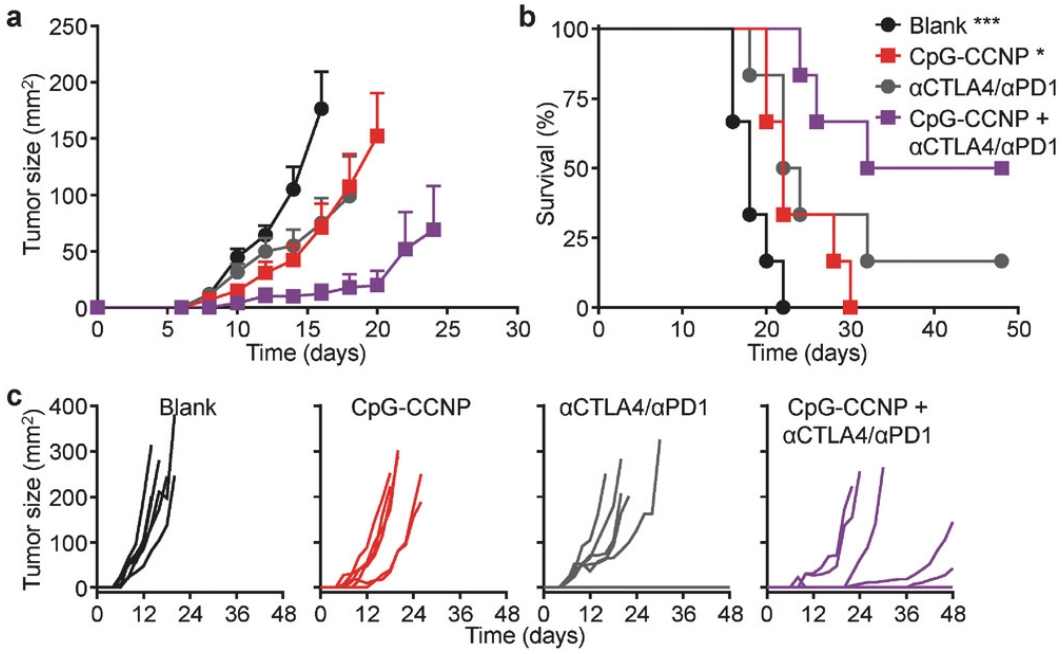

Figure 2.7: Therapeutic efficacy. a–c) After challenge with B16-F10 cells on day 0, mice were treated using CpG-CCNPs combined with a checkpoint blockade cocktail of anti-CTLA4 plus anti-PD1 (αCTLA4/αPD1), CpG-CCNPs alone, or the checkpoint blockade cocktail alone on days 1, 2, 4, and 7. Average tumor sizes (a), survival (b), and individual tumor growth kinetics (c) were plotted over time (n = 6; mean ± SEM). Reporting of average tumor sizes was halted after the first mouse died in each respective group. *$p < 0.05$, ***$p < 0.001$ (compared to CpG-CCNP + αCTLA4/αPD1 in survival plot); log-rank test.

2.4 Conclusions

In conclusion, we have reported on a biomimetic nanoparticulate anticancer vaccine formulation capable of activating multiantigenic immunity. The design leverages the unique advantages of recent nanoparticle technology, delivering both syngeneic cancer material along with a potent immunological adjuvant in a format that promotes effective antigen presentation. The final formulation is capable of generating strong antitumor responses *in vivo* and can work together with other immunotherapies such as checkpoint blockades to help control tumor growth. It is increasingly understood that presentation of tumor antigens alone, even in highly immunogenic contexts, may not be able to overcome the immunosuppressive tumor microenvironment [38, 40]. As such, efforts have shifted toward the rational design of combinatorial approaches that leverage multiple modes of action [41-43], including employing such strategies as adjuvant therapies to surgical resection [44]. In doing such, the potential adverse effects of immunomodulatory cocktails will also need to be considered [45]. The present nanoparticle-based cancer cell membrane coating strategy represents a generalizable and effective means of boosting endogenous immunity against autologous material, which may, in the future, be derived from a patient's own resected primary tumor as a means to prevent relapse. All of this is accomplished in a manner that is unique when compared to current strategies and can possibly pave the way for enhanced personalized anticancer vaccines.

Chapter 2, in full, is a reprint of the material as it appears in *Advanced Materials*, 2017, Ashley Kroll, Ronnie Fang, Yao Jiang, Jiarong Zhou, Xiaoli Wei, Chun Lai Yu, Jie Gao, Brian Luk, Diana Dehaini, Weiwei Gao and Liangfang Zhang. The dissertation author was a primary author of this paper.

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Chapter 3

Engineered Cell Membrane-Coated Nanoparticles Directly Present Tumor Antigens to Promote Anticancer Immunity

3.1 Introduction

It is understood now that cancer pathogenesis carries a significant immunological component [1], and tumors can utilize a number of mechanisms in order to achieve immune escape [2]. Cancer immunotherapies leverage this knowledge and seek to manipulate various aspects of the immune process in order to promote tumor destruction [3]. For example, approaches such as anticancer vaccination help to train T cells with the appropriate antigen specificities [4], while others such as checkpoint blockade therapies work by removing the mechanisms of inhibition on existing immune cell populations [5]. Each approach has its own benefits and challenges, although even the most promising immunotherapeutic modalities only work for a subset of patients, in large part due to the large heterogeneity among cancers [6]. Currently, most of the success in the clinic has been biased towards treatments that augment or supplement the effector stage of immunity [7, 8], perhaps because the manipulation of these downstream immune processes is more straightforward with fewer variables to consider. There is strong evidence, however, that combinatorial treatments affecting multiple aspects of immunity can promote strong antitumor immune responses [9, 10], and it is thus imperative that the development of immunotherapies continues along multiple fronts.

Vaccines are designed to generate antigen-specific immune responses and have historically been attractive due to their ease of use and potential for broad applicability [11]. Unfortunately, vaccines formulated against cancers are notoriously difficult to develop [12, 13]. This stems largely from the fact that tumor antigens are inherently lowly immunogenic, as they are usually based on normal antigens that are subtly mutated or differentially upregulated. To address this issue, vaccines must be formulated with potent immunological adjuvants, often in the form of toll-

like receptor agonists, in order to boost the immune response [14]. Delivery of antigen and adjuvant to professional antigen-presenting cells (APCs) results in the presentation of peptide epitopes in the context of major histocompatibility complex (MHC)-I, along with costimulatory markers such as CD80 or CD86 [15]. Together, these signals are necessary in order to promote activation of the cognate T cells that can target and destroy tumor cells expressing the corresponding antigen epitope. Manipulation of the antigen presentation process is not straightforward, and lack of potency is a common issue for anticancer vaccine formulations [16]. This is particularly true for most vaccines that are administered parenterally, where efficient delivery to the correct APC subsets is a major challenge [17]. These difficulties have necessitated the use of production workflows that require significant time and effort [18], greatly limiting their translational potential.

Researchers have sought to gain more control over the antigen presentation process by engineering artificial APCs (aAPCs) that can replace the function of their endogenous counterparts [19]. aAPCs can be cell-based, whereby living cells are engineered to express the appropriate MHC, as well as a costimulatory marker [20]. These modified cells have been shown to successfully engage with and activate T cells, with potential utility for adoptive cell therapy applications. More recently, there has been significant interest in developing particulate aAPCs [21]. For these synthetic platforms, the requisite biological signals, including peptide-loaded MHC multimer complexes and CD28 agonists, are conjugated onto the surface of synthetic microparticles or nanoparticles. In particular, nanoscale aAPC systems may hold significant potential given their ability to be used for *in vivo* applications [22, 23], offering significant advantages such as enhanced lymphatic transport after subcutaneous administration [24]. They could ultimately be employed as a means for directly stimulating T cells that reside in the body, obviating the need for *ex vivo*

stimulation. As aAPCs bypass the need for traditional antigen processing and presentation, such platforms could also replace vaccines for certain applications. Combined with sophisticated strategies for neoantigen identification [25], the technology could also be amenable to a high level of personalization in the future. Overall, aAPC platforms represent powerful tools for the direct activation of T cells, particularly when individual epitopes of interest can be identified.

Almost all cells in the body express MHC-I, which allows internal monitoring by the immune system in the case of infection or aberrancy. This includes cancer cells, which often employ strategies to subvert immune detection by manipulating their MHC expression [26]. It should therefore be possible to leverage this antigen presentation machinery for the direct stimulation of cancer-targeting T cell populations [27], given that the correct signals are provided. Here, we first engineer a model cancer cell line to express the costimulatory marker CD80, enabling it to present its own antigens in an immunostimulatory context. The membrane from these engineered cells is then collected and coated onto a nanoparticulate substrate [28], resulting in a biomimetic nanoformulation capable of direct antigen presentation to cancer-specific T cells (Figure 3.1). The anticancer utility of these engineered cell membrane-coated antigen-presenting nanoparticles is evaluated both *in vitro* and in animal models of disease. The described platform combines the advantages of natural cell-based aAPCs with those of synthetic nanoscale APCs into a single construct that is well-suited for *in vivo* use. These biomimetic antigen-presenting nanoparticles have the potential to promote multi-specific T cell activation and may ultimately be used for personalized therapies.

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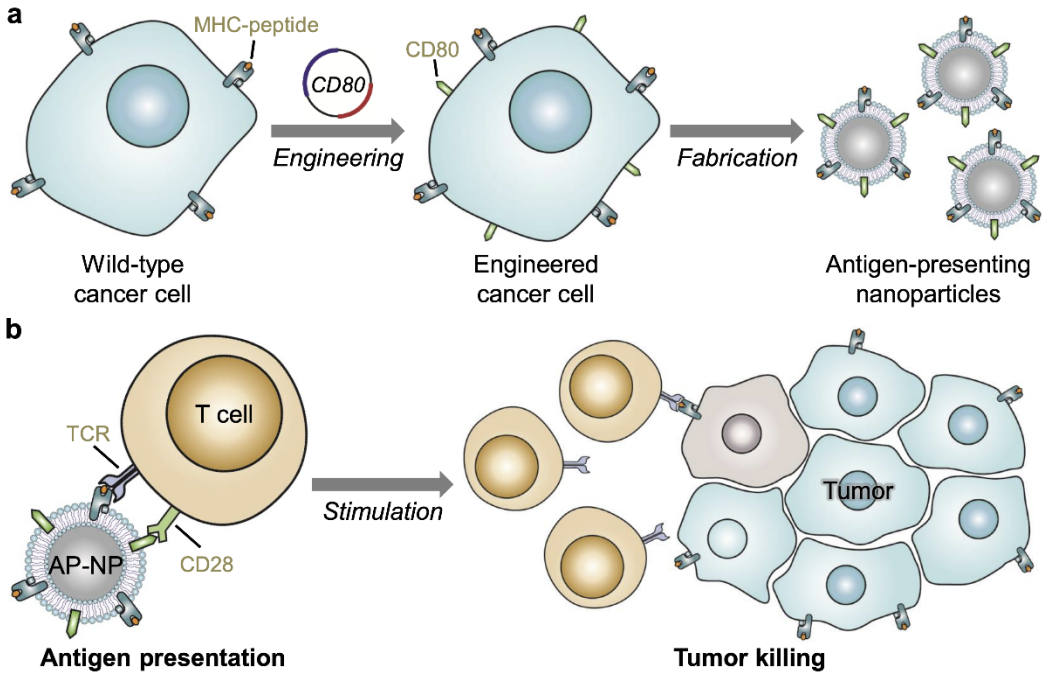

Figure 3.1: Schematic of engineered cell membrane-coated nanoparticles for direct antigen presentation. a) Wild-type cancer cells, which naturally present their own antigens via MHC-I, are engineered to express CD80, a co-stimulatory signal. The plasma membrane from these cells is then derived and coated onto polymeric nanoparticle cores. b) The resulting antigen-presenting nanoparticles (AP-NPs) can directly stimulate tumor antigen-specific T cells through engagement of the cognate T cell receptor (TCR) and CD28. Upon activation, the T cells are capable of controlling tumor growth by killing cancer cells that express the same antigens.

3.2 Experimental Methods

3.2.1 Cell Culture and Engineering

Wild-type B16-F10 (B16-WT) mouse melanoma cells (CRL-6475; American Type Culture Collection) were maintained in Dulbecco's modified eagle medium (DMEM; Mediatech) supplemented with 10% bovine growth serum (Hyclone) and 1% penicillin-streptomycin (Gibco).

To generate the B16-CD80 cell line, B16-WT cells were transfected with a plasmid encoding CD80 (pUNO1-mB7-1; InvivoGen) using lipofectamine 2000 (Life Technologies), followed by selection in media containing blastidicin (InvivoGen). Monoclonal selection was conducted by plating the blasticidin-selected cells in 96-well tissue culture plates at an average density of 0.5 cells per well, and the clone with the highest expression was expanded for further study. B16-CD80 cells were maintained in culture media supplemented with 4 μg/mL of blasticidin. To generate the B16-CD80/OVA cell line, a gene encoding for a cytoplasmic form of ovalbumin from pCI-neo-cOVA (a gift from Maria Castro; #25097; Addgene) was cloned into the pQCXIH retroviral expression vector (Clontech), and the resulting plasmid was used to transfect AmphoPhoenix cells (obtained from the National Gene Vector Biorepository). After 48 h, virus-containing cell culture supernatant was collected, mixed with polybrene (Sigma-Aldrich) at a final concentration of 4 μg/mL, and added to B16-CD80 cells. Viral transduction was facilitated by centrifuging the culture plate at 800 *g* for 90 min. After 48 h of transduction, selection was performed by culturing the cells in media containing hygromycin B (InvivoGen). A monoclonal cell line was obtained in a similar manner as above. B16-CD80/OVA cells were maintained in media containing 300 μg/mL hygromycin B and 4 μg/mL blasticidin. B16-OVA cells were generated using the same protocol starting with B16-WT cells and were maintained in media containing 300 μg/mL hygromycin B.

3.2.2 Western Blotting

The various cell lines were collected by scraping and lysed by sonicating in water for 5 min using a Fisher FS30D bath sonicator. Each sample was diluted to 0.7 mg/mL of protein content

in water based on a BCA protein assay (Pierce) and then mixed in a 3 to 1 volume ratio with NuPAGE 4× lithium dodecyl sulfate sample loading buffer (Novex). After heating for 10 min at 70 °C, the samples were loaded into 12-well Bolt 4-12% bis-tris gels (Invitrogen) and run at 165 V for 45 min in MOPS running buffer (Novex). The proteins were then transferred onto 0.45 μm nitrocellulose membrane (Pierce) in Bolt transfer buffer (Novex) at 15 V for 30 min. The blots were blocked with 5% milk (Genesee Scientific) in PBS with 0.05% Tween 20 (National Scientific), followed by incubation with a mouse anti-ovalbumin monoclonal antibody (3G2E1D9, Santa Cruz Biotechnology) as the primary immunostain and a horseradish peroxidase-conjugated secondary (Biolegend) as the secondary immunostain. Membranes were developed with ECL western blotting substrate (Pierce) in an ImageWorks Mini-Medical/90 Developer.

3.2.3 Surface Marker Characterization

Flow cytometry was employed to examine the MHC-mediated surface presentation of the SIINFEKL (OVA257-264) peptide and the expression of CD80 on the various cell lines. To facilitate the analysis of SIINFEKL presentation, the cells were incubated with 10 ng/mL recombinant mouse IFNγ (Biolegend) overnight to upregulate MHC-I expression [29]. Cells were collected from culture using 1 mM ethylenediaminetetraacetic acid (USB Corporation) in PBS and stained with PE-conjugated anti-mouse Kb-SIINFEKL antibody (25-D1.16, Biolegend) and Alexa647-conjugated anti-mouse CD80 antibody (16-10A1, Biolegend), or the corresponding isotype antibodies (Biolegend). Data was collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo.

3.2.4 *In Vitro* Biological Activity of Engineered Cells

All animal experiments were performed in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California San Diego. C57BL/6-Tg(TcraTcrb)1100Mjb/J transgenic mice (OT-I; The Jackson Laboratory) were euthanized and their spleens were collected. To obtain single cell suspensions, each spleen was physically extruded through 70 μm nylon cell strainers (Fisher Scientific), followed by red blood cell removal using RBC lysis buffer (Biolegend) per the manufacturer's instructions. The cells were then washed with 1× PBS and $CD8^+$ cells were isolated out by magnetic separation using CD8a (Ly-2) MicroBeads (Miltenyi Biotec) on MACS LS separation columns (Miltenyi Biotec) per the manufacturer's instructions. Splenocytes or isolated $CD8^+$ cells were resuspended using warm media consisting of 500 mL Isocove's modification of DMEM with 2 mM L-glutamine and 25 mM HEPES (Mediatech) supplemented with 50 mL USDA certified fetal bovine serum (Omega Scientific), 500 μL 55 mM β-mercaptoethanol (Gibco), 5 mL 200 mM L-glutamine (Gibco), and 5 mL penicillin-streptomycin. Either 2×10^5 $CD8^+$ cells or 3×10^6 splenocytes were incubated with 5×10^4 of the various engineered B16 cells for 24 h. For cytokine analysis, culture supernatant was collected and assayed using mouse IFNγ and IL-2 ELISA kits (Biolegend) according to the manufacturer's instructions. For surface marker analysis, $CD8^+$ cells or splenocytes in culture were collected and stained with one of the two following sets of antibodies: (1) PE/Cy7-conjugated anti-mouse CD3 antibody (17A2, Biolegend), APC-conjugated anti-mouse CD8a antibody (53-6.7, Biolegend), FITC-conjugated anti-mouse/human CD44 antibody (IM7, Biolegend), and Pacific Blue-conjugated anti-mouse CD62L antibody (MEL-14, Biolegend), or (2) PE/Cy7-conjugated anti-mouse CD3 antibody, Pacific Blue-conjugated anti-mouse CD8a antibody (Biolegend),

Alexa647-conjugated anti-mouse CD69 antibody (H1.2F3, Biolegend), and FITC-conjugated anti-mouse CD25 antibody (3C7, Biolegend). Data was collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo. All analysis was conducted on the $CD3^+CD8^+$ T cell population. To examine the ability of the engineered cells to activate T cells specific for a native antigen, splenocytes were derived from B6.Cg-*Thy1*^{a}/Cy Tg(TcraTcrb)8Rest/J transgenic mice (pmel-1; The Jackson Laboratory). Prior to the study, B16-OVA or B16-CD80/OVA cells were cultured in the presence of 10 ng/mL recombinant mouse IFNγ for 1 day. The engineered cells were then rinsed before incubating with 3×10^6 pmel-1 splenocytes for 48 h. Afterwards, surface marker expression was analyzed by staining with PE/Cy7-conjugated anti-mouse CD3 antibody, Pacific Blue-conjugated anti-mouse CD8a antibody, and Alexa647-conjugated anti-mouse CD69 antibody.

3.2.5 Nanoparticle Preparation

The cell membrane from B16-WT, B16-OVA, and B16-CD80/OVA cells was collected according to a previously published protocol [30]. Briefly, cells were suspended in a lysis buffer containing 30 mM Tris-HCl pH 7.0 (Quality Biological) with 0.0759 M sucrose (Sigma-Aldrich), 0.225 M D-mannitol (Sigma-Aldrich), and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich), followed by physical disruption using a Kinematica Polytron PT 10/35 probe homogenizer at 70% power for 15 passes. The membrane was separated by first centrifuging at 10,000 *g* and then centrifuging the resulting supernatant at 150,000 *g* to obtain a membrane pellet using a Beckman Coulter Optima XPN-80 ultracentrifuge. To prepare polymeric cores, 1 mL of poly(DL-lactic-*co*-glycolic acid) (50:50 PLGA, 0.67 dL/g, Lactel Absorbable Polymers) in

acetone at 10 mg/mL was added dropwise into 1 mL of water and the mixture was placed under a vacuum aspirator to evaporate the organic solvent. Membrane coating was carried out by mixing the preformed PLGA cores with the membrane at the appropriate ratios and sonicating the mixture for 2 min in a Fisher Scientific FS30D bath sonicator.

3.2.6 Nanoparticle Optimization and Characterization

To optimize the nanoformulation, [CD80/OVA]NPs were fabricated at membrane to polymer weight ratios of 0, 0.1, 0.2, 0.5, 1, and 2 in water and then adjusted to 1× PBS using 20× PBS (Teknova). Nanoparticle size and surface zeta potential were measured by dynamic light scattering using a Malvern ZEN 3600 Zetasizer. To evaluate stability over time, nanoparticles were suspended in 10% sucrose (Sigma-Aldrich) solution and stored at 4 °C for two weeks; size was monitored every 2 days. For electron microscopic visualization, freshly synthesized nanoparticles or nanoparticles stored for 1 week at 4 °C in 10% sucrose were first deposited onto carbon-coated 400-square mesh copper grids (Electron Microscopy Sciences), followed by negative staining with 1 wt% uranyl acetate (Electron Microscopy Sciences). Imaging was performed on a Tecnai Spirit transmission electron microscope. The pull-down assay was conducted by first suspending nanoparticles at 4 mg/mL in 1% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at 4 °C. Then, Alexa647-conjugated anti-mouse CD80 antibody, APC-conjugated anti-mouse Kb-SIINFEKL antibody (Biolegend), or the corresponding isotype antibodies were incubated with the nanoparticles for 30 min at 4 °C. After incubation, the nanoparticles were centrifuged at 21,000 *g*, and the fluorescence of the supernatant was measured. Binding was calculated as (initial fluorescence – supernatant fluorescence)/(initial fluorescence) and normalized to the value for the

corresponding isotype antibody.

3.2.7 *In Vitro* Biological Activity of Engineered Nanoparticles

To evaluate nanoparticle biological activity, 8×10^5 $CD8^+$ cells or 3×10^6 splenocytes from OT-I mice were cultured in the presence of 100 μg/mL of each nanoformulation for 3 days. CD25, CD69, CD44, and CD62L surface marker expression, as well as IFNγ and IL-2 secretion, were analyzed as described above. To measure biological functionality after storage, nanoparticles stored for 1 week at 4 °C in 10% sucrose were incubated with 8×10^5 OT-I $CD8^+$ cells at 100 μg/mL for 3 days. Expression of CD25 and CD69 on the $CD8^+$ T cells was measured as described above. For the cell proliferation study, 3×10^6 splenocytes derived from OT-I mice were labeled using CellTrace Violet (Invitrogen) according to the manufacturer's instructions and incubated with the various nanoformulations at a concentration of 0, 12.5, 25, 50, and 100 μg/mL. After 3 days of incubation, the cells were collected and stained with PE/Cy7-conjugated anti-mouse CD3 antibody and FITC-conjugated anti-mouse CD8a antibody (Biolegend). Data was collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo. All analysis was conducted on the $CD3^+CD8^+$ T cell population. To quantify fold expansion, splenocytes derived from OT-I mice were cultured in 24-well plates at a density of 4×10^6 cells per well. Nanoparticles were added to the wells at a final concentration of 100 μg/mL. On day 4, the cells were collected and stained with PE/Cy7-conjugated anti-mouse CD3 antibody and FITC-conjugated anti-mouse CD8a antibody for enumeration by flow cytometry. Fold expansion was calculated by normalizing the total number of $CD3^+CD8^+$ T cells on day 4 to the number on day 0. To measure cell killing, $CD8^+$ cells derived from OT-I mouse were activated in the presence of 50 μg/mL of

[CD80/OVA]NPs and 20 ng/mL of recombinant mouse IL-2 (Biolegend) for 3 days. On the day before the assay, target B16-WT and B16-OVA cells were plated at a density of 1×10^4 cells per well in a 96-well plate in the presence of 10 ng/mL of recombinant mouse IFNγ. To perform the assay, the target cells were first rinsed twice with culture media, and then 5×10^4, 1×10^5, or 2×10^5 activated $CD8^+$ cells were added, followed by incubation for 18 h at 37 °C. The cytotoxicity was measured by an LDH assay (Biolegend) per the manufacturer's instructions (Biolegend). To examine the ability of the nanoparticles to activate immunity against native antigens, 3×10^6 pmel-1 splenocytes were cultured with 100 μg/mL of nanoformulations fabricated using membrane derived from engineered cells pretreated with 10 ng/mL recombinant mouse IFNγ for 72 h. Expression of CD69 was examined as described above.

3.2.8 *In Vivo* Delivery

To evaluate the *in vivo* localization of the nanoparticles, 400 μg of [CD80/OVA]NPs labeled with 0.1 wt% 1,1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine dye (Biotium) were subcutaneously injected into the flanks of OT-I mice. The subcutaneous administration route was chosen over the intravenous route due to the propensity of the latter for generating tolerance [31]. At 0, 12, and 24 h, the inguinal lymph nodes were collected and cryosectioned, followed by fixation in 10% phosphate-buffered formalin (Fisher Chemical) for 15 min at room temperature. After fixation, the slides were blocked with 2% BSA in PBS for 30 min and stained with 7.5 μg/mL FITC-conjugated anti-mouse CD8a antibody in 2% BSA solution overnight at 4 °C. Finally, the slides were mounted in Vectashield mounting media (Vector Laboratories) and imaged on a Keyence BZ-X710 fluorescence microscope using the GFP and Cy5 filters.

3.2.9 *In Vivo* Biological Activity

Whole-body irradiation was performed on female C57BL/6 mice (Envigo) at a dosage of 6 Gy. After 1 day, 2.5×10^7 splenocytes derived from OT-I mice were labeled with CellTrace Violet and adoptively transferred into each irradiated mouse by intravenous injection. The next day, 450 μg of the various nanoformulations in 10% sucrose was injected subcutaneously into each the neck, left flank, and right flank. After another 3 days, the inguinal and axillary lymph nodes were collected and processed into single cell suspensions, after which they were stained with PE/Cy7-conjugated anti-mouse CD3 antibody, FITC-conjugated anti-mouse CD8a antibody, and Alexa647-conjugated anti-mouse CD69 antibody. Data was collected using a Becton Dickinson FACSCanto-II flow cytometer and analyzed using FlowJo. All analysis was conducted on the $CellTrace^+CD3^+CD8^+$ T cell population. To assess cytokine secretion, whole-body irradiation was performed on female C57BL/6 mice at a dosage of 6 Gy and, after 1 day, 2×10^7 splenocytes derived from OT-I mice were adoptively transferred. The next day, 450 μg of the various nanoformulations in 10% sucrose was injected subcutaneously into each the neck, left flank, and right flank. After another 4 days, the inguinal and axillary lymph nodes were collected and processed into a single cell suspension, which was cultured in 250 μL of media in a 96-well tissue culture plate. After 3 days of culture, the supernatant was collected and assayed using a mouse IFNγ ELISA kit according to the manufacturer's instructions.

3.2.10 *In Vivo* Antitumor Efficacy

To study prophylactic efficacy, whole-body irradiation was performed on female C57BL/6

mice at a dosage of 5.5 Gy. After 1 day, 1×10^7 splenocytes derived from OT-I mice were adoptively transferred into each mouse by intravenous injection. The following day, 400 μg of nanoparticles was subcutaneously injected into each the neck, left flank, and right flank. Tumor challenge was performed by subcutaneous administration of 4×10^5 B16-OVA cells into the lower right flank after another 5 days. To study therapeutic efficacy, female C57BL/6 mice were first challenged with 5×10^5 B16-OVA cells. Whole-body irradiation at a dosage of 5.5 Gy was performed on day 6 after tumor inoculation. On day 7, 1×10^6 $CD8^+$ T cells derived from OT-I mice (main therapeutic study) or 1×10^7 splenocytes derived from OT-I mice (whole cell lysate study) were adoptively transferred into each mouse by intravenous injection. Treatment was administered on days 8 and 13 by subcutaneous injection of 300 μg of nanoparticles into each the neck, left flank, and right flank. Whole cell lysate was prepared by resuspending B16-OVA cells in water and subjecting them to 3 freeze-thaw cycles. Mice were administered on days 8 and 13 with lysate at a dosage where the amount of membrane material was equivalent to the [CD80/OVA]NP sample [30], along with 10 μg of CpG 1826 (InvivoGen). To study therapeutic efficacy without irradiation and adoptive transfer, female C57BL/6 mice were first challenged with 3×10^5 B16-OVA cells. Treatment was administered on days 3 and 7 by subcutaneous injection of 300 μg of nanoparticles into each the neck, left flank, and right flank. Tumors were measured every other day and the experimental endpoint was defined as either death or tumor size greater than 200 mm^2.

3.3 Results and Discussion

To engineer cancer cells capable of displaying their own antigens under a stimulatory context, the wild-type B16-F10 (B16-WT) murine melanoma cell line was selected as the foundation. These cells are syngeneic to C57BL/6 mice and can readily be used to generate immunocompetent tumor models [32]. It has been confirmed that these cells express a certain degree of MHC class I [33], which can present peptide epitopes from endogenous antigens to $CD8^+$ T cells. For this study, the B16-WT cells were modified to overexpress two different genes. The first was a cytosolic form of ovalbumin (OVA) [34], which was selected as a model antigen given the wide range of immunological tools available to help facilitate its study. The second was the costimulatory marker CD80, which engages the CD28 receptor found on T cells [35]. When the two signals are presented together, CD80 and a peptide-MHC complex are generally sufficient to promote the activation of the cognate T cells. Stable cell lines were developed for both single knock-in clones, denoted B16-CD80 and B16-OVA, and a double knock-in clone, denoted B16-CD80/OVA. From western blotting analysis, it was confirmed that both B16-OVA and B16-CD80/OVA expressed the OVA protein, while no signal was detected for either B16-WT or B16-CD80 (Figure 3.2a). Flow cytometric analysis for CD80 confirmed that both B16-CD80 and B16-CD80/OVA cells displayed the costimulatory marker, whereas B16-WT and B16-OVA cells had little to no expression (Figure 3.2b). For the OVA-expressing clones, it was further confirmed that they were properly displaying the OVA-derived SIINFEKL peptide bound to H-2Kb (Kb-SIINFEKL) MHC-I (Figure 3.2c). This indicated that the cytoplasmic OVA was being properly restricted by endogenous cellular machinery for presentation to the immune system. When plotting CD80 expression versus Kb-SIINFEKL presentation, it was evident that the B16-CD80/OVA clone was positive for both signals required to activate anti-OVA cytotoxic T cell responses. (Figure 3.2d)

Next, the biological activity of both membrane-bound signals was evaluated to confirm that the engineered cells could promote the activation of antigen-specific T cells. Splenocytes derived from OT-I transgenic mice, whose $CD8^+$ T cells predominantly express the T cell receptor against Kb-SIINFEKL [36], were incubated with each of the B16-F10 variants. After 24 h of incubation, the $CD8^+$ T cells in the mixed-cell population were immunophenotyped using flow cytometry. Upregulation of CD69 is a hallmark of T cell activation [37], and the marker was highly expressed on $CD8^+$ T cells cocultured with B16-CD80/OVA cells. In contrast, B16-OVA cells produced a modest response, while both B16-WT and B16-CD80 cells had low expression. A similar trend was observed for CD25, an interleukin-2 (IL-2) receptor that aids in the clonal expansion of T cells [38]. In terms of memory T cells [39], only the B16-CD80/OVA cells were able to increase the proportion of $CD8^+$ T cells with the $CD44^{high}CD62L^{high}$ central memory phenotype, while the effect on the $CD44^{high}CD62L^{low}$ effector memory phenotype was less pronounced. In terms of cytokine secretion, IL-2 and interferon-γ (IFNγ), both of which are essential for the survival and function of $CD8^+$ T cells [40], were only found in high concentrations in the media of splenocytes cocultured with B16-CD80/OVA cells. The various engineered B16-F10 cells were then incubated and tested in a similar manner with purified OT-I $CD8^+$ cells, which should have a higher proportion of OVA-specific cytotoxic T cells (Figure 3.2e-l). The overall trends observed for this set of studies was largely consistent with the mixed splenocyte study, with the exception that the B16-CD80/OVA cells were able to clearly enhance the population of T cells with the $CD44^{high}CD62L^{low}$ effector memory phenotype. It was also confirmed that the CD80-modified cells could stimulate pmel-1 $CD8^+$ T cells (Figure 3.2m), which are specific against the native melanoma antigen gp100 present on B16-WT [41]. Overall, this data confirmed the

successful engineering of cancer cells capable of presenting their own antigens to activate specific T cell subsets.

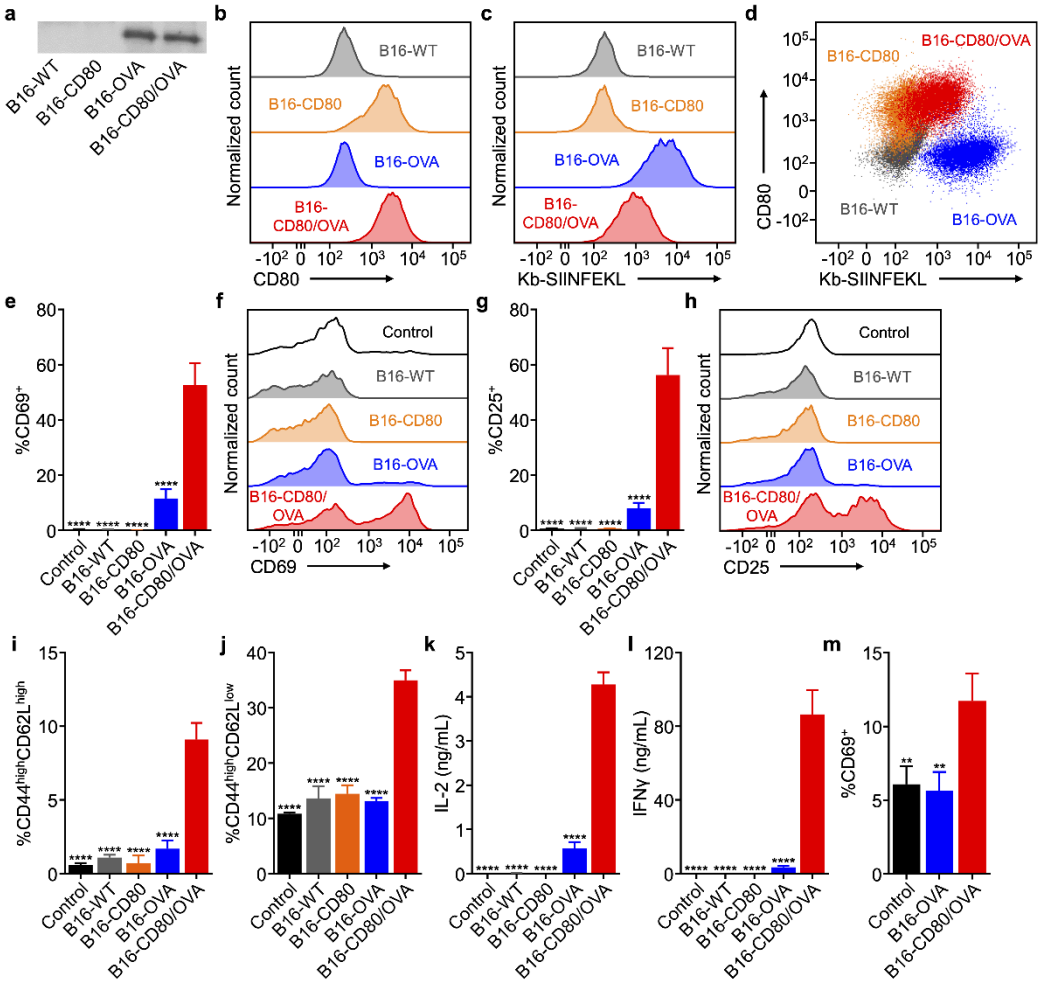

Figure 3.2: Characterization and biological activity of engineered cancer cells capable of direct antigen presentation. a) Western blot probing for OVA on B16-CD80/OVA cells and control cells. b,c) Expression of CD80 (b) and presentation of an MHC-I-restricted OVA peptide (Kb-SIINFEKL, c) by B16-CD80/OVA cells and control cells. d) Co-expression of CD80 and Kb-SIINFEKL by B16-CD80/OVA cells and control cells. e-h) Expression of CD69 (e,f) and CD25 (g,h) by OT-I $CD8^+$ T cells incubated with B16-CD80/OVA cells and control cells for 24 h (n = 3, mean + SD). i,j) Frequency of memory phenotypes $CD44^{high}CD62L^{high}$ (i) and $CD44^{high}CD62L^{low}$ (j) among OT-I $CD8^+$ T cells incubated with B16-CD80/OVA cells and control cells for 24 h (n = 3, mean + SD). k,l) Secretion of IL-2 (k) and IFNγ (l) by OT-I $CD8^+$ T cells incubated with B16-CD80/OVA cells and control cells for 24 h (n = 3, mean + SD). m) Expression of CD69 by $CD8^+$ T cells in a population of pmel-1 splenocytes incubated with B16- OVA or B16-CD80/OVA cells for 2 days (n = 3, mean + SD). $^{**}p < 0.01$, $^{****}p < 0.0001$ (compared to B16-CD80/OVA); one-way ANOVA.

The use of modified cancer cells *in vivo* would likely be accompanied with numerous safety concerns and face significant hurdles in terms of clinical translation. Thus, we sought to generate

a nanoformulation safe for *in vivo* use by leveraging cell membrane coating nanotechnology, which is a streamlined approach for the top-down fabrication of highly functional nanoparticles that mimic many of the functions of living cells [28]. These biomimetic nanoparticles have been used in a number of different applications, including drug delivery [42-44], detoxification [45-47], and vaccine design [30, 48]. In terms of immune modulation, a major advantage of the technology is that it can help to stabilize membrane vesicles and decrease their size, thus enhancing *in vivo* transport upon subcutaneous administration [49]. Having confirmed the functionality of both surface-bound signals on the engineered B16-CD80/OVA cells, their membrane was then derived using a procedure involving cellular disruption and differential centrifugation. The purified membrane was collected and coated onto the surface of preformed polymeric nanoparticle cores by a sonication process [50]. It should be noted that the nanoparticle core serves an important function to stabilize the membrane coating and prevent unwanted fusion [48], thus facilitating enhanced lymphatic transport [49]. The preparation of B16-CD80/OVA cell membrane-coated nanoparticles (denoted [CD80/OVA]NPs) was optimized by synthesizing the nanoparticles in water with varying weight ratios of the engineered cell membrane to polymer, followed by adjusting the resulting formulations to isotonic phosphate buffered saline (PBS) to match physiological conditions (Figure 3.3a). Without any membrane coating, the charge screening effect resulting from the presence of ions in the buffer caused the bare polymeric cores to aggregate significantly. This effect lessened at higher coating ratios, suggesting progressively better surface coverage. Based on the data, it was determined that the optimal membrane-to-core weight ratio was 1:2, and this [CD80/OVA]NP formulation was used for all subsequent studies.

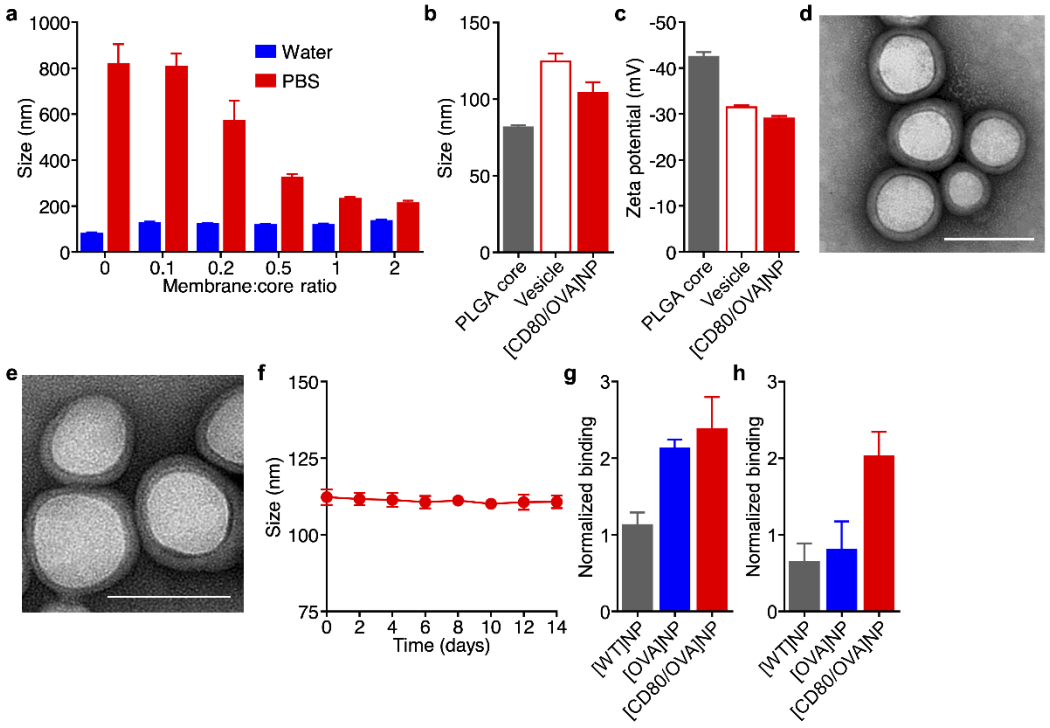

Figure 3.3: Fabrication and characterization of engineered antigen-presenting nanoparticles. a) Size of [CD80/OVA]NPs at different membrane to core weight ratios when suspended in water or PBS (n = 3; mean + SD). b,c) Hydrodynamic diameter (b) and surface zeta potential (c) of bare PLGA cores, B16-CD80/OVA membrane vesicles, and [CD80/OVA]NPs (n = 3; mean + SD). d,e) Transmission electron microscope images of [CD80/OVA]NPs immediately after synthesis (d) and after 1 week of storage (e). Scale bars = 100 nm. f) Size of [CD80/OVA]NPs over 2 weeks (n = 3; mean ± SD). g,h) Relative binding of antibodies against Kb-SIINFEKL (g) and CD80 (h) to [WT]NPs, [OVA]NPs, and [CD80/OVA]NPs (n = 3; mean + SD).

When measured by dynamic light scattering, the final [CD80/OVA]NPs were approximately 100 nm in size after coating, which was in between the sizes of the bare polymeric cores and the membrane vesicles (Figure 3.3b). Zeta potential measurements revealed that the surface charge of the membrane-coated nanoparticles was near the value of pure membrane vesicles, which were less negative than the highly charged polymeric cores (Figure 3.3c). While both these pieces of data suggested successful membrane coating, the nanoparticles were further observed under transmission electron microscopy after negative staining (Figure 3.3d). The imaging revealed a narrow size distribution, as well as a characteristic core–shell structure that further confirmed the membrane coating. The physical appearance of the nanoparticles remained

unchanged after 1 week of storage in solution (Figure 3.3e). To further test their stability in solution over time, the [CD80/OVA]NPs were suspended in isotonic sucrose, and they exhibited no increase in size during a 2-week observation period (Figure 3.3f). Finally, to verify that the nanoparticles retained the signals required for T cell activation, immunofluorescence pull-down assays were conducted to confirm the presence of intact CD80 and Kb-SIINFEKL (Figure 3.3g,h). The experiment showed that nanoparticles made using plasma membrane from the B16-CD80/OVA double knock-in clone ([CD80/OVA]NPs), expressed both markers, whereas nanoparticles fabricated from the B16-OVA single knock-in clone (denoted [OVA]NPs), was high only in Kb-SIINFEKL. As expected, nanoparticles made from B16-WT cells (denoted [WT]NPs), yielded baseline signals for both markers. While the amount of both the CD80 and Kb-SIINFEKL per [CD80/OVA]NP was dictated largely by the optimal membrane coating ratio, it is possible to modulate the density of each marker by sourcing the membrane from B16-CD80/OVA clones with different levels of expression.

With the successful fabrication of the [CD80/OVA]NP formulation, its biological activity was assessed *in vitro* to determine if it could activate antigen-specific T cells. The nanoparticles were incubated with OT-I splenocytes, and the effect on various $CD8^+$ T cell phenotypes, including $CD69^+$, $CD25^+$, central memory, and effector memory, was evaluated. A similar trend was observed as compared with whole cells, where only [CD80/OVA]NPs were able to elicit the phenotypic changes. Incubation with [OVA]NPs and [WT]NPs had minimal impact on OT-I $CD8^+$ T cell activation state. In terms of cytokine secretion, the results were striking, as signals for IL-2 and IFNγ were barely detectable for all sample groups other than the [CD80/OVA]NPs. The advantages were largely consistent when incubated with purified OT-I $CD8^+$ cells (Figure 3.4a-h), and again the double knock-in [CD80/OVA]NP formulation was able to positively modulate the

$CD44^{high}CD62L^{low}$ effector memory phenotype under this experimental setup. It was confirmed that the activity of the nanoparticles was largely retained even after storage in solution for 1 week (Figure 3.4i,j). When looking at their ability to promote antigen-specific T cell proliferation, [CD80/OVA]NPs were able to induce a significant amount of cell division as demonstrated by a dye dilution assay, whereas all of the control samples had a minimal impact on the state of the cells (Figure 3.4k). This effect was shown to be dependent on nanoparticle concentration, and [CD80/OVA]NPs at 100 μg/mL caused a majority of the T cells to experience proliferation (Figure 3.4l). The T cell activation properties of the nanoparticles was also confirmed by quantifying expansion, where the [CD80/OVA]NP-treated cells multiplied by nearly 9-fold in 4 days, whereas cell counts for all other groups dropped below the initial value at the beginning of the experiment (Figure 3.4m). OT-I $CD8^+$ cells activated using [CD80/OVA]NPs were able to preferentially kill cellular targets expressing the model antigen (Figure 3.4n). Overall, it is quite notable that the [CD80/OVA]NPs had significantly enhanced biological activity compared with [OVA]NPs, as this demonstrated that the T cell activation was not simply due to the introduction of OVA into the system. It was also confirmed that the CD80-modified nanoparticles could stimulate pmel-1 $CD8^+$ T cells specific against the melanoma antigen gp100 (Figure 3.4o).

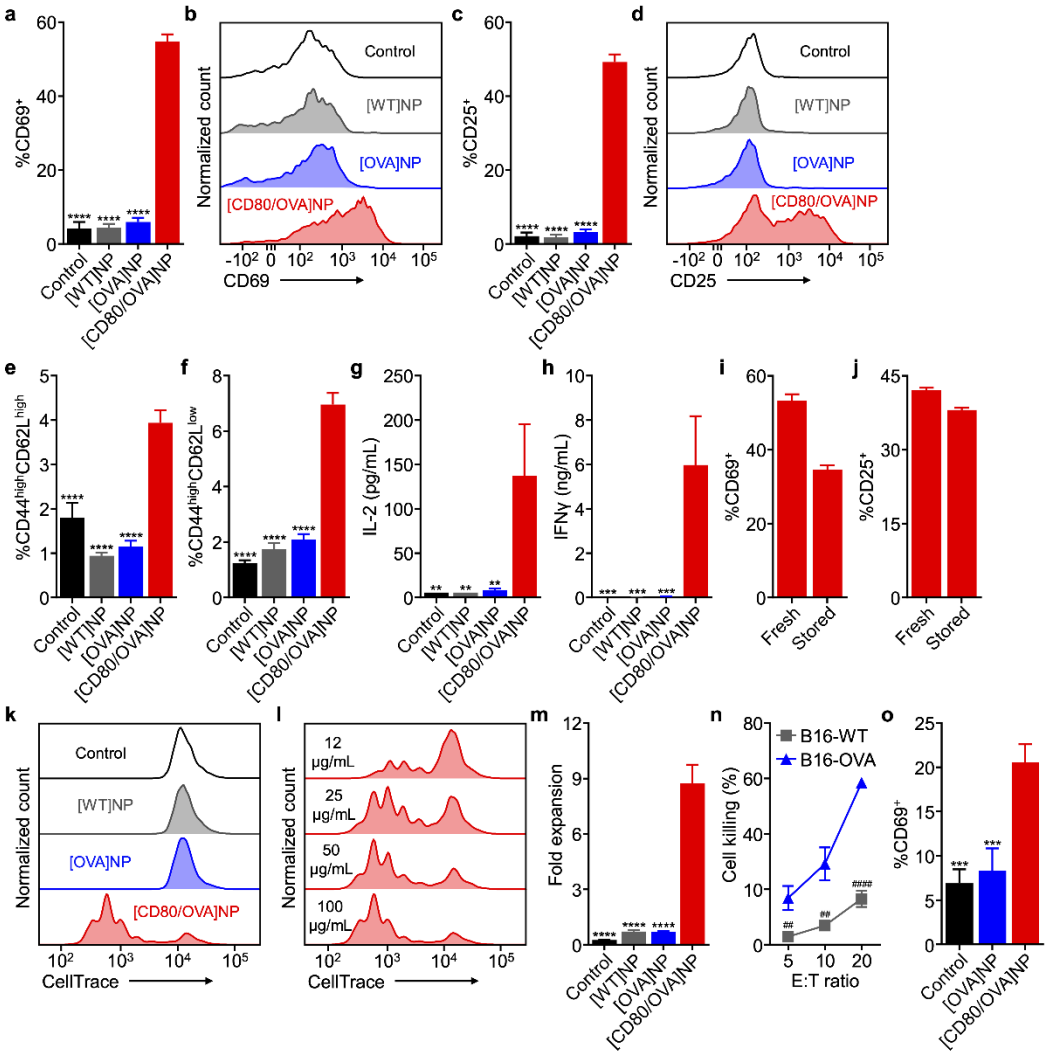

Figure 3.4: Biological activity of engineered antigen-presenting nanoparticles. a-d) Expression of CD69 (a,b) and CD25 (c,d) by OT-I CD8$^+$ T cells incubated with [CD80/OVA]NPs and control nanoparticles for 3 days (n = 3, mean + SD). e,f) Frequency of memory phenotypes CD44^{high}CD62L^{high} (e) and CD44^{high}CD62L^{low} (f) among OT-I CD8$^+$ T cells incubated with [CD80/OVA]NPs and control nanoparticles for 3 days (n = 3, mean + SD). g,h) Secretion of IL-2 (g) and IFNγ (h) by OT-I CD8$^+$ T cells incubated with [CD80/OVA]NPs and control nanoparticles for 3 days (n = 3, mean + SD). i,j) Expression of CD69 (i) and CD25 (j) by CD8$^+$ T cells in a population of OT-I splenocytes after 3 days of incubation with [CD80/OVA]NPs either freshly made or stored for 1 week (n = 3, mean + SD). k,l) Fluorescent signal dilution of CD8$^+$ T cells in a population of OT-I splenocytes labeled with CellTrace Violet after incubation with [CD80/OVA]NPs and control nanoparticles (k) or [CD80/OVA]NPs at various concentrations (l) for 3 days. m) Fold expansion of CD8$^+$ T cells in a population of OT-I splenocytes after incubation with [CD80/OVA]NPs and control nanoparticles for 4 days (n = 3, mean + SD). n) Cell killing by OT-I CD8$^+$ cells activated by [CD80/OVA]NPs for 3 days and then incubated with B16-OVA or B16-WT cells at various effector to target (E:T) ratios for 18 h (n = 3, mean ± SD). o) Expression of CD69 by CD8$^+$ T cells in a population of pmel-1 splenocytes incubated with [OVA]NPs or [CD80/OVA]NPs for 3 days (n = 3, mean + SD). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (compared to [CD80/OVA]NP); one-way ANOVA. $\#\#p < 0.01$, $\#\#\#\#p < 0.0001$; Student's *t*-test.

After confirming the activity of the antigen-presenting nanoparticles *in vitro*, we next performed a set of *in vivo* characterizations. To evaluate their transport characteristics, fluorescently labeled [CD80/OVA]NPs were subcutaneously administered into OT-I mice, and the draining lymph nodes were collected at various time points for histological analysis (Figure 3.5a). At the time of injection, it could be seen that the lymph node was absent any nanoparticle signal, while $CD8^+$ T cells were dispersed within various regions of the lymph node. At 12 h post-injection, the nanoparticle signal started to strengthen along the periphery of the lymph node. Finally, after 24 h there was a significant amount of nanoparticle fluorescence that could be visualized beyond the edges of the lymph node, and this signal was found to be adjacent to a significant number of $CD8^+$ T cells. To evaluate the biological activity of the nanoparticles after *in vivo* delivery, [CD80/OVA]NPs were administered to C57BL/6 mice that had been adoptively transferred with OT-I splenocytes. The CD69 activation marker was found to be significantly upregulated on adoptively transferred $CD8^+$ T cells in mice that were administered with [CD80/OVA]NPs, whereas those treated with [WT]NPs or [OVA]NPs had CD69 levels consistent with baseline (Figure 3.5b). This trend was also seen when looking at cytokine secretion, where cells derived from the lymph nodes of [CD80/OVA]NP-treated mice secreted significantly higher levels of IFNγ as compared to the control groups (Figure 3.5c).

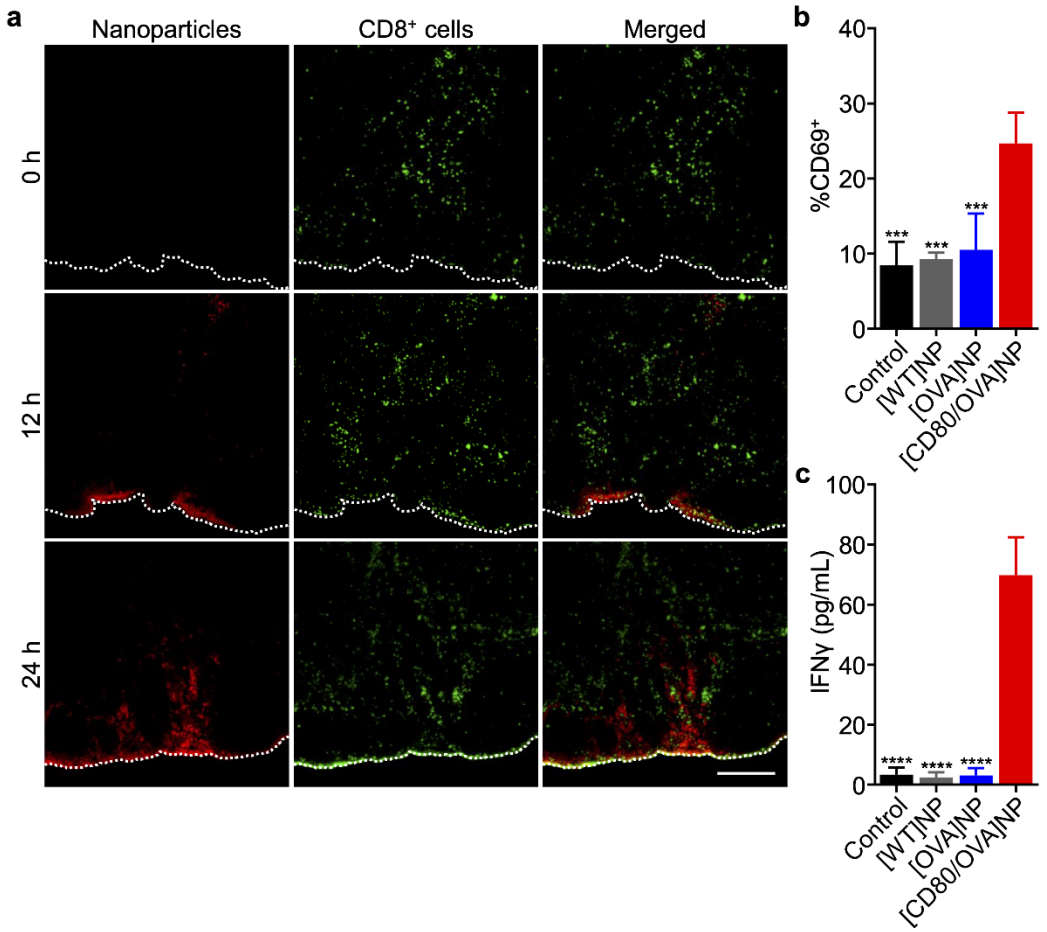

Figure 3.5: *In vivo* delivery and activity of engineered antigen-presenting nanoparticles. a) Immunofluorescence images of draining lymph node sections taken from OT-I mice at different periods after administration of dye-labeled [CD80/OVA]NPs. Red: [CD80/OVA]NPs, green: $CD8^+$ cells; scale bar = 250 μm. b) Expression of CD69 by OT-I $CD8^+$ T cells in the draining lymph nodes 3 days after administration of [CD80/OVA]NPs or control nanoparticles into C57BL/6 mice adoptively transferred with OT-I splenocytes (n = 4, mean + SD). c) Secretion of IFNγ by draining lymph node cells 4 days after administration of [CD80/OVA]NPs or control nanoparticles into C57BL/6 mice adoptively transferred with OT-I splenocytes (n = 3, mean + SD). ***$p < 0.001$, ****$p < 0.0001$ (compared to [CD80/OVA]NP); one-way ANOVA.

The ability of the [CD80/OVA]NP formulation to control tumor growth was first tested in a prophylactic setting on an immunocompetent tumor model developed using B16-OVA cells (Figure 3.6a). Mice were first irradiated, followed by adoptive transfer of OT-I splenocytes. The next day, nanoparticle formulations were administered subcutaneously, and tumor cells were implanted after another 5 days. When observing tumor growth, it could be seen that both [WT]NPs and [OVA]NPs had minimal impact on the growth kinetics when compared with the control group

administered with vehicle only (Figure 3.6b,c). On the other hand, mice treated with [CD80/OVA]NPs exhibited delayed tumor growth. This was also reflected in the survival data (Figure 3.6d), where the control, [WT]NP, and [OVA]NP groups had median survivals of 35, 34, and 35 days, respectively. In comparison, the [CD80/OVA]NP group had the best median survival of 44 days, with one mouse completely rejecting tumor challenge for the duration of the study. The antitumor activity was also evaluated in a more difficult to treat therapeutic scenario (Figure 3.6e). In this case, the tumor was implanted first, followed by irradiation for leukodepletion, adoptive transfer of OT-I $CD8^+$ cells, and then treatment with each of the nanoformulations. The [WT]NP and [OVA]NP formulations again had minimal impact on tumor growth, while the [CD80/OVA]NP formulation was able to delay the growth kinetics (Figure 3.6f,g). In terms of median survival, the blank control, [WT]NP, and [OVA]NP groups had values of 29, 31, and 27 days, respectively, while the [CD80/OVA]NP group had an extended median survival of 37 days (Figure 3.6h). It was also confirmed that therapeutic efficacy could be achieved in the absence of leukodepletion and adoptive transfer, as [CD80/OVA]NP treatment was able to delay the growth of established B16-OVA tumors in unmanipulated mice. When benchmarked against a whole cell lysate vaccine adjuvanted with CpG 1826, the antigen-presenting nanoformulation was able to better control tumor growth and prolong survival. In this case, the improved efficacy of the [CD80/OVA]NPs may likely be attributed to their ability to present more relevant antigenic material to the immune system [51].

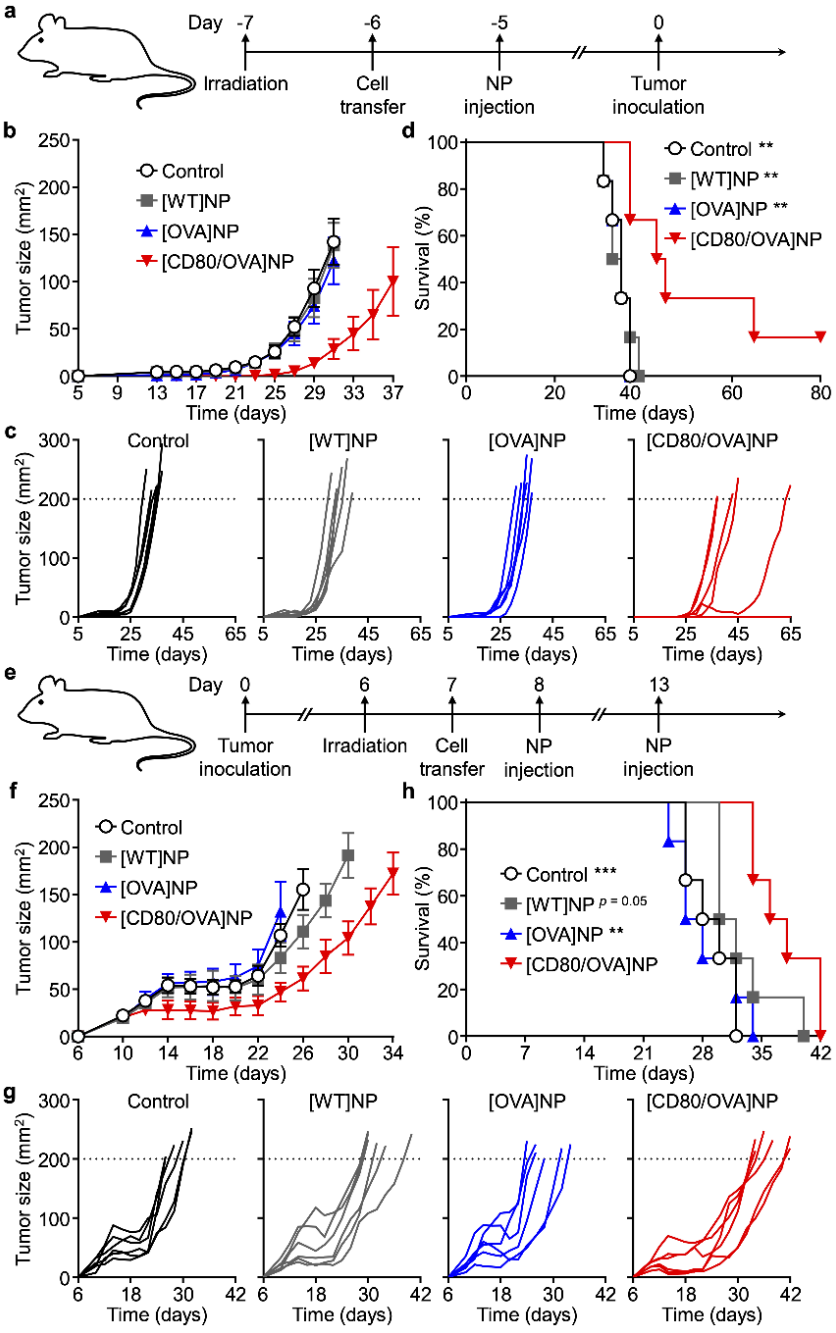

Figure 3.6: *In vivo* prophylactic and therapeutic efficacy. a) Experimental timeline for prophylactic efficacy study. b-d) Average tumor sizes (b), individual tumor growth kinetics (c), and survival (d) over time for the prophylactic efficacy study (n = 6; mean ± SEM). e) Experimental timeline for therapeutic efficacy study. f-h) Average tumor sizes (f), individual tumor growth kinetics (g), and survival (h) over time for the therapeutic efficacy study (n = 6; mean ± SEM). ** $p < 0.01$, *** $p < 0.001$ (compared to [CD80/OVA]NP in survival plot); log-rank test.

Throughout both the *in vitro* and *in vivo* assessment of our platform, only the experimental group expressing both CD80 and the OVA antigen was able to generate significant biological activity. This indicates that the observed effect was not simply due to endogenous processing of the antigenic material, but rather it was more likely a result of direct antigen presentation by the nanoparticles. In its current form, [CD80/OVA]NP was only able to promote a modest survival benefit, which may be attributed to the fact that the process for eliciting antitumor immunity is highly complex. The notion that effective antigen presentation alone cannot be expected to overcome the various immunosuppressive strategies employed by tumor cells is supported by the current landscape of antitumor vaccination, where durable responses are hard to achieve despite generation of T cell subsets with the correct specificities [52]. It is for this reason that researchers are actively exploring the combination of vaccines with other immunotherapies to more comprehensively activate immunity on multiple fronts [53], and this is a strategy that will likely benefit aAPC platforms. In addition to issues posed by the tumor microenvironment, the membrane protein expression profile of the parent cells may also present its own set of challenges. The B16-F10 cell line employed in the present study is known to express low amounts of MHC-I while also expressing programmed death-ligand 1 [33, 54], and these immunosuppressive mechanisms may combine to undermine the immune-activating stimulus provided by CD80. While the main goal of the present work was to demonstrate that immune activity can be modulated *via* genetically engineered cell membrane-coated nanoparticles, the platform could be improved through further engineering of the cancer cells to address immune evasion mechanisms or to introduce additional immune-activating surface markers. Other avenues for improving efficacy could involving optimizing nanoparticle size to maximize lymphatic drainage [55] or to pretreat the cells with IFNγ to upregulate MHC expression [29]. Overall, the strategy outlined in this article serves as a

blueprint for how to engineer complex, multimodal cell–cell interactions using biomimetic nanotechnology, and there are countless opportunities for modulating cellular function natively *via* their surface markers in a manner that is unique from traditional therapies.

3.4 Conclusions

In conclusion, we have constructed a biomimetic nanoscale aAPC platform capable of directly activating T cells against tumor antigens based on the direct presentation of epitopes found on cancer cells. This was achieved by engineering cancer cells to express costimulatory markers in order to leverage their endogenous antigen presentation machinery. The membrane from these cells, which contained the requisite signals for T cell stimulation, was then stabilized onto a nanoparticulate substrate to enable *in vivo* application. It was demonstrated that the double knock-in [CD80/OVA]NP formulation was able to control tumor growth in murine models. One of the key advantages of this biomimetic approach towards antigen presentation is its ability to bridge the gap that exists between current cell-based and synthetic nanoparticle-based anticancer immunotherapies. On one hand, the non-living nature of the biomimetic antigen-presenting nanoparticles eliminates concerns associated with the derivation, manipulation, and re-administration of patient-derived cells, which should simplify manufacture and quality control. On the other hand, the biomembrane component readily enables the presentation of multiple tumor antigens without requiring the specific identification of the relevant epitopes. Further, there has been evidence suggesting that the fluidity afforded by lipid membrane structures can enhance antigen presentation efficiency [56, 57]. It is also notable that the anticancer immunity in the present study was generated in the absence of other immunostimulatory compounds, such as

adjuvants, cytokines, or checkpoint blockades, which may be included in the future within the nanoparticle core to enhance treatment potency by providing additional immunological signals. With regards to clinical translation, the well-established workflows for modifying patient-derived cells in chimeric antigen receptor T cell therapy can be adapted for engineering autologous cancer cells prior to fabricating the antigen-presenting nanoparticles [58]. Ultimately, the platform represents an effective means of producing tumor-targeting immune cell subsets and could be combined with other modes of immunotherapy to produce a more comprehensive solution for generating robust antitumor responses in the clinic.

Chapter 3, in full, is a reprint of the material as it appears in *Advanced Materials*, 2020, Yao Jiang, Nishta Krishnan, Jiarong Zhou, Sanam Chekuri, Xiaoli Wei, Ashley Kroll, Chun Lai Yu, Yaou Duan, Weiwei Gao, Ronnie H. Fang and Liangfang Zhang. The dissertation author was a primary author of this paper.

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Chapter 4

Conclusions

4.1 Cancer Cell Membrane-Coated Nanoparticles for Anticancer Vaccination

This chapter reported on the fabrication of a biomimetic anticancer nanovaccine that can co-deliver tumor antigens and adjuvants. This anticancer vaccine utilizes natural cancer cell membrane as the multivalent tumor antigen source to generate specific immune responses to a heterogenous portfolio of cancer antigens and prevent immune evasion commonly seen in single-antigen vaccines. Adjuvants encapsulated in the polymeric core can engage dendritic cells in an immunostimulatory manner to elicit a strong immune response and prevent immune tolerance of natural tumor antigens with low immunogenicity. Formulated as a nanoparticle, the anticancer vaccine is around 120 nm in size. This nanometer size range is ideal for phagocytosis by antigen-presenting cells as well as draining into lymph nodes, where the immune priming occurs. It is demonstrated that both *in vitro* and *in vivo*, the nanovaccine can efficiently drive dendritic cell uptake and maturation, and lead to T cell activation and expansion. Furthermore, when combined with additional immunotherapies such as checkpoint blockades, the nanovaccine demonstrates substantial therapeutic effect. Overall, the work represents the rational application of nanotechnology for immunoengineering and can provide a blueprint for the future development of personalized, autologous anticancer vaccines with broad applicability.

4.2 Engineered Cell Membrane-Coated Nanoparticles Directly Present Tumor Antigens to Promote Anticancer Immunity

The recent success of immunotherapies has highlighted the power of leveraging the immune system in the fight against cancer. In order for most immunotherapies to succeed, T cell subsets with the correct tumor-targeting specificities must be mobilized.

This chapter presented a biomimetic nanoparticle platform that can be used to directly stimulate T cells without the need for professional antigen-presenting cells. Our T cell-activating nanoparticles are fabricated using a cell membrane coating derived from cancer cells engineered to express a T cell co-stimulatory marker. Combined with the peptide epitopes naturally presented on the cell membrane surface, the final formulation contains the two necessary signals to promote tumor antigen-specific T cell immune responses. It is demonstrated that both *in vitro* and *in vivo*, the nanoparticle can significantly activate and expand specific T cell subsets. Primed T cells can secrete immunostimulatory cytokines, mount cytotoxicity to tumor cells carrying the cognate antigens, and inhibit tumor growth in both prophylactic and therapeutic settings. Overall, the reported approach represents an emerging strategy that can be used to develop multiantigenic, personalized cancer immunotherapies.

4.3 Future Outlook

In the past several decades, researchers have leveraged increases in the knowledge of tumor immunology to develop therapies capable of augmenting endogenous immunity and eliciting strong antitumor responses. In particular, the goal of anticancer vaccination is to train the immune system to properly utilize its own resources in the fight against cancer. Recently, there has been a significant push in vaccine design toward the use of nanotechnology which offers the advantage of flexibility to purposefully program immune responses. This new generation of nanovaccines,

especially the novel biomimetic platforms covered in the Chapter 2 and 3 of this dissertation, has shown to elicit strong, multiantigenic antitumor responses, and can be translated to the clinic in the future as personalized immunotherapies with enhanced potency and specificity.

Toward clinical translation, there are two important considerations we could not overlook. First, there have been significant interests in recent years to use combination therapies in the clinical space. The biomimetic nanoparticles presented above could be employed in tandem with other cancer treatment regimens, such as surgical resection, chemotherapy, and radiotherapy as well as other immunostimulatory compounds, such as cytokines and checkpoint blockades, in order to simultaneously address various tumor evasive mechanisms. Second, the scaled manufacturing of biomimetic nanoparticles would be a challenge for future researchers to take on. Thanks to the prosperity of antibody drug and cell therapy for the past decade, there has been significant advancement in culturing and engineering both cell lines and autologous cells on a large scale. The relevant workflows and techniques could be adapted for handling cells and cell membrane materials to efficiently manufacture biomimetic nanovaccines.