Nano-vaccines and dendritic cell processing for anti-cancer therapies

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ABBREVIATIONS

Clinical breast exam – CBE

Breast self-examination – BSE

Triple-negative breast cancer – TNBC

Breast conserving surgery – BCS

Partial breast irradiation – PBI

Selective estrogen receptor modulators – SERM

Human epidermal growth factor receptor 2 – HER2

poly(ADP-ribose) polymerase 1 – PARP1

Epidermal growth factor receptor – EGFR

Ribonucleic acid – RNA

Circulating tumor cell – CTC

Fig – Figure

Epithelial-mesenchymal transition – EMT

miRNA – microRNA

phosphoglucose isomerase/autocrine motility factor – PGI/AMF

NAD(P)H Quinone Dehydrogenase 1/Pyruvate kinase L/R – NQO1/PKLR

C-terminal binding protein – CtBP

vascular endothelial growth factor – VEGF

Tumor necrotic factor-related apoptosis-inducing ligand – TRAIL

Fluid shear stress – FSS

Hepatitis B – HBV

Human papillomavirus – HPV

Dendritic cell – DC

Antigen-presenting cell – APC

Metastatic-castration resistant prostate cancer – mCRPC

Granulocyte macrophage-colony stimulating factor – GM-CSF

Prostatic acid phosphatase – PAP

Mechanosensitive ion channel – MSC

Calcium – Ca^{2+}

Nuclear factor-κB – NF-κB

Polyethylene glycol – PEG

Pan-DR helper T-lymphocyte epitope – PADRE

Cervical intraepithelial neoplasia – CIN

Virus-like particle – VLP

Tetanus, diphtheria, and pertussis – Tdap

Tumor microenvironment – TME

Interleukin – IL

Tumor necrosis factor – TNF

Regulatory T cell – Treg

Cytotoxic lymphocyte – CTL

Cytotoxic T-lymphocyte associated protein 4 – CTLA-4

Programmed cell death protein 1 – PD-1

Damage-Associated Molecular Pattern – DAMP

Pattern recognition receptor – PRR

Major histocompatibility complex – MHC

T cell receptor – TCR

Hereditary non-polyposis colorectal cancer – HNPCC

Immunoglobin – Ig

Ki-ras2 Kirsten rat sarcoma viral oncogene homolog – KRAS

Bacteriophage MS2 (Emesvirus zinderi) – MS2

Keyhole limpet hemocyanin – KLH

Tumor-derived exosome – TEX

Radiotherapy – RT

Mucin 1 – MUC1

Sialyl-TN – STn

Polyinosinic-Polycytidylic acid stabilized with polylysine and carboxymethylcellulose – poly-ICLC

RAS Like Proto-Oncogene A – RALA

Protein stem cell antigen – PSCA

Microneedle – MN

Ras related protein – Ral-A

Antibody- dependent cellular cytotoxicity – ADCC

Human immunodeficiency virus – HIV

Enzyme-linked immunoassay – ELISA

Monoclonal antibody – mAb

Globo H – GH

Stage specific embryonic antigen-4 – SSEA-4

Tumor-associated carbohydrate antigen – TACA

Lipid nanoparticle – LNP

Human leukocyte antigen – HLA

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine – DOPE

Interferon – IFN

Nanoparticle – NP

Trivalent live attenuated influenza vaccine – LAIV

Trivalent inactivated influenza vaccine – TIV

Glycolipopeptide – GLP

Regioselectively addressable functionalized template molecules – RAFT

Tumor-associated antigen – TAA

Ductal carcinoma *in situ* – DCIS

Helper T cell – Th

Fluoro-substituted STn analogue was coupled with a nontoxic cross-reactive material of

diphtheria toxin 107 – F-Stn-CRM197

Induced pluripotent stem cell – iPSC

Tumor nano-lysate – TNL

Deoxyribonucleic acid – DNA

Cell- line-derived xenografts – CDX

Patient-derived xenografts – PDX

Genetically engineered mouse models – GEMM

Polydispersity index – PDI

Heat shock protein – HSP

Bioluminescence imaging – BLI

Transmission electron microscopy – TEM

Phosphate buffered saline – PBS

Vanderbilt Institute of Nanoscale Science and Engineering – VINSE

Nano-particle tracking analysis – NTA

Phosphotungstic acid – PTA

Mass Spectrometry Research Center – MSRC

Propidium iodide – PI

Department of Animal Care – DAC

In vivo imaging system – IVIS

Lipopolysaccharide – LPS

Hank's balanced salt solution – HBSS

Paraformaldehyde – PFA

Red blood cell – RBC

Annexin V – AV

Propidium iodide – PI

Poly-l-lysine – PLL

Department of Animal Care – DAC

Translational Pathology Shared Resource – TPSR

Complete blood count – CBC

Peripheral blood mononuclear cell – PBMC

Hematocrit – HCT

Platelet distribution width – PDW

Platelet count – PLT

Red cell distribution width – RDW

Mean corpuscular hemoglobin concentration – MCHC

Mean corpuscular hemoglobin – MCH

Mean corpuscular volume – MCV

White blood cell – WBC

Shear rate – G

Angular velocity – ω

Angle of the cone – θ

FSS – τ

Viscosity – μ

Bovine serum albumin – BSA

Mouse serum albumin – MSA

2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose – 2-NBDG

Dimethyl sulfoxide – DMSO

Chemokine receptor type – CCR

Bone marrow dendritic cell – BMDC

Tumor-specific antigen – TSA

Prostate specific antigen – PSA

Penicillin-streptomycin – P/S

Vanderbilt-Ingram Cancer Center – VICC

Caucasian – CA

Non-Hispanic – NH

Not reported – NR

Lymph node – LN

Treatment in course 1/2/3 – C1/2/3

Plasmacytoid dendritic cell – pDC

Toll-like receptor – TLR

Conventional dendritic cell – cDC

National Health Institute – NIH

National Science Foundation – NSF

Extracellular vesicle – EV

Chimeric antigen receptor – CAR T

Multivesicular endosome – MVE

Phosphatidylethanolamine – PE

Dynamic light scattering – DLS

Scanning electron microscope – SEM

ALG-2-interacting protein X – Alix

Tumor susceptibility gene – TSG101

Natural killer – NK

Programmed death-ligand 1 – PD-L1

Cancer-associated fibroblast – CAF

Molecular weight – MW

MW cutoff – MWCO

Polyethersulfone – PES

Size exclusion chromatography – SEC

Mesenchymal stem cell – MSC

Fast Protein Liquid Chromatography – FPLC

Multi-angle light scattering – MALS

Lysosome-associated membrane protein – LAMP

Hepatocellular carcinoma – HCC

Adipose tissue-derived MSC – AMSC

Platelet-derived growth factor receptor – PDGF-R

Adeno-associated vector – AAV

Glioblastoma – GBM

Tumor-Associated Macrophage/Microglia – TAM

Hydrogenated soy phosphatidylcholine – HSPC

Membrane fusogenic liposome – MFL

Macrovesicles – MV

Paclitaxel – PTX

Gold nanorod – GNR

Multiple drug resistance – MDR

Pancreatic cancer – PaCa

Good manufacturing practice – GMP

Tumor exosome-loaded DCs – DC-TEX

CHAPTER 1:

Introduction

In the United States, the most common cancer among women is breast cancer, with 1 and 8 women expected to be diagnosed with the disease in her lifetime.[1,2] Current preventative measures for breast cancer include early detection methods such as clinical breast exam (CBE), breast self-examination (BSE) and regular screening after age 40 via mammography.[3,4] Although breast cancer mortality heavily declined after 1989 with mammography screenings, these declines have slowed down in recent years due to stagnated mammography rates.[1,5] While early stages of breast cancer are often associated with 5-year survival rates as high as 99%, these survival rates are severely reduced to 27% with more advanced, metastatic stages.[6] Metastasis accounts for around 90% of all cancer-related deaths, representing a huge need for successful anti-metastatic therapies and preventative measures.[7]

Standards of treatment

Standard of care for breast cancer includes surgery, radiotherapy, chemotherapy and targeted therapeutics.[8] Often as a part of these treatment regimens, neoadjuvant therapies are given prior to tumor removal, while adjuvant therapies are given after primary treatment.[9] Neoadjuvant and adjuvant therapies each have their advantages and are used depending on the specific treatment regimen, although some studies have identified adjuvant therapy to correspond to increased survival for triple-negative breast cancer (TNBC).[9–11]

Surgery

The most common surgical treatment for early-stage breast cancer is breast conserving surgery (BCS), which removes the primary tumor while saving as much of the breast as possible.[12] BCS has been identified as having similar long-term outcomes as the more invasive yet conventional treatment of mastectomy in combination with neoadjuvant chemotherapy while maintaining better

aesthetic results for patient quality of life.[13] Despite advances in breast cancer detection and surgical techniques, 10-40% of women require more than one BCS to completely clear the tumor, and postoperative complications often reduce patient self-esteem and delay follow-up therapies.[12,14]

Radiation

Breast cancer radiation consists of regional nodal and axillary radiation, partial breast irradiation (PBI), and more recently, hypofractionation, which uses fewer doses of higher intensity radiotherapy.[15,16] Currently, radiotherapies are applied uniformly to cancer patients, therefore, more research should be performed identifying patient need and assessing risks prior to radiation treatments.[16] There is ongoing research into neoadjuvant radiochemotherapies, which sequentially apply radiation doses and boast improved local control, but currently compromise cosmetic outcomes.[15]

Chemotherapy

Anthracycline and taxane-based therapies have been shown to have a high response rate for breast cancer patients, but overall do not demonstrate large improvements in survival.[17] Targeted biological agents and taxanes used in combination with antimetabolites have improved survival benefits in clinical trials.[17] Metastatic breast cancer is often sensitive to chemotherapies, exhibiting controlled patient symptoms and prolonged survival.[18] Nevertheless, it has been noted that certain molecular subtypes of metastatic breast cancer respond to chemotherapies better than others, so further exploring these subtypes may be necessary for effective therapeutics.[19]

Targeted therapeutics

One of the major battles of breast cancer targeted therapies is fighting against resistance.[20] These resistance methods include mutation of the target, activation of upstream or downstream

pathways and activation of alternative pathways.[20] Estrogen and estrogen receptors are frequent therapeutic targets as they often drive breast cancer progression.[20–22] Selective estrogen receptor modulators (SERM) and aromatase inhibitors have been used to reduce hormone levels to treat estrogen-dependent breast cancers.[20] However, many patients develop resistance to anti-estrogen treatments.[21] Non-hormone-dependent targets include human epidermal growth factor receptor 2 (HER2), which is commonly overexpressed in breast cancers.[20,23,24] Trastuzumab was the first drug approved by the FDA for the treatment of HER2 in 1998.[25] Drug-antibody conjugates have been developed based on trastuzumab, which aids in the delivery of the drug emtansine to cancer cells, stunting their growth through microtubule inhibition.[21,26]

TNBC, which accounts for 15-20% of all breast cancers, lacks hormone receptors and upregulated HER2 and is therefore more difficult to treat with targeted therapeutics.[27,28] HER1 has been investigated as a potential target in TNBCs as well as the use of poly(ADP-ribose) polymerase 1 (PARP1) inhibitors, but more recent studies have demonstrated the success of combined cetuximab and cisplatin chemotherapy, indicating potential epidermal growth factor receptor (EGFR) sensitivity of these cells.[20] Nevertheless, chemotherapy remains the most common treatment regimen for TNBC until better targets are identified.[20,28] Interestingly, six potential subsets of TNBC have been identified via ribonucleic acid (RNA) microarray – immunomodulatory, mesenchymal, mesenchymal stem-like, luminal androgen receptor, and two basal-like – and these may respond differently to specific therapies.[29] There are enormous benefits to the development of novel gene expression prognostic tests for breast cancer such MammaPrint, MapQuant Dx, Oncotype DX, PAM50, and Theros Breast Cancer Index.[30]

Metastasis

Five steps contribute to a successful metastatic cascade: (1) invasion and migration, (2) intravasation, (3) dissemination, (4) extravasation, and (5) colonization.[31] Tumor cells leave a primary tumor, squeezing through blood vessels to enter the bloodstream where they then travel as circulating tumor cells (CTCs) and have the potential to form a secondary tumor at a distant site (**Fig. 1.1**).[32] Studies have shown that only 0.1% of CTCs survive in circulation, and of these only 0.01% form successful secondary tumors.[33] Finding ways to target these rare CTCs in circulation can prove essential to anti-metastatic therapies.[34–36]
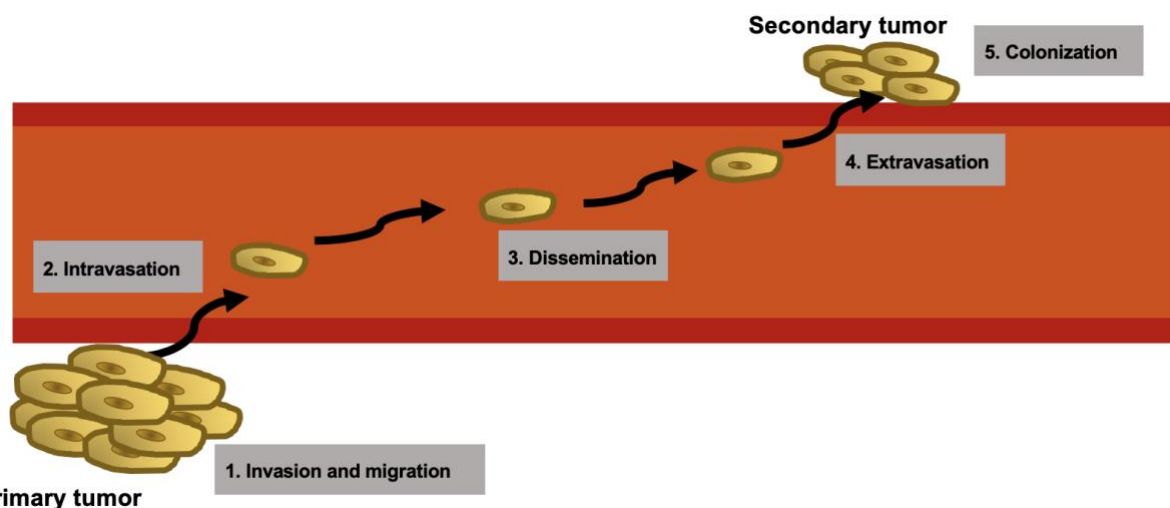
Figure 1.1 Metastatic cascade. For the successful formation of metastases, tumor cells come from a primary tumor, enter circulation, and travel through the bloodstream. Once in the circulation, these cells can form a secondary tumor in a distant microenvironment.

Breast cancer is often associated with poor prognosis as it metastasizes to lymph nodes and distant organs.[37] It is imperative to find a way to prevent metastasis in early stage breast cancer, and treat it in later stages. As in most cancers, metabolic reprogramming plays a role in breast cancer metastasis, and is therefore a potential target for preventing progression.[38] Epithelial-mesenchymal transition (EMT) is induced by metabolic rewiring and is a significant mechanism that gives cancer cells metastatic potential and aggressiveness.[37,39,40] Anti-metastatic therapies often block EMT induction to prevent metastasis as well as drug resistance.[40] For instance,

miR200s have been used as targets to block EMT induced by phosphoglucose isomerase/autocrine motility factor (PGI/AMF).[41] The NAD(P)H Quinone Dehydrogenase 1/Pyruvate kinase L/R (NQO1/PKLR) network and C-terminal binding protein (CtBP) are also identified as necessary for EMT, and are potential anti-metastatic therapeutic targets.[40] Angiogenesis, a necessary factor for successful metastasis, is mediated by vascular endothelial growth factor (VEGF).[20,37,42–44] VEGF has been explored as a target for breast cancer therapies and while by anti-angiogenesis treatments alone did not reduce overall survival, they have shown more success when used as a neoadjuvant with chemotherapy.[45–48]

Mouse models

Breast cancer and subsequent metastases can be studied using a variety of models including 2D, 3D and in vivo models.[49] 4T1 cells represent a syngeneic in vitro model of mammary carcinoma[14]. This model has demonstrated high metastatic and invasive potential of TNBC and can be orthotopically transplanted in the mammary fat pad of mice via subcutaneous injection for in vivo studies.[50–52] With a high disposition for forming metastasis in the brain, bones, lungs and liver, the 4T1 model is very useful for understanding CTCs and developing anti-metastatic therapies.[49]

TRAIL therapies

For patients with metastasis, therapies focus primarily on improving the patient's quality of life and reduce symptom burden.[53] However, progress is underway for the development of successful targeted anti-metastatic therapies, including TRAIL-based therapies.[34,54–56] TRAIL, or tumor necrotic factor-related apoptosis-inducing ligand, is a therapeutic that binds to death receptors on cancer cells and spares healthy cells (**Fig. 1.2A**).[57] While early TRAIL therapies were halted due to poor targeting efficacy in vivo, it has been further explored with conjugation to liposomes.[58]

Liposomal TRAIL treatments, and other anti-metastatic therapies designed to act in the bloodstream, can be modeled via cone-and-plate viscometers, which mimic the physiological fluid shear stress (FSS) of blood flow that CTCs are exposed to in vivo (**Fig. 1.2B**).[34,54–56,59] Previous research in the King Lab demonstrated that TRAIL-liposomal therapy was able to target CTCs and prevent metastasis in the 4T1 model.[56] In that study, mice received primary tumor resections in combination with doses of either soluble TRAIL, unbound liposomes, or TRAIL-liposomes decorated with adhesion molecule, E-Selectin, to allow for binding to leukocytes. Mice receiving E-selectin-TRAIL liposomes survived significantly longer than mice receiving soluble TRAIL or naked liposomes, and 2 of 10 mice in the treatment group remained completely tumor free for the remainder of the study.[56]
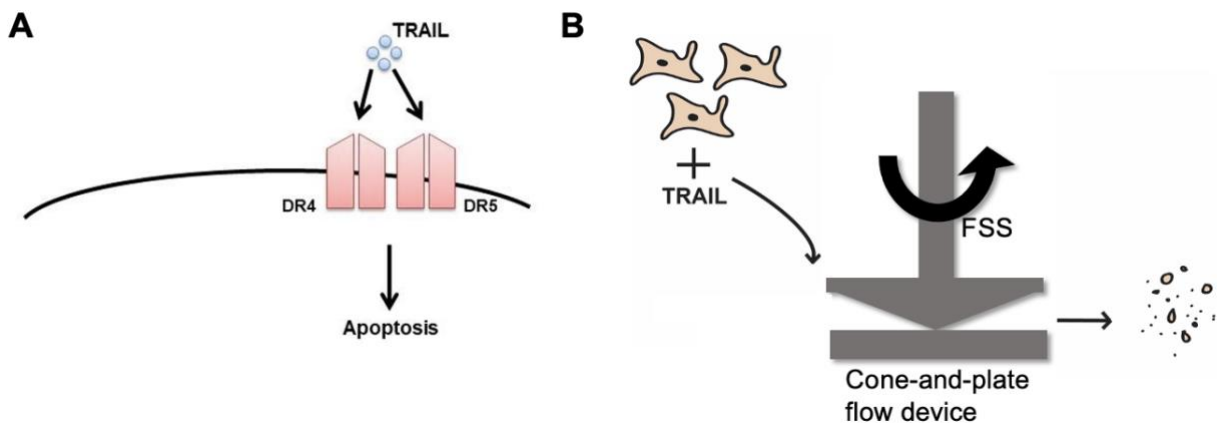
Figure 1.2 TRAIL therapies. A. Tumor necrosis factor related apoptosis inducing ligand (TRAIL) binds to death receptors 4 and 5 on cancer cells. TRAIL spares noncancerous cells from apoptosis. **B.** Cone-and-plate viscometer setup combining CTCs with TRAIL, resulting in cell destruction.

Cancer cell fragmentation is a byproduct of TRAIL therapeutics in vitro, and these cell fragments are subsequently released into the bloodstream in vivo.[60] Given that studies investigating TRAIL as a therapeutic did not observe complete ablation of 100% of all CTCs, it is likely that some evade destruction by TRAIL.[34,54–56] Remaining CTCs and cancer cell fragments likely initiate an

adaptive immune response, which may further explain how some mice remained completely tumor free following treatments.[56]

Preventative vaccines

For over 200 years, vaccines have been used to prevent and, in some cases, eradicate a variety of infections and diseases.[61] Cancer remains one disease state that, despite decades of developments in understanding and therapeutics, few advances have been made in prevention beyond increasing awareness and screening measures.[1] There are only five FDA-approved cancer vaccines that are used in clinical practice, all preventing one of two sexually transmitted viral infections, hepatitis B (HBV) and human papillomavirus (HPV).[62] These viruses are known to promote cancers such as oralpharyngeal, liver and cervical cancer.[63–66] These vaccines have been largely successful, and the development has led to a rapid decline in associated cancers in regions where there is more widespread vaccination.[62]

Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) that are an essential link between the adaptive and innate immune systems.[67] DCs uptake foreign antigens, generate subsequent MHC-peptide complexes and migrate to lymphoid organs to formulate an immune synapse and stimulate T cells.[68,69] With these unique abilities, DCs have been the focus of many studies for both preventative and therapeutic vaccines for cancer.[70–75] In 2010, Provenge (Sipuleucel-T) was the first FDA-approved cancer vaccine for metastatic castration-resistant prostate cancer (mCRPC).[76] This vaccine activates a patient's DCs ex vivo using granulocyte macrophage-colony stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP); these activated DCs are reintroduced into the patient, where they home to lymphoid organs and stimulate T cells.[76] Despite these exciting advances in DC-based ex vivo therapeutics, treatment costs remain high and new data have shown that Provenge has limited effectiveness.[77–79]

Mechanosensitive ion channels

Cellular responses to mechanical stimuli are facilitated by mechanosensitive ion channels (MSCs), which open upon sensing a mechanical force and allow for an influx of ions.[80–82] The resulting biochemical signal of MSCs drives many key cellular functions.[83–85] Forces have been observed to play a major role in activating a variety of cell types through these channels, from cancer cells to endothelial cells to immune cells.[86,87] Shear stress is one such force that MSCs like TRPV4 and Piezo1 can sense and subsequently open to allow for the influx of calcium (Ca^{2+}).[83,84,88–92] A ubiquitous secondary messenger, Ca^{2+} leads to a cascade of cellular responses once inside the cell.[88–91] The MSC Piezo1, specifically, plays a role in cell cycle progression, differentiation and proliferation.[85] Piezo1 has been observed to enhance tumorigenesis in prostate cancer cells, with the influx of Ca^{2+} activating Akt and mTOR, facilitating cancer cell survival, proliferation and migration.[93]

Immune cell activation

Blood flow applies a shear stress to immune cells in the circulation.[94] Transcription of nuclear factor-κB (NF-κB), an important regulator of cytokine expression in immune cells, is initiated by an influx of Ca^{2+}.[95–99] Studies have demonstrated that T cells are sensitive to shear stress, as observed by increased activation and proliferation.[100–103] For DCs, cyclic strain has been applied in one study and consequently MHC II and co-stimulatory molecule expression were increased.[104] While not widely explored in the context of applied shear stress, NF-κB is significant for DC survival and maturation, and for the progression of cell cycle.[81,105,106]

Conclusions

Breast cancer is a major epidemic that necessitates better ways to either prevent or control the disease. Despite early detection methods, there remains a gap in cancer prevention. Additionally,

while often successful at treating early-stage cancers, current therapeutics are expensive and have low efficacy for patients with metastatic cancer. Successful therapeutics often take advantage of the immune system to elucidate a preventative response or promote specific targeting of cancer cells. Here, we develop, characterize, and analyze a preventative, DC-targeting vaccine for TNBC, and develop and explore a DC-based anti-cancer vaccine.

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CHAPTER 2:

Prophylactic Cancer Vaccines Engineered to Elicit Specific Adaptive Immune Response

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ABSTRACT

Vaccines have been used to prevent and eradicate different diseases for over 200 years, and new vaccine technologies have the potential to prevent many common illnesses. Cancer, despite many advances in therapeutics, is still the second leading causes of death in the United States. Prophylactic, or preventative, cancer vaccines have the potential to reduce cancer prevalence by initiating a specific immune response that will target cancer before it can develop. Cancer vaccines can include many different components, such as peptides and carbohydrates, and be fabricated for delivery using a variety of means including through incorporation of stabilizing chemicals like polyethylene glycol (PEG) and pan-DR helper T-lymphocyte epitope (PADRE), fusion with antigen-presenting cells (APCs), microneedle patches, and liposomal encapsulation. There are currently five cancer vaccines used in the clinic, protecting against either human papillomavirus (HPV) or hepatitis B virus (HBV), and preventing several different types of cancer including cervical and oral cancer. Prophylactic cancer vaccines can promote three different types of adaptive responses: humoral (B cell, or antibody-mediated), cellular (T cell) or a combination of the two types. Each vaccine has its advantages and challenges at eliciting an adaptive immune response, but these prophylactic cancer vaccines in development have the potential to prevent

or delay tumor development, and reduce the incidence of many common cancers.

INTRODUCTION

Vaccines have improved the human condition since Edward Jenner developed the first vaccine to prevent smallpox over 200 years ago, paving the way for the prevention and even eradication of many common ailments [1]. Cancer is the second leading cause of death in the United States, with 1.89 million people projected to be diagnosed with cancer in 2021 alone [2], [3]. While there are many successful therapeutics for cancer treatment, advances in prophylactic vaccination against cancer have been limited.

Preventative, or prophylactic, cancer vaccines have the potential to reduce cancer prevalence and improve prognosis by inducing an immune response to prevent the development of specific cancers. Currently, five vaccines are used in clinical practice and approved by the FDA. These vaccines protect against two cancer-promoting viral infections, hepatitis B (HBV) and human papillomavirus (HPV) [4]. HPV is a sexually transmitted infection, with several of its forms associated with different types of cancers, the most common being cervical and oral cancer. Individuals vaccinated with the HPV vaccine are protected from cancer by preventing HPV development; since HPV can promote the onset of cervical cancer, HPV prevention is expected to lead to its decline [4]. In Scotland, women vaccinated for HPV showed an 89% reduction in cervical intraepithelial neoplasia (CIN) grade 3 or worse when compared to non-vaccinated women. Similar reductions were shown in CIN grade 1 and grade 2 [5]. Furthermore, vaccination against HBV, a risk factor for hepatocellular cancer, of infants in Taiwan has shown reduced cancer prevalence. Rates of hepatocellular cancer in vaccinated Taiwanese children age 6-14 years fell approximately 70% [4].

While preventative vaccines are commonly implemented, preventative vaccines designed to

protect against cancer are a relatively new development. The goal of preventative cancer vaccines is not to treat, but to prevent the development of a tumor. Cancer vaccines are often defined as therapeutic vaccines, which are different from prophylactic vaccines in that they elicit an immune response to an existing tumor and to residual cancer cells following other treatments [6]. Therapeutic vaccines against cancer elicit immune responses following the onset of disease. For example, proposed therapeutic vaccines against breast cancer can target human epidermal growth factor receptor 2 (HER2), utilizing T cells to elicit a targeted immune response [7]. While strides are being made in therapeutic vaccines for cancer, many different strategies have been proposed for the development of prophylactic cancer vaccines (**Fig. 2.1**).
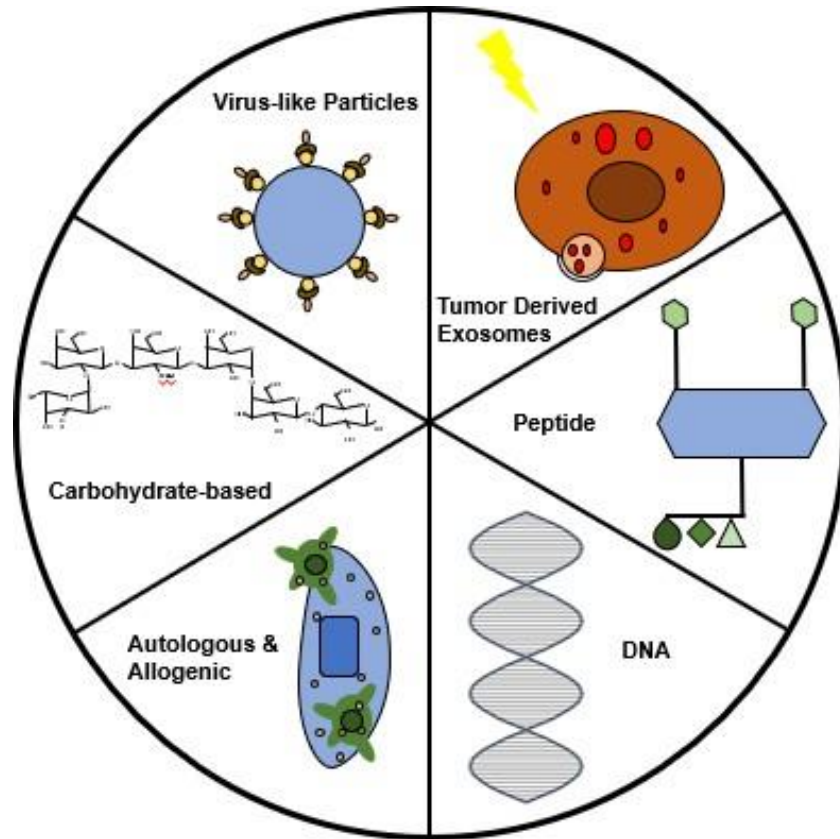
Figure 2.1. Summary of vaccine strategies. Each vaccine strategy has shown promise. Further investigation into each strategy could lead to clinically-relevant prophylactic cancer vaccines. In this figure, the virus-like particle (VLP) represents the HER2-VLP, which has elevated levels of anti-HER2 antibody to protect against breast cancer. The carbohydrate displayed is the chemical structure of Globo H, which has often been used in therapeutic vaccines, but shows potential for prophylactic vaccine development. The allogenic vaccine displays dendritic cells (DCs) recognizing the tumor antigen, which can allow for immune cell activation. The double helix of DNA is the building block for all DNA vaccines. The peptide vaccine shows four epitopes engineered for display, which caused upregulation of CD4+ and CD8+ T cells in addition to increases in IgG antibodies in vaccinated mice. The exosome shows TEX synthesis via radiotherapy that prevented breast cancer via CD8+ T cells.

cost benefits. Preventative treatments reduce morbidity and mortality, and current vaccines from childhood vaccines like Tdap (Tetanus, diphtheria, pertussis) to influenza vaccines have led to economic benefits in low- and middle-income countries [8]. Successfully engineered cancer vaccines could decrease health care costs associated with cancer. It has been estimated that the total cost of cancer in the United States would increase 34% in just fifteen years, from $183 billion in 2015 to $246 billion in 2030 [9]. Thus, decreasing the cost of cancer treatments through preventative vaccines could result in dramatic decreases in healthcare costs associated with cancer.

Vaccines have demonstrated the ability to successfully eradicate previously common diseases, controlling the spread of 12 diseases, such as smallpox and yellow fever [10]. Disease eradication is a common and efficient way to improve public health [10]. The successful development of preventative cancer vaccines could decrease the prevalence of cancer, reducing cancer-related deaths. HPV vaccines have already reduced the prevalence of cervical cancer. In Scotland, an 89% reduction in grade 3 or worse cervical intraepithelial neoplasia (CIN) was seen for women vaccinated at 12-13 years old [11]. Cervical cancer prevention with HPV vaccines provide promise that vaccines can be developed for other cancers to achieve similar results. Several possible strategies for cancer prevention will be discussed in this review, with each presenting distinct advantages and challenges (**Table 2.1**). Age-related immune decline is seen across all vaccine engineering strategies as a major challenge.

Table 2.1. Advantages and disadvantages of different prophylactic cancer vaccine strategies currently being investigated.

Vaccine Strategy	Advantages	Disadvantages
Virus-like Particles	Overcome B cell tolerance [54] Humoral and cellular responses [109]	Must be highly stable for proper downstream applications [50]
Carbohydrate-based	Ease of synthesis [60] Target unique target glycans [57] Humoral and cellular responses [110]	Poor immunogenicity [62]
Peptide	High stability against degradation *in vivo* [77] Ease of synthesis [78] Humoral and cellular responses [101,102]	Inefficient immune response [75]
Lipid Nanoparticle	Overcome genetic material degradation [65] Easily synthesized [66]	Difficult to evaluate and predict *in vivo* effectiveness to identify proper dosage and side effects [66]
DNA	Stable at ambient temperatures [87] Ease of preparation [87] Humoral and cellular responses [90]	Inadequate immunogenicity [88]
Tumor-derived Exosomes	Play natural role in tumor progression [91]	Primarily cellular response [95]
mRNA	Low manufacturing cost [96] Potential high potency Possible non-invasive administration [98]	*In vivo* stability [96] Primarily cellular response [98]
Autologous Tumor Cell	Personalized formulations [111] Humoral and cellular responses [113]	Requires patient tumor cells [111] Mainly therapeutic currently [112]
Allogenic Tumor Cell	Clinical trials for therapeutic version [114] Humoral and cellular responses [99,117]	Limited current effectiveness [115] Mainly therapeutic currently [115]

Role of the Immune System in Cancer

A unique aspect of cancer is its ability to survive in the presence of an immune system, making immunotherapy a challenging yet promising therapeutic for cancer. This property stems from two essential hallmarks of cancer: tumor-promoting inflammation and avoiding immune destruction

[12]. Tumors utilize the immune system by generating an inflammatory response conducive to tumor growth [12]. The tumor microenvironment (TME) consists of neoplastic tissue, which is highly disorganized and grows uncontrollably [13]. Neoplastic progression is supported by inflammation of cytokines like interleukin-1 (IL-1) and tumor necrosis factor (TNF) [14], [15]. Necrotic cells within the TME stimulate proliferation of neighboring cells through the release of IL-1α, and angiogenesis is driven by IL-1β [16], [17]. IL-18 induces vascular cell adhesion expression, supporting invasion and metastasis [18]. TNF-α promotes tumor development by regulating factors such as cytokines, chemokines, adhesion molecules, and matrix metalloproteinases (MMPs) [19].

Tumors evade immune detection, and therefore destruction, through a variety of means including regulatory cells, down-modulating antigen presentation, tolerance, and immune suppression [12], [20]. Not only does the hypoxic tumor promote regulatory T cell (Treg) homing to the TME, but tumor-derived CD4+CD25+FoxP3+ Tregs have been found to be more suppressive of cytotoxic lymphocytes (CTLs) than normal Tregs [21]–[23]. Tumors are able to evade CTL recognition by down-modulating essential components of antigen processing and presentation such as the MHC I pathway. [20]. Tolerance is induced by tumor cells, since they do not express co-stimulatory molecules that are needed to activate T cells or antigen-presenting cells (APCs) [24]. Furthermore, cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are upregulated on cancer cells, inhibiting a T cell response [25]. The combination of these various traits of cancer contribute to the difficulty of the immune system to independently stop tumor development, making a prophylactic vaccine a useful approach for cancer prevention.

Cancer Vaccines and the Immune System

The goal of a prophylactic cancer vaccine is to elicit an adaptive primary immune response, to allow for a rapid and strong secondary response if carcinogenesis occurs [26], [27]. The

mechanism behind these preventative vaccines can be viewed as specific immunity to modified self-antigens, therefore producing an immune response to cells that have undergone malignant transformation [28]. Cancer vaccines can be developed to recognize and prevent cancer-promoting viruses or neoantigens, which are peptides found on tumor cells that are associated with spontaneous cancers [29].

Microbes and other foreign bodies included in a vaccine alert the host immune system via presentation of Damage-Associated Molecular Patterns (DAMPs), which cause innate immune cells such as APCs to produce cytokines necessary for activating T cells (**Fig. 2.2**). This activation
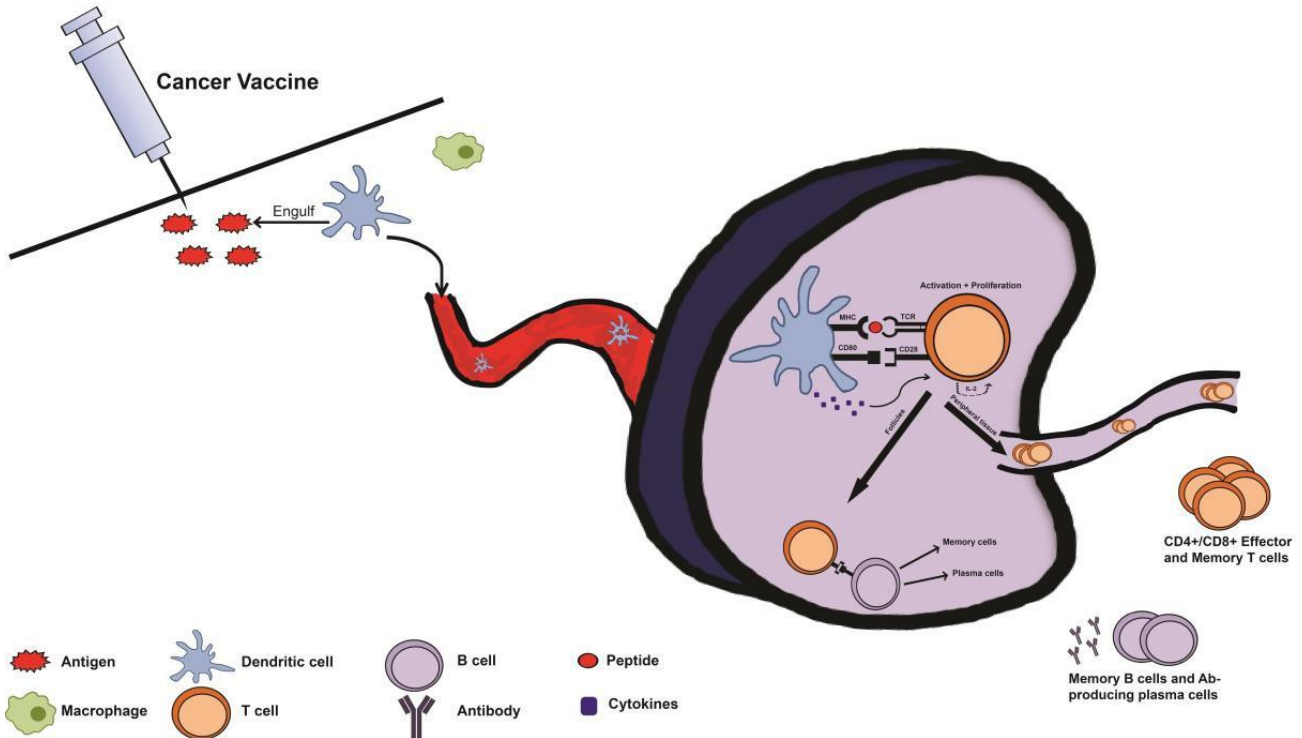
Figure 2.2. Immune system response with prophylactic cancer vaccine administration. Following administration of a cancer vaccine, antigen-presenting cells (APCs) from the innate immune system such as macrophages and dendritic cells (DCs) recognize the injected antigen as foreign via pattern recognition receptors (PRRs), and uptake the antigen. Subsequently, the APCs transport the antigen, migrating to a lymph node and processing and presenting the antigen via major histocompatibility complex (MHC) pathway. Once in the lymph node, an immune synapse will form as the APC presents the antigen to an immature T cell at the T cell receptor (TCR). T cells will be activated by this interaction, with the aid of cytokines and co-stimulatory signals from the APC. Upon activation, T cells proliferate via IL-2 production and differentiate into effector T cells depending on cytokines and MHC type from the APC. These T cells can then contribute to the activation of B cells or travel to distant sites as effector or memory T cells. This primary response following vaccination produces memory cells so that secondary exposure to cancer-associated antigens results in a rapid and robust secondary immune response.

ultimately leading to lasting immunity [26]. By producing this adaptive response, a vaccine develops memory for protection from an antigen [27]. Adaptive immune responses can consist of T cell-mediated cellular responses, B cell-mediated humoral responses, or combinations of the two [30]. By activating T and B cells, a vaccine can produce memory T and B cells, which are essential for stopping another attack or antigen exposure [30]. These memory cells proliferate, causing a stronger, faster response upon a second exposure [27].

While there are many preventative cancer vaccines being developed in the research setting, there are only five clinically-relevant cancer vaccines, three for HBV and two for HPV [4]. These vaccines are successful because they avoid major issues in the development of a therapeutic vaccine: an immunosuppressive tumor microenvironment, low immunogenicity of the antigen, and a disease with high incidence [31]. Successful prophylactic cancer vaccines take advantage of the immune system to provide lasting benefits of cancer prevention. Cancer vaccines can be used to prevent the formation of virally onset cancers or spontaneous cancers by initiating immune responses against a virus or neoantigen target.

Safety concerns and challenges of prophylactic cancer vaccines

It is essential that preventative vaccines, given to healthy patients, do not cause any adverse side effects such as an autoimmune response [32]. Therefore, high risk individuals—those with increased risk of a specific cancer—are often the best candidates for such vaccines. [32]. Patients with syndromes such as hereditary non-polyposis colorectal cancer (HNPCC) have a genetic predisposition for specific cancers, motivating the development of a viable preventive measure [33]. Since many of the prophylactic vaccines developed involve unnecessary exposure to cancer antigens, these vaccines must be engineered to ensure antigens do not increase cancer risk. This could also pose a problem for public acceptance and successful implementation of prophylactic cancer vaccines into the clinic. Other safety concerns include off-target effects and toxicity related

to any possible vaccine materials [34]. Successful engineering of prophylactic vaccines must consider the issue of safety earlier in development than therapeutic vaccines, as preventative vaccines are intended for healthy individuals.

There are several challenges that prophylactic vaccines must overcome that are described in this review. These obstacles include poor immunogenicity of common vaccine formulations, and poor stability *in vivo*. Furthermore, prophylactic vaccine trials may need to be proceeded by therapeutic vaccine development, as vaccine dosages for healthy clinical trial participants must be low. Immune system decline in elderly patients is another challenge faced by prophylactic cancer vaccines, as adaptive immunity is paramount to vaccine success. With 70% of cancer-related deaths occurring in patients 65 and older, this poses a significant problem that must be addressed [35].

HUMORAL CANCER VACCINES

The different types of cancer vaccines are summarized in **Table 2.2**. Humoral, or antibody-mediated, vaccines invoke B cell responses to prevent disease and have the ability to last for decades, a goal of preventative cancer vaccines. For instance, smallpox vaccines can cause the maintenance of vaccinia-specific IgG+ (Immunoglobin G) memory B cells for more than 50 years [36]. Another benefit of humoral vaccines is the possibility for secondary tumor antigen targeting. One phenomenon, known as epitope or antigen spreading, is an important concept in vaccine development, where an immune response develops for epitopes that are different than the disease-causing epitope, allowing for more complete and robust protection from disease [37, 38]. Studies have shown epitope spread can increase the effectiveness of previously developed therapeutic cancer vaccines [39]. For example, Sipuleucel-T, which is FDA approved, is a therapeutic autologous immunotherapy vaccine for metastatic castration-resistant prostate cancer that results in elevated levels of antigen spread. This results in higher levels of IgG against

secondary tumor antigen, increasing overall survival [37]. Epitope spread has also been associated with tumor regression [40]. In one study, highly specific intramolecular epitope spreading was partly responsible for preventative effects of a vaccine against KRAS (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog)-induced lung cancer [41].

Table 2.2. Summary of the most promising prophylactic cancer vaccine formulations and possible antigens and targets associated with each vaccine strategy.

Vaccine Strategy	Example Vaccines Developed	Antigens/Targets
Virus-like Particles	Cervarix and Gardasil – Commercially available vaccines for HPV [52] HER2-VLP Vaccine – Vaccine for HER2-Postive breast cancer [55] MS2 mRNA-based VLP – Vaccine for prostate cancer [109]	HER2 – protein [55]
Carbohydrate-based	4-KLH Vaccine – Vaccine for colorectal cancer targeting STn [63]	Sialyl-TN – oncofetal antigen [62]
Peptide	KRAS-targeting Peptide Vaccine – Vaccine for lung cancer [85] MUC1-poly-ICLC Vaccine – Vaccine for colorectral cancer [103]	KRAS – proto-oncogene [85] MUC1- glycoprotein and TAA [103]
DNA	RALA-pPSCA-loaded MNs – Vaccine for CRPC [90] HPV Polynucleotide Vaccine – Vaccine for HPV16 and HPV18 [119]	Ral-A – protein [90] E6/E7 – oncoprotein [120]
Tumor-derived Exosomes	RT-TEX Vaccine – Vaccine for mammary carcinoma [95]	Multiple targets [95]
mRNA	Nasal Encapsulated mRNA Vaccine – Vaccine encoding for tumor antigen [98] MS2 mRNA-based VLP – Vaccine for prostate cancer [109]	Multiple targets [98, 109]
Allogenic Tumor Cell	Tumor Nano-Lysate Vaccine – Vaccine for triple negative breast cancer [117]	Multiple targets [117]

Humoral vaccines offer other advantages in the form of neutralization and antibody- dependent

cellular cytotoxicity (ADCC). These mechanisms protect cells from viral infection instead of controlling previously infected cells. Neutralizing antibodies function by binding to the virus, alerting the immune system to the presence of a foreign body and preventing the virus from infecting a cell [42]. Antibody neutralization of the virus HPV can prevent infection by multiple mechanisms, such as prevention of cell surface binding and disruption of virus internalization [43]. Humoral protection via neutralization of oncoviruses is an effective strategy to prevent some cancers, such as cervical cancer. ADCC, which utilizes innate immune cells to provide antitumor activity by linking antibodies to target cells, is also a vital part of the humoral response. Natural killer cells play a major role in ADCC, as they are responsible for provoking the immune response and direct cytotoxicity of cells infected by a virus and tumor cells [44]. One study found that the success of a preventative human immunodeficiency virus (HIV) vaccine could be partially attributed to an ADCC response [45]. Several cancer vaccines, including a MUC1- based cancer vaccine, have successfully elicited an ADCC response [46].

Verifying successful humoral response requires accurate quantification of antibodies and protein expression in patient plasma and tissue samples. Research has shown that many patients have a natural immunity to mesothelin, a glycoprotein expressed in several common cancers. ELISA analysis of IgG antibodies in patient serum and immunohistochemistry analysis of mesothelin protein expression of tumor specimens can be used to evaluate the effectiveness of vaccines and find potential antigen targets [47]. A significant disadvantage of humoral vaccines stems from elderly patients having particularly weak humoral immune responses. Aging is associated with decreased B cell levels, which are essential for humoral vaccine success. *In vitro* and *in vivo* studies on tetanus toxoid showed decreases in IgG secretion in elderly patients, with younger patients not only having more B cells, but more potent B cells [48]. Following immunization, younger patients also displayed elevated IgG levels for up to a year, while elderly patients returned to baseline levels after only 6 months [48]. Since the age distribution of cancer patients

skews towards the elderly, this is particularly concerning for the development of humoral cancer vaccines. Thus, when engineering cancer vaccines, especially for cancers originating from the lung, prostate and colon, which are common in elderly patients, it is vital to consider decreased humoral response [49].

Virus-like Particles

Virus-like particles (VLPs) are protein structures with multiple subunits [50]. Several VLPs have been engineered to prevent cancer by eliciting a humoral immune response, often through increasing IgG levels. In the past, VLPs have been used to treat many different viruses, as VLPs closely resemble the structure of the virus it is being used to prevent but lack virus-specific genetic material [50], [51]. Specifically, commercially available vaccines against HPV are VLP-based, including Cervarix and Gardasil, in addition to the HBV vaccine [52]. However, stability remains a large concern for VLP vaccine development, as VLP vaccine success depends on downstream effects leading to a need for high stability [50]. VLPs have been investigated to treat viruses vaccines must consider the issue of safety earlier in development than therapeutic vaccines, as preventative vaccines are intended for healthy individuals.

A VLP vaccine has been engineered to treat and prevent human epidermal growth factor receptor-2 (HER2)-positive breast cancers [54]. To synthesize this VLP, S2 insect cells were transfected to express SpyCatcher-HER2 fusion antigen and incubated with Spytagged VLPs for a stable antigen coating. Transgenic mice, which can spontaneously develop HER2-positive mammary carcinoma, were vaccinated with this HER2-VLP. Vaccinated mice showed no tumor growth until one year of age, while untreated mice developed tumors after only two months. Elevated levels of anti-HER2 antibody were found in vaccinated mice for at least 24 weeks [54]. HER2-VLP induced a stronger antibody response and provided better protection against tumor onset than a previously studied DNA vaccine, which had been more effective than passive administration of

trastuzumab in HER2 transgenic mice. Furthermore, the anti-HER2 antibodies induced by the vaccine showed comparable affinity to that of monoclonal antibody (mAb) trastuzumab, a HER2-targetting antibody, and the vaccine showed decreased tumor onset when compared to mAb trastuzumab passive administration [55]. The HER2-VLP vaccine inhibited not only tumor onset, but tumor growth, suggesting both preventative and therapeutic effects achieved by the vaccine [54].

Importantly, the HER2-VLP vaccine overcame B cell tolerance, a phenomenon which occurs when B cells die to prevent autoreactive antibody synthesis and is a frequent obstacle for humoral vaccine development [55]. VLP vaccines can overcome this issue since they exhibit multivalent display of self-antigen [54]. One study demonstrated that multivalent VLP induced higher IgG titers and overcame the effects of anergy [56]. This outcome is likely due to VLP multivalency increasing the ability to create stable signaling domains, causing an increase in B cell activation [56]. Using anti-HER2 antibodies from the mice, IgG antibodies elicited strong binding to HER2-positive human tumor cell lines, but no binding was detected on HER2-negative cell lines.

Carbohydrate-based Vaccines

Using carbohydrate structures to induce an immune response is a promising direction in the field of vaccines [57]. Cell-surface glycans are targeted by carbohydrate-based vaccines [57]. While most carbohydrate-based vaccines are currently limited to therapeutics for infectious diseases, applications for preventative cancer vaccines have been proposed and studied [57]. Specifically, the hexasaccharide Globo H (GH) has been proposed to both treat and prevent cancer [58]. Globo H is a carbohydrate located on the outer membrane of epithelial cells and is often overexpressed in a variety of tumor specimens, including breast, ovarian, and lung cancer. [59]. Huang et al. proposed synthesizing a GH and linking it to a carrier protein as a therapeutic treatment for SSEA4-expressing breast cancers [58]. Although this is strictly therapeutic, mice treated with this

vaccine elicited IgG antibodies against the stage specific embryonic antigen-4 (SSEA-4) ganglioside, which can be overexpressed in breast cancer [58].

GH can be synthesized via glycal chemistry, one-pot synthesis or enzymatic synthesis. Among these methods, enzymatic synthesis is the cheapest and easiest, and requires enzymes overexpressed in *Escherichia coli* [60]. Using sugar nucleotide regeneration and glycosyltransferases, GH can be synthesized in just three steps [60]. The GH vaccine engineered by Danishefsky et al. not only induces anti-GH antibodies, but also anti-SSEA3 and anti-SSEA4 antibodies. These three glycoproteins are overexpressed on over 16 cancer types [61]. Additionally, a glycolipid adjuvant was designed, which targeted CD1d receptors found on dendritic and B cells to cause a shift to IgG production. This process induces a switch from IgM, which is usually the sole response induced by carbohydrate-based vaccines [61]. While this vaccine functions currently as a therapeutic, Danishefsky et al. indicate the possibility of using this vaccine in a preventative manner. The proposed design lays a framework for successful engineering of future carbohydrate-based vaccines. Unique glycan markers associated with cancer can be identified for use as a target, and then a carbohydrate compound can be designed using chemical and immunological processes to effectively leverage the target for cancer prevention [61].

A common problem with carbohydrate vaccines is poor immunogenicity of tumor-associated carbohydrate antigens (TACAs). Sialyl-TN (STn) is an oncofetal antigen found in specific cancers and has been used as an adjuvant to boost the immunogenicity of TACAs [62]. One study couples three fluoro-substituted STn analogues to the metalloprotein keyhole limpet hemocyanin (KLH). Fluorine-modified STn compounds can be used to increase immunogenicity and thereby increase the strength of the immune response [63]. Previously, it has been shown that 4-KLH, a fluorine-modified STn antigen, results in increased IgG levels when compared to anti-modified-STn [64].

Both therapeutic and preventative effects were observed *in vivo*. 4-KLH-vaccinated mice inoculated with colorectal cancer showed increased anti-STn antibodies when compared to a 1-KLH vaccine. 4-KLH also showed some preventative effects when compared to the control [63]. This result could provide the framework for STn-KLH vaccines as a means to prevent cancer formation when used with the proposed fluorine modification strategy.

Lipid Nanoparticle Vaccines

Lipid nanoparticle (LNP) vaccines have the potential to effectively deliver genetic information for cancer prevention. Delivery of mRNA and DNA to the body has potential to prevent cancer, but degradation is often a problem for delivery of naked genetic material [65]. Use of LNPs can help overcome these problems for preventative cancer vaccines. LNPs are easily synthesized and can protect mRNA or DNA from degradation [66]. However, there are challenges associated with LNP vaccine development. Assays to effectively predict *in vivo* responses do not currently exist, as current assays only measure second-order effects. LNPs may complete their goal successfully, but if certain pathways are not activated, these effects will be undetectable to current assays. This presents challenges when evaluating different formulations, dosages, and side effects [66].

CELLULAR CANCER VACCINES

Cellular vaccines induce CD8^{+} and CD4^{+} T cell activity [67]. For many successful vaccines, memory T cell induction is vital to eliciting a sufficient immune response to stop disease formation. This response requires large-scale changes in both the properties and number of T cells [68]. The idea for cellular vaccines against cancer originated from successful T cell-mediated vaccines for viral infections. T cell-mediated vaccines can have both preventative and therapeutic benefits. For example, a vaccine engineered to prevent HPV and cervical cancer development induces CD8^{+} T cells, which provides lasting protection against HPV and associated diseases [69]. While

engineering new vaccines, it is important to consider that a sufficient dose is required to induce a T cell response strong enough to prevent disease, so a high dosage must not be toxic. Another concern with engineering cellular vaccines is overexertion of T cells [70]. An overexerted immune system can cause T cell exhaustion, and ultimately, dysfunction. T cell exhaustion is the result of sustained expression of inhibitory receptors, low effector function, and an altered transcriptional state. It leads to decreased immune control of tumors and infections, and poor memory formation [70]. Exhaustion can occur during chronic infection and cancer, making it a significant problem that must be addressed when engineering cellular vaccines against cancer [70]. Another concern with cellular vaccines, as with humoral vaccines, is age-associated decline. T cell-mediated immunity declines with age due to alterations in the thymus, signal transduction and human leukocyte antigen (HLA) Class II expression on monocytes [71]-[73]. Aging is also associated with decreased T cell reactivity to foreign antigens [71]. Despite these concerns, the successful engineering of preventative cellular vaccines against oncogenic viral infections, which cause 15% of cancers worldwide, offers promise for similar solutions to cancer prevention [74].

Peptide Vaccines

Peptide vaccines use engineered short peptide fragments to induce a specific immune response [75], [76]. Longer amino acid chains may also be used, but shorter chains are most common [76]. Peptide vaccines can be engineered to have stability against degradation *in vivo* and are cost effective and easy to synthesize [77], [78]. Nevertheless, they suffer from poor immunogenicity [75]. A peptide vaccine designed to prevent breast cancer was formulated with stabilizing chemical pan DR epitope (PADRE), a carrier epitope used to engineer synthetic and recombinant vaccines [79]. A nanoliposomal vaccine was designed using DOPE-containing liposomes and engineered with three different peptides (AE36, E75, and E75-AE36) used in combination with PADRE. Vaccinated mice showed higher CD4+ and CD8+ T cell induction when compared to mice treated with liposomal short peptides without PADRE and mice treated with non-liposomal

peptides. Furthermore, increased IFN-γ (interferon- γ) levels were observed, which promotes adaptive immune mechanisms [80], [81]. IFN-γ also plays a role in promoting tumor surveillance, although the exact mechanism is unknown. Previous studies have hypothesized that IFN-γ may even be the basis for immune surveillance. Nevertheless, it is clear that IFN-γ plays a role in directing tumor surveillance to chemically-induced tumors, as well as tumors caused by genetic defects [81].

Transmembrane protein GP2 has also been explored for use in peptide vaccines [82]. GP2 vaccines have been explored as viable means to prevent breast cancer reoccurrence for HER2/neu+ patients. A polymorphism leading to a mutant GP2 protein called 2VGP2 was found at codon 655 of the HER2/neu protein and has been identified as a common mutation associated with higher risk of breast cancer [83]. Autologous DCs from blood samples from HLA-A2 breast cancer patients were pulsed with synthesized GP2 and used to stimulate T cells *in vitro*. Cytotoxicity experiments showed killing of breast and ovarian cancer cells via GP2-stimulated CD8$^+$ T cells. These experiments confirm GP2 immunogenicity and show its potential as a peptide vaccine against HER2/*neu*$^+$ breast cancer [84].

A KRAS-targeting peptide vaccine has been engineered to prevent lung cancer. KRAS is considered a proto-oncogene, with mutant KRAS a common driver of cancer [85]. A KRAS peptide vaccine was developed with four peptides corresponding to different regions of the protein, and CpG, R848, and anti-CD40 were used as adjuvants. This vaccine increased IFN-γ and granzyme B levels in CD8$^+$ T cells, and when combined with avasimibe, resulted in infiltration of CD8$^+$ T cells in tumors and prevented KRAS-driven lung tumorigenesis. Thus, this vaccine may be a starting point to develop vaccines to prevent premalignant lung legions with mutant KRAS from progressing to malignant lesions [86].

DNA Vaccines

DNA vaccines are appealing due to the ability to mimic natural infections, ease of production, and stability at ambient temperatures [87]. Several DNA vaccines have been developed to prevent prostate cancer but have had mixed results in clinical trials, DNA vaccines often failing due to inadequate immunogenicity [88], [89]. A DNA vaccine was proposed to prevent castration resistant prostate cancer (CRPC), using RALA/pPSCA nanoparticles (NP) incorporated into a dissolvable microneedle (MN) patch. RALA codes for Ras-related protein Ral-A, a protein implicated in several cancers, and pPSCA is a plasmid encoding prostate stem cell antigen. RALA/pPSCA-loaded MNs caused endogenous production of prostate stem cell antigen, and induced a response against TRAMP-C1 tumors ex vivo and anti-tumor immunity *in vivo*. In prophylactic experiments, unvaccinated mice developed palpable tumors within seven days of tumor implantation, whereas vaccinated mice showed delayed tumor growth. On average, tumor development took 16.2 days for RALA/pPSCA-loaded MNs-treated mice, with one mouse remaining tumor free through the duration of the experiment. This study shows that the use of microneedles to administer a DNA vaccine could be a promising strategy to prevent cancer formation [90].

Tumor-Derived Exosomes

Exosomes are microvesicles released by cells in physiological and pathological settings; exosomes are enclosed by a lipid bilayer with proteins from the origin cell. These cargo exosomes can assist in tumor progression and cancer metastasis through delivery of parental cell proteins and nucleic acids to target cells [91]. The proteins or nucleic acids in cargo exosomes that contain antigens associated with the parental cancer cells could, therefore, become targets for prophylactic vaccination. Tumor growth is promoted by tumor-derived exosomes (TEX), exosomes released from tumor cells. They signal to both cancerous and normal cells throughout the body and play a role in cancer progression [92]. A vaccine against breast cancer has been

engineered using TEX. TSA, a BALB/c mouse-derived mammary carcinoma, was exposed to Sham radiotherapy (RT) to develop TEX [93], [94]. A vaccine of TEX from untreated cells (UT-TEX) was also used. The RT-TEX vaccine induced a tumor-specific CD8$^+$ response, with 2 of 6 mice vaccinated showing no tumor growth and 4 showing reduced tumor growth compared to UT-TEX-vaccinated mice. RT-TEX-vaccinated mice had a higher number of CD8$^+$ T cells in the tumor, many of which were specific to an immunodominant antigen in the tumor. This supports the idea that TEX produced via irradiated cancer cells is a viable strategy for cancer prevention [95].

mRNA Vaccines

mRNA vaccines have the advantage of low-cost manufacturing and potential for high potency. However, stability *in vivo* is a large concern for successful engineering of mRNA vaccines against cancer [96]. Even so, mRNA-based vaccines independent of VLP carriers are in development. Previous methods have injected DCs transfected with mRNA with promising results, but this method is costly [97]. Another study recommended nasal administration of an mRNA vaccine for the prevention of cancer. Nasal administration is promising due to its non-invasive format and high patient compliance. Tests were performed for nasal administration of naked and nanoparticle encapsulated mRNA for tumor prevention using a mouse model. The mRNA was encoding for a tumor antigen. While the naked mRNA administration did not prevent tumor growth, nasal administration of mRNA encapsulated in nanoparticles was effective for tumor prevention. Therapeutic effects were also observed in additional experiments. Splenocytes recovered from the mice revealed anti-cancer CD8+ T cells in mice treated with the encapsulated mRNA vaccine. As one of the few mRNA vaccine studies available for cancer vaccination, this study shows possible effectiveness of mRNA vaccines for cancer prevention in addition to showing possible effectiveness of nasal administration of prophylactic and therapeutic cancer vaccines [98].

COMBINED CELLULAR AND HUMORAL CANCER VACCINES

As discussed above, there are advantages to both humoral and cellular vaccines. However, many vaccines induce both a humoral and cellular immune response. Vaccines can cause a biased immune response towards one type of adaptive immunity while inducing both T and B cell immunity [99]. The benefit of a combined humoral and cellular response can be seen in many non-cancer vaccines. For instance, for influenza prevention, the trivalent live attenuated influenza vaccine (LAIV) induces both B cell and T cell responses. Conversely, the trivalent inactivated influenza vaccine (TIV), which only invokes a T cell response, has been found to be immunologically inferior [100].

Combined Peptide Vaccines

While many peptide vaccines induce only a cellular immune response, others can induce both humoral and cellular responses. A mimotope peptide-based vaccine was developed using BAT monoclonal antibodies, which have immune modulatory and anti-tumor effects [101]. Mimotopes are peptides that can bind to an antibody directed against a certain antigen [102]. For this vaccine, BAT-binding peptides A and B were used as mimotopes. Vaccinated mice displayed increased IgG antibody production, which competed with BAT binding on Daudi cells, a human B lymphoblast. The IgG antibodies caused similar immune stimulation to BAT, indicating a humoral component to the vaccine. The observed cellular response included increased cytolytic activity, and the vaccine prevented tumor growth *in vivo* in a mouse model [101].

A self-adjuvanting multivalent glycolipopeptide (GLP) has also been developed as a vaccine [102]. The GLP vaccinse displays four components on a molecular delivery system: TACA B cell epitope, CD4$^+$ Th peptide epitope, CD8$^+$ CTL peptide epitope, and immunoadjuvant palmitic acid. This GLP vaccine was administered in combination with PADRE and regioselectively addressable

functionalized template molecules (RAFT). *In vivo*, vaccinated mice did not develop tumors over a 90-day period, while unvaccinated mice developed tumors around 35 days after tumor inoculation. Serum collected from BALB/c mice showed IgG antibodies specific to breast cancer, and upregulation of CD4+ and CD8+ T cells, indicating both humoral and cellular responses [102].

Another combined peptide vaccine has undergone clinical trials for the prevention of colorectal cancer. A MUC1-poly-ICLC vaccine has been tested for patients with advanced adenomatous polyps, which are a precursor to colorectal cancer. MUC1 is a glycoprotein and tumor-associated antigen (TAA) for colorectal cancer. Around 43% of patients showed elevated anti-MUC1 IgG levels following vaccination, and long-term memory was observed. T cell response and memory were also measured following a booster vaccine [103]. Thus, this vaccine formulation could be useful for prophylactic vaccination in some patients with advanced adenomatous polyps [104].

A vaccine for patients with ductal carcinoma *in situ* (DCIS) has been developed to prevent progression. DCIS is often associated with HER-2/*neu* overexpression. Patients were given 4 doses of the Her-2/*neu*-pulsed dendritic cells. This vaccine resulted in lower HER-2/*neu* expression and T and B lymphocyte accumulation in the breast. Tumorlytic antibodies were observed. This vaccine lowers HER-2/*neu* expression in addition to decreasing residual DCIS following resection. These results suggest possible prophylactic value for this vaccine formulation [105].

Virus-like Particles

VLPs have been studied as a way to induce a combined humoral and cellular response. An mRNA-based VLP was developed to target prostate cancer, which has shown responsiveness to mRNA-based vaccines in previous studies [106], [107]. Obtaining sufficient *in vivo* potency for

nucleic acid vaccines has been difficult, as repeated use of viral vectors results in a dampened immune response [108]. A recombinant bacteriophage MS2 mRNA-based VLP was developed using pESC yeast epitope tagging vectors, and PEG precipitation for synthesis to induce both a humoral and cellular response [109]. VLP-vaccinated C57BL/6 mice exhibited elevated levels of IgG antibodies and increased antigen-specific cytotoxic T lymphocytes. Further investigation found that the initial Th2 (helper T) response was converted to Th1 by target proteins. Mice were injected with TRAMP-C2 cells, a murine model of prostate cancer, and vaccinated mice were effectively protected from tumor development [109]. This vaccine offers many advantages when compared to other nucleic acid vaccines, such as easy preparation using recombinant protein technology, and production of a strong humoral and cellular response.

Carbohydrate-based Vaccines

Carbohydrate-based vaccines have been engineered to induce a combined humoral and cellular response. A fluoro-substituted STn analogue was coupled with a nontoxic cross-reactive material of diphtheria toxin 107 (F-Stn-CRM197) for cancer prevention. When combined with Freund's adjuvant, F-STn-CRM197 had significantly higher IFN-γ- and IL-4-releasing splenocytes compared to control. Vaccinated mice showed elevated levels of anti-STn IgG antibodies, which were further elevated with Freund's adjuvant. The F-STn-CRM197 vaccine increased cellular and humoral immune responses when compared to a STn-CRM197 vaccine. This immune response resulted in increased cancer cell lysis. The data of Song et al. suggests the utility of this vaccine as a cancer prophylactic, building a basis for future carbohydrate-based cancer vaccine development [110].

Autologous Tumor Cell Vaccines

Autologous tumor cell vaccines are derived from a patient's own tumor, and although this personalized formulation has been exclusively therapeutic, it holds potential as building blocks for

preventative vaccines [111]. Agenus' autologous tumor cell vaccine, AutoSynVax, has controlled tumor growth and produced lasting immune responses in recent pre-clinical and phase I clinical trials [112]. While this vaccine is therapeutic, the development of autologous tumor cell vaccines to prevent recurrence is underway to prevent cancer recurrence in the setting of high-risk cancer patients. For instance, the use of autologous induced pluripotent stem cells (iPSCs) has been proposed for the development of an autologous tumor cell vaccine [113]. Mice vaccinated with iPSCs combined with a CpG adjuvant were inoculated with B16F0 melanoma cells four weeks later. Vaccinated mice showed decreased tumor progression, and spleen analysis revealed increased tumor- specific effector and memory helper T cells. Additionally, there were more mature APCs found in the vaccinated group. While antibody analyses were not included in the study, increased IgG responses were measured in therapeutic experiments, indicating the possibility that this formulation could successfully induce both a humoral and cellular response. This study showed that prophylactic immunization with non-genetically engineered iPSC-based vaccines produce immune responses to melanoma. These vaccines have the potential for tumor immunity to a larger number of cancer types, which is supported by the large number of tumor antigens presented. Both humoral and cellular effects were observed. The use of autologous iPSCs was suggested as they may provide an accurate and personalized panel of a patient's tumor immunogens [113].

Allogenic Tumor Cell Vaccine

Allogenic tumor cell vaccines differ from autologous vaccines in that they are derived from another patient's cells. Melacine, an allogenic tumor cell vaccine for treatment of melanoma, has undergone phase I, II, and III clinical trials, and survival benefits for patients were observed [114]. The overall success of allogenic tumor cell vaccines has been limited to therapeutic immunotherapy [115]. A preventative vaccine has been proposed using a vaccine derived from the fusion of allogenic DCs with tumor cells [99]. DC-tumor fusion vaccines allow for the

presentation of a broad spectrum of tumor-associated antigens [116]. This vaccine was engineered using PEG-mediated fusion between DCs and inactive gastric cancer cells. The fused cell (FC) vaccine was combined with CTLs to prevent gastric cancer. Vaccinated mice showed slowed tumor growth compared to the control, with 9 of 10 remaining tumor-free and surviving for 90 days. The vaccine successfully induced cytotoxic T lymphocyte cloning through induction of antigenic determinants, resulting in anti-tumor effects. Furthermore, IL-7 and IL-15 levels increased following immunization, indicating immune memory formation. Elevated levels of IFN-γ and IL-10, which enhances B cell survival and antibody production. This study verified antigen-presenting and tumor-targeting effects from DC-based tumor vaccines and provides a template for future vaccine development [99].

Our group recently fabricated a preventative vaccine for triple negative breast cancer [117]. This vaccine was developed by sonicating 4T1 breast cancer cells and delivering the tumor nano-lysate (TNL) to BALB/c mice via tail vein injection 10 d before tumor inoculation. Tumor growth and metastasis were significantly delayed, and survival was increased for mice in the vaccinated group compared to the unvaccinated group. While the TNL-vaccinated mice ultimately developed tumors, the success of this simple process motivates future studies to engineer similar vaccines to produce a preventative response [117].

DNA Vaccines

The idea of deoxyribonucleic acid (DNA) vaccines has received much attention over the past decade [118]. A DNA vaccine to induce both cellular and humoral responses *in vivo* has been proposed to prevent HPV infection, specifically high risk HPV16 and 18, or HPV16-E7-expressing tumors [119]. These viruses, encoding for oncoproteins E6 and E7, promote cervical cancer development [120]. The proposed polynucleotide vaccine uses a designed DNA sequence coding for an E6/E7 fusion protein. Vaccinated mice had complete tumor prevention when injected with

TC-1 cells, a tumor cell line derived from primary lung epithelial cells that are E6- and E7-expressing [121]. Vaccination resulted in an E7-specific antibody response that lasted at least five months. E6- and E7-specific T cells could be identified after 5 months [119]. Despite the many obstacles to successful DNA vaccines, this vaccine serves as evidence that the use of prophylactic cancer vaccines is possible and should be further studied [119].

CONCLUSIONS

Preventative vaccines have helped to eradicate many diseases. Cancer remains one of the leading causes of death and healthcare burden in the United States. The development of prophylactic cancer vaccines has the potential to save lives and reduce healthcare costs by going beyond treating cancer to preventing it altogether. These vaccines are currently in a variety of development stages, from concept design and research, to implementation, and clinical practice.

Some of these vaccines produce humoral or cellular immune responses, with associated advantages and disadvantages. While, humoral vaccines allow for long-term immune protection and may be used to target secondary tumor antigens, B cell tolerance can limit their effectiveness. Cellular vaccines can have both preventative and therapeutic benefits, but T cell exhaustion is a common problem that needs to be addressed. Engineered vaccines that induce both humoral and cellular immune responses could represent an innovative solution to address these shortcomings. However, all cancer vaccines must consider age-related immune decline, a problem magnified by the elevated age distribution of cancer patients. Aging is associated with decreased B cell prevalence and potency, attenuating the effectiveness of humoral immune responses. Furthermore, multifactorial phenomenon, including changes to the thymus, cause a decrease in T cell reactivity, resulting in reduced cellular immunity. These challenges require new, innovative solutions. One possible solution is combining cancer vaccine administration

with immune augmentation treatments. Vaccinated patients with high-risk of cancer development may be given continuing doses, with increasing frequency as they age.

Many strategies have been discussed in this review to prevent tumor development via cellular, humoral or a combined immune response. VLPs and carbohydrate-based vaccines have been designed to induce humoral responses or a combined humoral and cellular immune response. VLP vaccines are able to overcome B cell tolerance due to their multivalent display of self-antigens, but stability must be addressed. Carbohydrate-based vaccines, which have the advantage of targeting unique glycan markers, often show poor immunogenicity. Peptide vaccines and DNA vaccines are able to induce a cellular response or a combination of humoral and cellular. Peptide vaccines are usually engineered to have high stability against degradation *in vivo* and are easy to synthesize but suffer from inefficient immune responses. DNA vaccines are easy to produce and stable, but exhibit inadequate immunogenicity. mRNA vaccines have the potential for high potency but lack *in vivo* stability. Autologous and allogeneic tumor cell vaccines utilize both cellular and humoral immunity, but most current uses are therapeutic in nature. Maximizing the potential of the immune system may be necessary to successfully engineer preventative cancer vaccines, requiring the utilization of both humoral and cellular immunity. Further research into these strategies will lead to improved prophylactic cancer vaccines.

Despite the benefits of each type of vaccine, DNA and mRNA vaccines are garnering increased attention. With new technologies being developed, it seems that DNA and mRNA vaccines may offer the most promise for future research. DNA and mRNA vaccines may be developed to specifically target tumor antigens and promote specific immune responses against tumor onset. New technologies in LNPs and other nanomaterial carriers may help overcome stability problems associated with mRNA vaccines, increasing potency of the vaccine.

Currently, there are very few prophylactic cancer vaccines on the market. Gardasil and Cervarix prevent HPV, while Energix-B, Recombivax HB and Hiberix-B prevent HBV, two viruses commonly associated with cancer development [122]. These vaccines have been highly successful, and the number of cervical cancer patients has decreased as HPV and HBV vaccination has become more prevalent. Following these successful viral vaccines, there is great potential in preventing cancers caused by viruses, which account for 15% of all cancers. Development of vaccines to prevent the remaining 85% of cancer types is underway. However, significant obstacles remain in the development of vaccines for these cancers not caused by viruses. While vaccines against HPV and HBV stop cancer through viral protection, preventing cancers with no known viral etiology will be much more challenging. Researchers must identify viable targets, engineer successful delivery mechanisms, and find long-lasting immune effects. Many current technologies allow for preventative success in non-human tests, but one of the major problems will be finding solutions at clinically relevant doses. Further issues include prevention of immune response to self-antigens. This challenge may be overcome by identifying cancer-specific membrane expression or pre-malignant tumor properties to target. One possible way to overcome this issue is through targeting of neoantigens, a type of tumor-specific antigen, as they are recognized as non-self by the immune system [29]. Another possible target includes tumor-associated antigens, although those are more difficult since they are also found in healthy cells. Finding possible targets to prevent tumor onset is critical for successful vaccine development for spontaneous cancers.

Despite these obstacles, current research points to vaccine strategies that could be viable for cancer prevention. Success in animal models offers a promising template for clinical development. Several strategies discussed in this review seem viable for future development; additional insights may come by engineering solutions that combine multiple approaches.

However, the benefits of prophylactic vaccine development justify these difficulties. Prophylactic cancer vaccines could be administered to high-risk groups. For example, those with familial risk of triple negative breast cancer would be ideal candidates for vaccination with a breast cancer vaccine. Patients with genetic predisposition for cancer, such as those with hereditary non-polyposis colorectal cancer (HNPCC), a genetic predisposition to colorectal cancer, would also make ideal candidates for vaccination. Successful development of prophylactic cancer vaccines will lead to new challenges: when to administer vaccines, ideal vaccine patients, and proper monitoring of vaccine success in patients. Eliciting strong and lasting immune response is critical for the success of prophylactic vaccine implementation. Furthermore, immune responses must be directed at targets unique to tumor cells during the early stages of carcinogenesis. Likely, vaccines with the most success will elicit both humoral and cellular responses as they work together to strengthen anti-tumor response upon tumor onset. This will include T cell memory, antibody generation, and possible responses by other immune cells, such as dendritic cells. Vaccines can be engineered to induce these responses. Successful immune induction will likely include engineered peptides or carbohydrates combined with stabilizing chemicals. Development and fabrication of both primary components and stabilizing chemicals, such as PEG, PADRA, or liposomal encapsulations, could lead to the prevention of spontaneous cancer formation.

With a foundation for preventative cancer vaccines established, and approved vaccines to prevent two cancer-associated viruses, there is hope that more types of cancer will be prevented by engineering vaccines to evoke a specific immune response. Targeting and promoting the adaptive immune system to respond to a preventative, anti-cancer vaccine will be crucial to the adoption of more successful, prophylactic cancer vaccines.

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CHAPTER 3:

Fabrication and Characterization of Tumor Nano-Lysate as a Preventative Vaccine for Breast Cancer

This chapter is adapted from *Fabrication and Characterization of Tumor Nano-Lysate as a Preventative Vaccine for Breast Cancer* published in *Langmuir* in 2020. The work has been reproduced with the permission of the publisher and co-authors Nidhi Jyotsana, Davis W. Crews, Zhenjiang Zhang and Michael R. King.

ABSTRACT

Brest cancer is the most common cancer among women in the United States, with late stages associated with the lowest survival rates. The latest stage, defined as metastasis, accounts for 90% of all cancer-related deaths. There is a strong need to develop antimetastatic therapies. TRAIL, or TNF-related apoptosis inducing ligand, has been used as an antimetastatic therapy in the past, and conjugating TRAIL to nanoscale liposomes has been shown to enhance its targeting efficacy. When circulating tumor cells (CTCs) released during metastasis are exposed to TRAIL-conjugated liposomes and physiologically relevant fluid shear stress, this results in rapid cancer cell destruction into cell fragments. We sought to artificially recreate the phenomenon using probe sonication to mechanically disrupt cancer cells and characterized the resulting cell fragments, termed "tumor nano-lysate", with respect to size, charge, morphology, and composition. Furthermore, an in vivo pilot study was performed to investigate the efficacy of tumor nano-lysate as a preventative vaccine for breast cancer in an immunocompetent mouse model.

INTRODUCTION

One in eight women in the United States will be diagnosed with breast cancer in her lifetime, and breast cancer can also afflict men, although it is less common. Later stages of breast cancer, accompanied by the lowest 5-year survival rates, are defined as distant or metastatic. Metastasis accounts for 90% of all cancer-related deaths.[1-3] While there has been improvement in the 5-year survival rate for breast cancer, there is still a significant disparity; in fact, while survival rates are 99% for patients diagnosed with local disease, they drop to 27% for metastatic breast cancer.[4] In the process of metastasis, a cancer cell derived from a primary tumor intravasates into the bloodstream as a circulating tumor cell (CTC). CTCs travel through the circulation with the potential to form a secondary tumor in a distant microenvironment.[5] Primarily, metastatic therapies focus on quality of life and prolonged survival for patients, given the severity of the stage of the disease.[2,6,7] However, some novel treatments for metastatic patients have emerged. Current therapies include virotherapy and chimeric antigen receptor (CAR) T and stem cell therapies.[8,9] Additionally, there is a wide array of therapies that hold potential for antimetastatic therapy, such as the delivery of engineered exosomes.[10]

TRAIL therapy can also be used to target CTCs in the bloodstream to prevent the development of cancer meta- stasis.[11-13] TRAIL functions by binding to death receptors 4 and 5 on cancer cells to induce apoptosis while sparing healthy, noncancerous cells.[14] Conjugating TRAIL to nanoscale liposomes increases its targeting efficiency for CTCs both in vitro and in vivo.[11-13] Cone and plate flow devices were used to apply a physiologically relevant shear stress to cancer cells in vitro to simulate the fluid shear stress of the circulation that CTCs traveling through the bloodstream experience. Through modeling blood flow, cone and plate flow devices are also able to apply a sustained shear rate that increases the sensitivity of cancer cells to TRAIL.[15,16] In images obtained following these experiments, it was shown that CTCs exposed to

fluid shear stress (FSS) and TRAIL-conjugated liposomes were disrupted into cancer cell fragments.[16] Normal cells will not produce a similar product, since TRAIL produces apoptosis in cancer cells while sparing most normal cells, and FSS alone will not cause the same effects in such healthy cells.[17] As cancer cell fragments are released into the blood in vivo in response to liposomal TRAIL therapy, it is expected that these bodies are exposing the tumor-associated antigens to cells in the immune system to potentially elicit an immune response.

Breast cancer treatments can be modeled and tested in vitro and in vivo with a variety of murine models that include cell- line-derived xenografts (CDX), patient-derived xenografts (PDX), syngeneic models, genetically engineered mouse models (GEMM), and humanized models.[18] 4T1 breast cancer is a syngeneic mouse model of mammary carcinoma that is highly invasive and metastatic and can be transplanted orthotopically into the mammary fat pad of a BALB/c mouse for in vivo studies.[19,20] A strength of the 4T1 model is that it allows for the use of an immunocompetent mouse, which is extremely beneficial for studies involving host response and vaccines.[21-23] The metastatic predisposition of the 4T1 model has made it a significant model for circulating tumor cells and antimetastatic therapies, and we have used it for our FSS and TRAIL therapies.[12]

We have shown that TRAIL and FSS can kill 4T1 CTCs in vitro and in vivo with a variety of murine and humanized models.[11-13] Surgical removal of the primary tumor is a common practice in breast cancer treatment. However, postsurgical metastasis poses an immense setback in cancer therapy. Considering that 90% of cancer-related deaths are due to metastasis, antimetastatic therapeutic strategies that can target disseminating tumor cells in the circulation before they can form secondary tumors hold preclinical and clinical potential for cancer patients. Our recent work uses a liposomal formulation functionalized with the adhesion receptor E- selectin and the apoptosis-inducing ligand TNF (tumor necrosis factor)-related apoptosis-inducing ligand (TRAIL)

to reduce metastasis following tumor resection in an aggressive triple-negative breast cancer (TNBC) mouse model.[11−13] We demonstrated that minimal administration of E-selectin−TRAIL liposomes can target metastasis in a TNBC model, with primary tumor resection to mimic clinical settings. Our study indicated that TRAIL liposomes, alone or in combination with existing clinically approved therapies, may neutralize distant metastasis of a broad range of tumor types.[11−13] We have found that TRAIL and FSS can kill 4T1 CTCs in vitro.[12] With three doses of TRAIL-conjugated liposomes and removal of the primary tumor, we were able to target CTCs and prevent metastases in vivo in the 4T1 model.[12] Because mice showed no signs of metastases following treatment, we believe that the cell fragments produced during the therapy may aid in immune memory and prevention of tumor development from any remaining CTCs. A downside of using these therapies to evoke immune memory is the potential for issues of resistance and harmful side effects, so finding a way to generate similar cell products without TRAIL treatment would be beneficial.[24]

With the goal to recreate this phenomenon artificially, we used probe sonication to mechanically disrupt cancer cells. We characterized these nanoscale cancer cell fragments, which we have termed "tumor nano-lysate" (TNL). The TNL were characterized by size, charge, morphology, and composition. We also performed an in vivo pilot study to investigate the efficacy of the tumor nano-lysate as a preventative vaccine for 4T1 breast cancer in a mouse model.

RESULTS

Preparation and Characterization of TNL

The fluid shear stress (FSS) of blood flow can be modeled by using a cone and plate flow device.[25] Using this system, we have recapitulated the stresses that circulating tumor cells (CTCs) experience in the bloodstream in vivo and used this to test the efficacy of antimetastatic treatments for a variety of cancer types.[11,13] Combining 4T1 cancer cells with TRAIL therapy and

applying FSS via cone and plate flow device result in cancer cell fragments in vitro. With the ultimate goal to recreate the phenomenon of cancer cell fragmentation that results from the combination of FSS and exposure to TRAIL, we sought to lyse cancer cells artificially. Cells can be lysed by a variety of means, including chemical and mechanical methods of lysis.[26,27] Lysing cells artificially would offer many advantages over the use of cell fragments derived from exposing 4T1 cells to TRAIL and FSS. These advantages include avoiding potential issues such as cell resistance to TRAIL and negative side effects such as hepatotoxicity.[24]

With the goal to mimic the exposure to forces applied by shear stress and a desire to prevent further changes to the cells beyond lysis, we chose mechanical lysis via ultrasonic frequencies. In previous literature, when lysing cells via sonication, 40−50% amplitude was recommended, as were short pulses of sonication followed by time for cooling.[28,29] The sonication process was optimized by using different sonicating and cooling times as recommended by the literature.[28,30−33] After sonication, samples were measured by polydispersity index (PDI) and size. Sonicating times that provided fragments larger than the desired size range of 175− 275 nm or with a PDI larger than 0.75 were not selected. Because short pulses and cooling cycles were suggested, we selected a series of 2 and 10 s pulses, finding that they had similar size range and PDI. Three pulses of 10 s sonication and 10 s cooling, as suggested by the literature, resulted in the least amount of visible foaming and provided ample cooling time, as it was not hot to the touch.[29,33,34] TNL was kept on ice during sonication and cooling to keep the samples cooled. For the mechanical disruption of our cancer cells, 4T1 breast cancer cells suspended in PBS were mechanically disrupted via probe sonication for a combined total of 30 s of sonication at 50% amplitude, with time for cooling, and kept on ice (**Fig. 3.1A**). At these settings, we were able to minimize both sample foaming and heating.

Cancer Cells Disrupted by Sonication Become Nanometer Sized and Highly Dispersed

The resulting sonicated cancer cells were characterized by size via Zetasizer and NanoSight and then compared to cells fragmented after exposure to TRAIL and FSS. Zetasizer measurements revealed a high polydispersity (PDI) in sample size for both conditions, which was expected due to randomness of the lysis during the sonication process (**Fig. 3.1B**). For 4T1 cells exposed to FSS and TRAIL, the average polydispersity index was 0.54, while the average polydispersity index for sonicated cells, or tumor nano-lysate (TNL), was 0.50. PDI is defined as the weight-average divided by the number-average molecular weight and characterizes the breadth of molecular weight distribution.[35] A high PDI lies close to 1.0 and indicates a highly dispersed size range of measured samples. Therefore, these results indicate that the samples were not monodispersed and that a Zetasizer is not the optimal measurement tool for determining the average particle size. As a result, the NanoSight, with an ability to provide more accurate size range and concentrations of nanoparticles, was used to characterize the particle size distribution of each sample.

With the Zetasizer, TRAIL+FSS lysate was measured to be 200−230 nm in diameter, while TNL were measured to be 185−275 nm. With the aid of the NanoSight, the sonicated cancer cell product was measured to have a standard error of 19.7 nm and have an average diameter of 205 ± 48.2 nm. Similarly, cancer cell product exposed to FSS and TRAIL had a standard error of 17.9 nm and was measured to have an average diameter of 199 ± 35.6 nm (**Fig. 3.1C−E**). From this information, we were able to conclude that we could closely recapitulate the size range of fragmented cancer cells resulting from the in vitro FSS modeled system.

Measured Surface Charge of Sonicated Cancer Cells is Slightly Negative

The zeta potential was measured and compared for the sonicated cancer cells and cancer cells exposed to TRAIL and FSS. Using electrophoretic light scattering via Zetasizer, we determined the surface charge for each sample. It was observed that the zeta potential was slightly negative
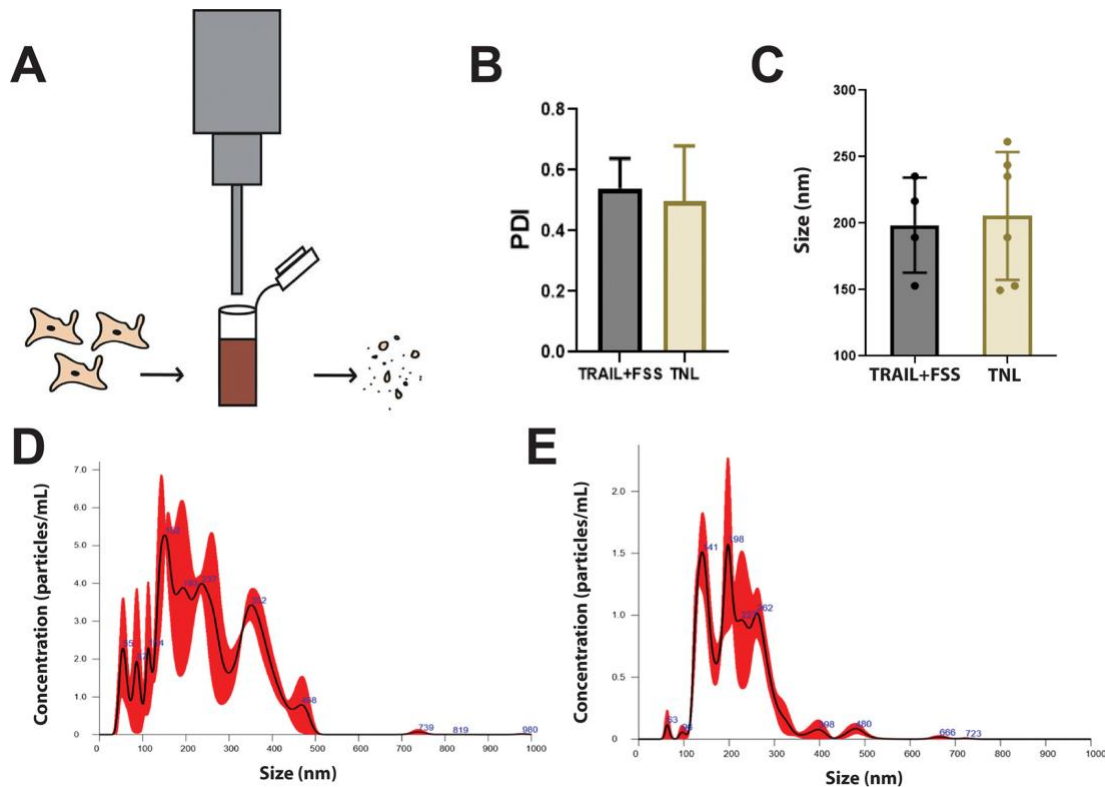
Figure 3.1. Preparation, polydispersity, and size characterization of TNL. (A) Sonication process for producing cancer cell fragments or tumor nano- lysate. (B) Polydispersity index (PDI) of 4T1 cells exposed to TRAIL+FSS and sonication (TNL) had a standard error of 0.04 and 0.06, respectively. The average PDI for the TRAIL+FSS group and TNL group was 0.54 ± 0.10 and 0.50 ± 0.18, respectively. (C) Average particle size as measured by NanoSight, comparing TRAIL+FSS. (D) Representative figure of average TNL size as measured by NanoSight. (E) Representative figure of average size of cancer cell fragments following exposure to TRAIL+FSS. Statistical significance was calculated by using the unpaired t test.

for both samples, although the standard deviation of each sample was relatively high (**Fig. 3.2A-B**). The mean zeta potential for 4T1 cells exposed to TRAIL and fluid shear stress (FSS) was −3.7 ± 1.5 mV and −4.1 ± 1.3 for 4T1 cells exposed to sonication (TNL). The negative charge of cell fragments is unsurprising given that the surface charge of cells is also negative, and mechanical lysis should not alter that property.[27,36] While the standard deviation for 4T1 cells exposed to either TRAIL+FSS or sonication was high, interestingly, there was not a significant difference between the standard deviations of the zeta potential values.
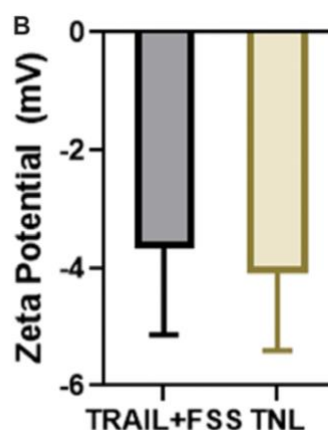
Figure 3.2. Zeta potential measurements for cells exposed to TRAIL+FSS and sonication. (A) Table of values for mean zeta potential (mV) and standard deviation for zeta potential (mV) for 4T1 cells exposed to TRAIL and FSS and 4T1 cells exposed to sonication (TNL). The standard error was 0.6 mV for the TRAIL+FSS group and 0.5 mV for the TNL group. (B) Quantification of zeta potential (mV) for 4T1 cells exposed to TRAIL and FSS and 4T1 cells exposed to sonication (TNL).

Imaging of Sonicated Cancer Cells Reveal Membrane-Bound Vesicles

The cell fragments derived from the sonication of cancer cells, referred to as tumor nano-lysate (TNL), were imaged by using transmission electron microscopy (TEM). Using microscopy, we were able to visualize what appeared to be membrane-encapsulated vesicles among the tumor nano-lysate (**Fig. 3.3**). TEM shows two of the smaller-sized TNL particles, at around 100 nm in size. The creation of these membranous features has been observed in the past when sonicating cells via probe sonication, with an ability to form liposome-like structures as a result of the disruption of the cell membrane and then spontaneous re-formation of a bilayer due to electrostatic interactions.[37]
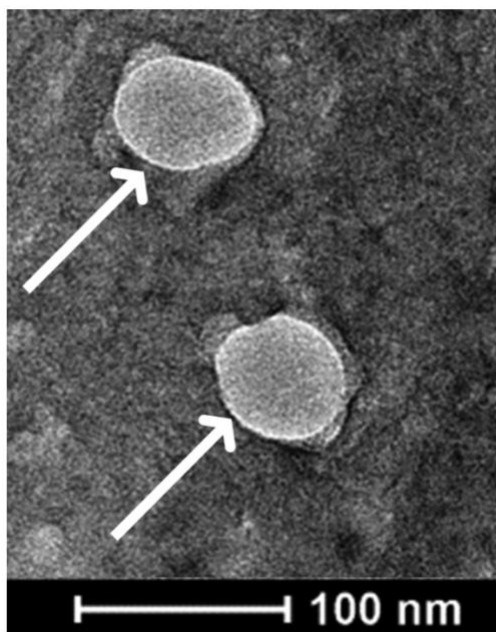
Figure 3.3. Transmission electron microscopy image of 4T1 breast cancer cells after sonication. White arrows indicate the presence of membrane-bound vesicles in the sample of sonicated cells.

Sonicated Cancer Cells Contain Protein Contents Similar to 4T1 Cells Lysed by RIPA Buffer

The sonicated cancer cells were then characterized for composition and contents. Mass spectrometry was used to examine the protein contents, comparing the sonicated cancer cells to a sample of control 4T1 cells lysed with RIPA lysis buffer. Fragments derived from TRAIL+FSS treated cells were not used as a comparison group since there was insufficient protein for accurate measurements via mass spectrometry. Among the most common proteins in the sample of sonicated cancer cells were albumin, cytoplasmic actin, α-enolase, elongation factor 1-α1,
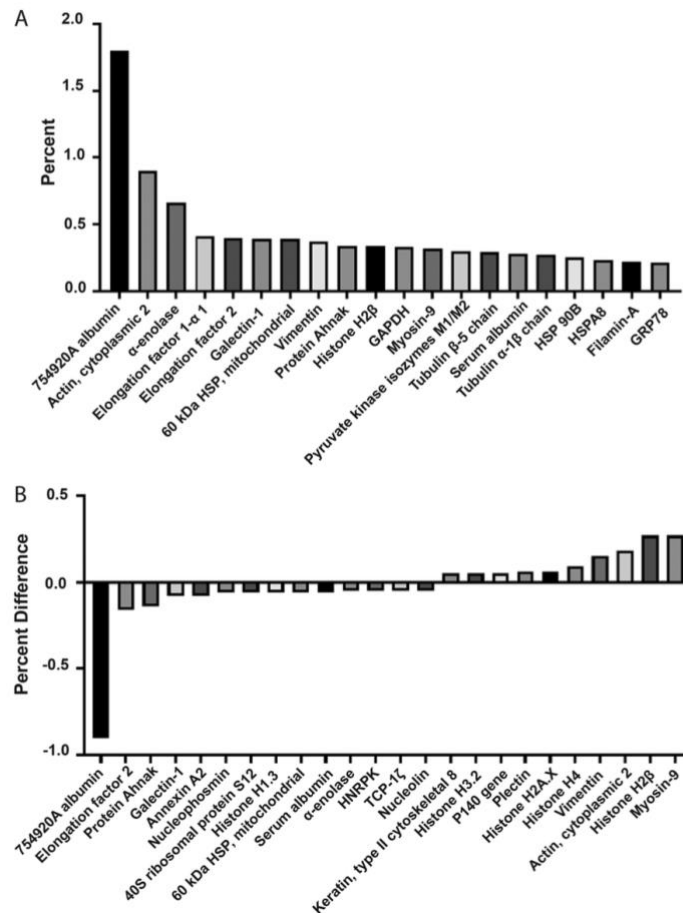
Figure 3.4. Content characterization of tumor nano-lysate via mass spectrometry. (A) The 25 highest protein concentrations for sonicated cancer cell sample. Albumin is present in large amounts because of the RPMI cell culture media. (B) Greatest percent differences between TNL and control of 4T1 cells lysed by RIPA buffer. The low differences in concentration indicate that the tumor nano-lysate is not significantly different than the proteins found in the control.

galectin, mitochondrial heat shock protein (HSP) 60 kDa, and vimentin (**Fig. 3.4A**). The greatest percent deviations of protein concentrations of the TNL from the RIPA buffer control were calculated from the 1317 analyzed proteins of each sample. The extremely low differences in concentration demonstrate that the proteins from tumor nano-lysate are not significantly different than the proteins found in the RIPA control. (**Fig. 3.4B**). One of the most common proteins, Myosin-9, is expected as it is commonly upregulated in cancer cells as it is a protein responsible for growth and embryonic development and plays a role in aiding in cancer cell motility.[38,39] The group of heat shock proteins (HSP mitochondrial, HSPA8, and HSP90B) is also expected, as these are often diagnostic markers of cancer and implicated in a variety of cancer-promoting phenomena.[40]

Sonicated Cancer Cells Do Not Affect the Viability of Healthy Epithelial or Primary Tumor Tissue in Vitro

Apoptosis assays were performed to test the toxicity of the sonicated cancer cells and determine whether the formulation would induce an unwanted effect of cell death. An Annexin V apoptosis assay compared the effects of cells treated with tumor nano-lysate (TNL) to cells treated with a vehicle control (PBS). First, healthy mouse mammary epithelial tissue (EpH4-Ev) cells were exposed to TNL suspended in PBS. At 1 mg TNL/10 mg live EpH4-Ev cells, EpH4-Ev cells exposed to TNL showed an average cell viability of 75 ± 5.3% compared to 79 ± 3.3% for the PBS-treated control, with standard errors 2.38% and 1.47%, respectively. At 2 mg TNL/5 mg live EpH 4- Ev cells, cell viability was 75 ± 4.0% for TNL-treated cells and 79 ± 2.0% for PBS-treated cells. Standard errors were 1.79% and 0.89%, respectively. Both the higher and lower concentrations of TNL revealed no significant increase in apoptosis or loss of cell viability when compared to PBS (**Fig. 3.5A**). These TNL dosages are much higher than the dose used in the in vivo study, thus representing a conservative toxicity test.

In addition to healthy cells, we also investigated the apoptotic effects of TNL on breast cancer cells in vitro. When 4T1 breast cancer cells were exposed to TNL suspended in PBS, the resulting Annexin V apoptosis assay showed no significant toxic effects on these primary tumor cells compared to the PBS-treated control (**Fig. 3.5B**). At 1 mg TNL/10 mg live 4T1 cells, TNL-treated 4T1 cells showed an average viability of 85 ± 1.8%, compared to 86 ± 2.0% for the PBS control, with standard errors calculated to be 0.74% and 0.89%, respectively. The higher 2 mg TNL/5 mg live 4T1 cells dose produced similar results, with an average cell viability of 84 ± 3.6% for TNL-treated cells and 83 ± 6.2% for PBS-treated cells. Standard errors were 1.45% and 2.55%, respectively. These results support the idea that this tumor nano-lysate may well act as a preventative method by exposing the immune system to tumor-associated antigens, rather than as a mechanism of directly targeting the primary tumor itself.
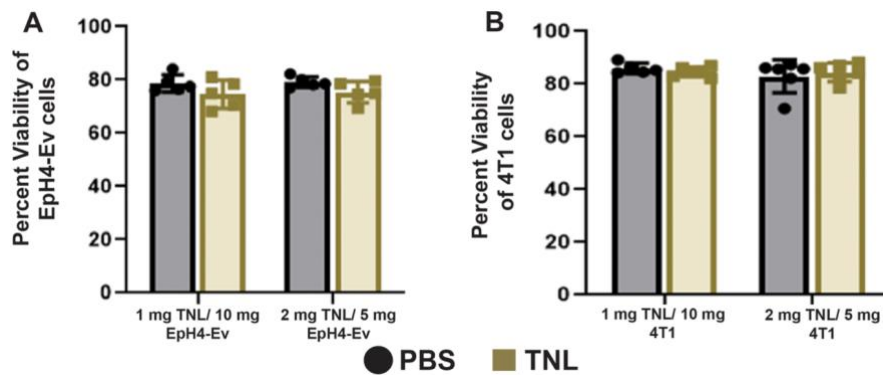
Figure 3.5. Apoptosis assay of healthy epithelial and tumor breast tissue. (A) No significant difference in cell viability was observed between EpH4- Ev epithelial tissue exposed to either dosage of TNL when compared to EpH4-Ev cells exposed to PBS buffer. (B) Similarly, there was no significant difference in cell viability for 4T1 breast cancer cells exposed to either concentration of TNL when compared to 4T1 cells exposed to PBS buffer. These experiments were performed in duplicate.

One Dose of TNL Vaccine Prior to Tumor Inoculation Reduces Tumor Growth, Delays Metastasis, and Increases Survival for Mice Challenged with Breast Cancer Xenograft

Having developed TNL that physically mimic the naturally created apoptotic bodies resulting from TRAIL treatment in the presence of FSS, we proceeded to test their biological activity in vivo. We hypothesized that the tumor-associated antigens from the 4T1 cells would induce an adaptive immune response. Prior to the animal trial, an immunological assay for T cell activation was performed to ensure that the TNL would not put undue strain on the immune system of the mice. This study tested for the upregulation of CD25 and CD69 in lymphocytes, T cell markers for late and early activation, respectively. Splenocytes harvested from BALB/c mice were exposed for 24 h to three different conditions: (1) PBS vehicle control, (2) 4T1 cells exposed to fluid shear stress and TRAIL (FSS+TRAIL) suspended in PBS, and (3) tumor nano-lysate suspended in PBS (TNL). No significant difference was found between the three conditions, indicating that these treatments were sufficiently safe for the pilot study (**Fig. 3.6A**).

Ten days before tumor inoculation with 4T1-Luc breast cancer cells, mice in the tumor nano-lysate group received one dose of TNL suspended in PBS, while the remaining mice were untreated. Following tumor inoculation, mice were monitored via bioluminescence imaging (BLI), and the tumor size was measured via calipers once palpable (**Fig. 3.6B**). BLI data showed more rapid tumor growth for control mice that did not receive the injection of TNL (**Fig. 3.6C**). Results of the pilot study revealed significantly reduced tumor volume for mice that received a dose of tumor nano-lysate compared to control (**Fig. 3.6D**). Metastasis from the primary tumor was also observed much earlier for untreated mice, delayed an average of 10 days for mice that received one dose of the vaccine (**Fig. 3.6E**). The median overall survival was extended an average of 7 days for the vaccinated group, and they did not require humane sacrifice as early as their untreated counter- parts (**Fig. 3.6F**). Although survival was delayed, eventually the mice in both groups required humane sacrifice. There were no visible changes to the appearance, behavior,

or health of the mice that would be evidence of adverse side effects. Thus, a single pretreatment with TNL can delay the onset of primary tumor development, delay metastasis, and prolong survival in mice.
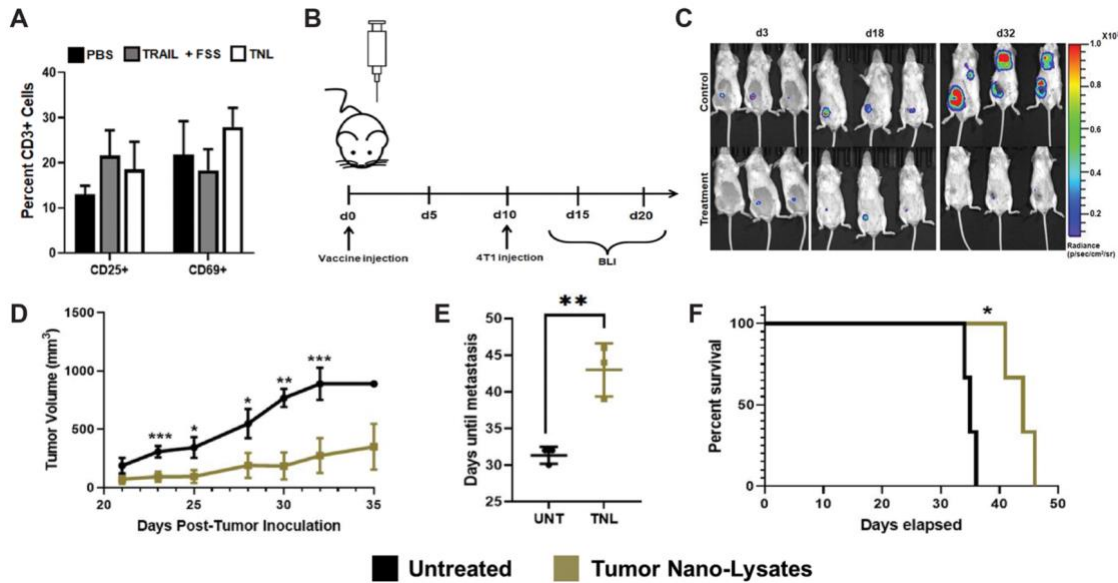
Figure 3.6. In vivo pilot study for vaccinated mice inoculated with 4T1 tumor. (A) In vitro assay comparing T cell activation for PBS vs TNL-treated splenocytes. (B) Timeline for in vivo study (C) IVIS imaging of mice using bioluminescent and Luc-expressing 4T1 cells. (D) Tumor size over time for measurements taken via caliper. (E) Days of metastasis observation post-tumor inoculation. (F) Survival curve. Statistical significance determined by two-tailed ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

DISCUSSION

We were able to successfully create nanoscale lysate derived from tumor cells using sonication. This TNL was found to have a negative zeta potential, with its size and charge similar to cancer cell particles created through the application of TRAIL and fluid shear stress via cone and plate flow device. Although the standard deviation of the zeta potential was high compared to the charge, this is to be expected. While the lipid bilayer defining the TNL particle structures should

resemble the negatively charged surface of the parent cell plasma membrane, the relatively high variance in zeta potential that was observed may reflect the variety of cytoplasmic contents that have associated with the TNL surface during the chaotic sonication process. As desired, we were able to closely recapitulate these characteristics of cell fragments resulting from exposing 4T1 cells to TRAIL and FSS. To further validate this study, performing a rechallenge experiment on mice given our TRAIL therapy would provide further insight to compare the two conditions. Nevertheless, our method does not require the use of TRAIL, limiting potential issues such as resistance and side effects that could result from the therapeutic.

Following sonication, the lysate was observed to form membrane-bound vesicles. The proteins present in the TNL sample, consistent with those found in 4T1 cells lysed with RIPA buffer, could prove effective for evoking a protective response once exposed to memory cells in the immune system. These characteristics likely make TNL favorable to uptake by antigen presenting cells (APCs) or other immune cells, which may aid in adaptive immunity in vivo.

While little research has been dedicated to the characterization of cell lysate in the past, artificially developed apoptotic bodies are the closest resemblance to TNL and fragments derived from TRAIL+FSS.[41,42] They have shown similar wide size ranges and morphologies as the cell fragments in this study, although microvesicles show nearly identical average size and morphology to our TNL.[42]

No evidence of toxicity was found when healthy breast tissue was exposed to lower or higher (1:10−2:5 mg TNL/mg EpH 4-Ev) concentrations of tumor nano-lysate when compared to PBS control. Similarly, when 4T1 breast cancer cells were exposed to two different concentrations of TNL derived from 4T1 cells subjected to sonication (1:10−2:5 mg TNL/mg 4T1), no toxic effects or decrease in cell viability was found. This evidence suggested that TNL could proceed with in a

preliminary in vivo study and that any observed effect would likely occur through immune cell response rather than a direct effect on the primary tumor cells. While the success of our in vitro toxicity assays prompted an in vivo study, we plan on performing future mouse studies to analyze the in vivo effects of TNL. These studies should investigate localized and systemic reactions that could take place upon exposure to TNL. However, we do not expect that cell lysate would be toxic, since they are recreating the apoptosis resulting from TRAIL+FSS treatments; 50−70 billion cells undergo apoptosis each day in the adult human, which does not result in toxicity in patients.[43] Cell lysate and apoptotic bodies from a variety of cell types including T cells, stem cells, skin cells, brain cells, and peripheral blood mononuclear cells (PBMCs) have been injected in vivo in several recent studies, and none of the studies raised toxicity concerns.[34,43−45]

Although mice treated with a single dose of the TNL vaccine ultimately developed primary tumors and metastases, this study represents an important step forward and indicates that these mechanically produced cell fragments hold potential as a preventative vaccine for triple negative breast cancer.

It would be interesting to further explore the mechanisms behind the effectiveness of these tumor-derived lysate. These should consist of studies on immune cell activation in response to TNL exposure. Preliminary studies suggest that a B cell response drives the response (data not shown), and further investigations into this mechanism should be conducted. In addition, an analysis of immune cell infiltration to the tumor site may reveal discrepancies in the tumor microenvironments between mice in the control and vaccinated groups. A future study to test if sonicated normal cells would produce a similar preventative effect could act as a control to more conclusively elucidate whether TNL are successful due to the specific presentation of tumor-specific antigens.[46]

MATERIALS AND METHODS

Production of Sonicated Cancer Cells. 4T1 breast cancer cells (ATCC, CRL-2539) were cultured in RPMI 1640 growth media (Thermo Fisher Scientific) with 10% fetal bovine serum and 1% penicillin−streptomycin. The cells were plated, grown to 80% confluency, and lifted from the plate surface. Cells were centrifuged at 1200 rpm for 5 min, with the cell pellet resuspended in phosphate buffered saline (PBS). Cells in the pellet were counted and diluted with PBS to achieve a final concentration of 1 million cells in 1 mL of PBS. Using a Fisherbrand Sonic Dismembrator Model 50, we sonicated cells at 1/2 max sonication 3 times for 10 s each, for a total of 30 s of sonication time. Temperature rise of the sample in the test tube was attenuated by using a beaker full of ice. Immediately after sonication, cells were placed on ice. The sonicated cancer cells, termed tumor nano-lysate (TNL), were stored at −20 °C.

Size, Dispersity, and Charge Measurements. The Vanderbilt Institute of Nanoscale Science and Engineering (VINSE) Analytical Laboratory was used for size and dispersity measurements. Nano-particle tracking analysis (NTA) for particle size and concentration was performed using a Malvern Panalytical NanoSight NS300. Samples were further diluted with PBS to 1:100. A DLS Malvern Nano ZS (Zetasizer Nano ZS) was used to measure sample size, dispersity, and electrostatic charge. Size and dispersity measurements were obtained using Malvern Disposable microcuvettes. A disposable folded capillary cell was used to take zeta potential measurements.

Morphology. An Osiris transmission electron microscope (TEM) from the Vanderbilt Institute for Nanoscale Science and Engineering Advanced Imaging facility was used to analyze the morphology of the tumor nano-lysate material. Samples were prepared by using negative staining, placing the sample on a copper grid, and adding phosphotungstic acid (PTA) solution.47 This process surrounds the sample with electron dense materials, revealing the surface contrast between darker stain and lighter specimen.

Contents Characterization. A complete mass spectrometry analysis was performed for all of the identified proteins in the control sample and the tumor nano-lysate sample. Mass spectrometry analysis was performed at the Vanderbilt Mass Spectrometry Research Center (MSRC) Core at the Vanderbilt University School of Medicine. Scaffold Proteome Software identified 1317 proteins. The top 20 proteins were analyzed and compared for this study. Albumin was identified as the highest concentration of protein, although that is due to the presence of cell culture medium. The full file containing all of the proteins identified will be provided on request.

Annexin V Assay. EpH4-Ev (ATCC CRL-3063) and 4T1 (ATCC CRL-2539) cells were used to test the toxicity of tumor nano-lysate on normal epithelial and triple negative breast cancer cells, respectively. Annexin V assay was performed to test the apoptotic effects of TNL on each cell type. 50000 cells were plated and exposed to 1 mg TNL/10 mg live cells or 2 mg TNL/5 mg live cells, equivalent to 5 and 20 µL of TNL solution (1 million 4T1 cells/ 1 mL PBS), respectively. Cells in the control groups were exposed to 5 or 20 µL of PBS buffer. Cells were lifted and centrifuged at 1200 rpm for 5 min, and the pellet was resuspended in 150 µL of PBS with calcium and magnesium. 5 µL of annexin and 5 µL of propidium iodide (PI) were added to the samples, with unstained, annexin V, and PI controls. After 15 min of incubation in the dark at room temperature, 100 µL of PBS was added to each of the samples and then run through a Guava EasyCyte flow cytometer (Millipore, Billerica, MA). Flow cytometry data analyses were performed using FlowJo software.

Immune Assay. Splenocytes were isolated from the spleen of healthy BALB/c mice following an established protocol.48 Cells were plated in 24-well plates at 50000 cells per well. Three wells were dedicated to controls (unstained, FITC isotype, and APC isotype). Each sample was tested in triplicate. 5 µL of PBS was added to six wells for vehicle controls for the two different markers

(CD25 and CD69). Similarly, 1:10 mg TNL/mg splenocytes, equivalent to 5 µL of TNL (1 million sonicated 4T1 cells in 1 mL of PBS), were incubated in six wells. Finally, 1:10 mg TRAIL+FSS lysate/mg splenocytes were incubated in six wells of the plate. After 24 h, the cells were lifted and centrifuged at 1200 rpm for 5 min and resuspended in 100 µL of PBS. All cells were then stained for 1 h without light exposure with 2 µL of CD3 and 2 µL of CD25 or CD69. Samples were processed through a Guava EasyCyte flow cytometer, and data were analyzed by using FlowJo software.

In Vivo Pilot Study. Eight-week-old female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were cared for by veterinary staff at the Department of Animal Care (DAC) at Vanderbilt University. Mice in the control group (n = 3) and experimental group (n = 3) experienced identical housing conditions and care regimens. This pilot study was approved by Institutional Review Board, protocol #M1700009-00. Mice were euthanized at humane end points. These end points were determined based on tumor size and recommendation by veterinary staff at the DAC.

4T1 Orthotopic Tumor Model and TNL Treatment. Mice were injected with one dose of TNL (10^5 sonicated 4T1 cells in 100 µL of PBS) via the tail vein 10 days prior to tumor inoculation. For tumor inoculation, 30000 4T1 breast cancer cells transfected with luciferase were suspended in 50 µL of PBS and injected into the anatomical right fourth mammary fat pad of each mouse. During the inoculation, mice were anesthetized for 5−10 min via continuous inhalation of isoflurane. Luciferase expression in the mice was monitored via bioluminescence imaging on an in vivo imaging system (IVIS Lumina III, PerkinElmer Inc.) twice per week, following a subcutaneous injection of 100 µL of D-luciferin (150 mg/kg). Calipers were used to measure the length (l) and width (w) of the tumor, with tumor volume estimated as (l × w2)/2. Metastases were considered as bioluminescent signals in locations other than the mammary fat pad. These indicate

cancer spread to distant organs. Days when mice were observed to begin showing signs of metastases were recorded and compared.

Data Analysis. Data are presented as mean ± standard deviation, and statistical significance is determined in GraphPad Prism by two- tailed ANOVA, unless otherwise indicated. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

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Chapter 4:

Tumor nano-lysate activates dendritic cells to evoke a preventative immune response

Jenna A. Dombroski, Abigail R. Fabiano, Samantha V. Knoblauch, Schyler J. Rowland, Katherine N. Gibson-Corley, and Michael R. King. Tumor nano-lysate activates dendritic cells to evoke a preventative immune response.

ABSTRACT

A tumor nano-lysate "TNL" preventative vaccine for triple-negative breast cancer has previously been developed and characterized. This study aims to gain a better understanding of the immune response behind the success of the vaccine in vivo, through use of ex vivo and in vivo assays. Here, we analyze the activation of various immune cells and find that antigen-presenting cells such as dendritic cells are being activated. These cells were further explored to determine the pathway by which activation is occurring, and it was observed that TNL are phagocytosed by dendritic cells and activate NF-kB and c-Fos pathways, resulting in enhanced cytokine release. An in vivo temporal analysis was performed to understand the immune response over time, and changes were observed for up to one week. A multiple dose study was performed comparing mice that were vaccinated with three doses of TNL and a PBS control. The differences between the groups at the end of the study demonstrate the potential for TNL as a preventative therapeutic.

INTRODUCTION

Breast cancer remains an epidemic in the United States, with 1 in 8 women diagnosed with the disease in her lifetime.[1] While 5-year survival rates as high as 99% are associated with early

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stage breast cancers, survival rates for later stage, metastatic breast cancers are reduced to as low as 27%.[2] These statistics indicate a need for successful anti-metastatic therapeutics and preventative measures for breast cancer. Current preventative measures include regular screening via breast self-examination (BSE), clinical breast exam (CBE), and mammography after the age of 40.[3,4]

Currently, there are only five FDA-approved preventative cancer vaccines used in clinical practice, and these protect against sexually transmitted viral infections human papillomavirus (HPV) and hepatitis B (HBV).[5] These viruses commonly promote liver, cervical and oral cancers, and when implemented the vaccines have successfully prevented the virus and corresponding disease onset.[6–9] While strides have been made in research and development of non-viral preventative cancer vaccines, many challenges remain.[10]

Previously, we developed a tumor nano-lysate (TNL) preventative vaccine for triple-negative breast cancer.[11] TNL was fabricated via membrane disruption of 4T1 breast cancer cells using probe sonication, and the vaccine was characterized by size, protein contents, and morphology and analyzed for toxicity in vitro.[11] A pilot study was also performed analyzing the effectiveness of the vaccine when used with the 4T1 model in vivo, and for TNL-vaccinated mice, reduced tumor growth was observed, onset of metastasis was delayed, and survival was increased.[11] Although TNL-vaccinated mice ultimately developed tumors, the results of the study demonstrated the potential of TNL as a preventative vaccine.[11] In this study, we aim to understand the immunological response to TNL, which will provide a better understanding of what is occurring in vivo and potentially uncover ways to improve the success of the formulation.

MATERIALS AND METHODS

Cell culture: Immortalized dendritic cell (DCs) line DC2.4 cells (Sigma-Aldrich Catalog No. SCC142) were grown in culture media consisting of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1X L-Glutamine, 1X non-essential amino acids, 1X HEPES buffer solution and 0.0054X β-Mercaptoethanol. Cells were maintained at 37°C with 5% CO_2, and experiments performed at ~70-80% confluency.

Reagents: For preparation of DC2.4 cell culture media, RPMI-1640, FBS, non-essential amino acids and HEPES buffer (Gibco), and β-Mercaptoethanol (Sigma-Aldrich) were used. Hank's balanced salt solution (HBSS) w/w/o calcium and magnesium was purchased (Gibco). Lipopolysaccharide (LPS) solution was purchased from Thermo Scientific. ODN 1585 - TLR9 ligand (CpG) was purchased from InvivoGen. Anti-mouse PE-CD40 (5C3) was purchased from BD. Anti-mouse PE-MHC (Class I H-2Kk) was purchased from Antibodies-Online.com. Anti-mouse PE-CD70 (FR70), PE-CD80 (16-10A1), PE-I A/I E (M5/114.15.2) (MHC II), and PE mouse anti-Ki-67 (567720) were used for flow cytometry (BD). Anti-mouse PE-CD83 (Michel 17), PE-CD197 (CCR7) (4B12), PE-phospho-NFkB p65 (Ser529) (NFkBp65S529-H3), PE-phospho-c-Fos (AP-1) (Ser32) (cFosS32-BA9), and PE (MA5-36891) were obtained from Thermo Scientific. H-2Ld MuLV gp70 Tetramer-SPSYVYHQF-PE was obtained from MBL International. For immune cell labeling, CD3, CD4 and CD19 were purchased (BD), CD8 was purchased (Thermo Fisher), and CD335, CD68 and CD11c were purchased (BioLegend). Anti-RAC1 (MBS9200589) was purchased through MyBioSource. Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006) was used to analyze cytokine release (R&D Systems). VECTASHIELD® Antifade Mounting Medium (H-1000-10) was purchased from Vector Laboratories and used for confocal microscopy. Triton X-100, DAPI (D9542-10MG), Tween® 20, viscous liquid, and Poly-L-lysine solution were purchased from Sigma-Aldrich. ActinRed 555 ReadyProbes Reagent and secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 goat anti-mouse IgG (H+L) were purchased from Invitrogen for confocal

imaging. 10% Normal Goat Serum (Life Technologies) and 32% paraformaldehyde aqueous solution, electron microscopy grade (Electron Microscopy Sciences) were purchased for imaging. Ficoll-Paque PLUS was purchased from GE Healthcare. Syringe/plungers (BD), cell strainers (Celltreat), and red blood cell (RBC) lysis buffer were purchased for splenocyte isolation. Syringes for mouse injections were purchased from BD.

TNL fabrication: TNL were fabricated via sonication as previously described.[11]

Flow cytometry analysis and antibody staining: For intracellular proteins phospho-NF-κB and -cFos, cells were fixed with 4% paraformaldehyde (PFA) for 10 min. Cells were washed with HBSS and permeabilized with 100% ice cold methanol. Cells were washed again and stained for 15 min in the dark with antibodies fluorescently tagged with PE fluorophore while suspended in 1% BSA. A Guava easyCyte 12HT flow cytometer (Millipore) was used to measure fluorescence intensity, with FlowJo software used for gating and analysis. For extracellular proteins analyzed after 24 h, cells were lifted, washed, and incubated with primary antibodies pre-conjugated with fluorophores in 1% BSA and then washed once more prior to analysis. For RAC1 staining, cells were first stained with the primary antibody and then stained for 15 min with an Alexa 488 secondary antibody and washed prior to analysis via flow cytometry. For these analyses, DC2.4 cells were plated overnight and were treated for 4 h or 24 h with freshly made TNL at a concentration of 1:10 mg TNL/mg DC2.4 (TNL_{Low}) or 2:1 mg TNL/mg DC2.4 (TNL_{High}), and equivalent PBS volumes for a vehicle control. When stimulators were used as positive controls, LPS was at a concentration of 10 μg/mL and CpG was at a concentration of 2 μM.

Splenocyte isolation and analysis: Primary cells were isolated from the spleens of healthy BALB/c mice using a plunger and cell strainer, and red blood cell (RBC) lysis buffer.

Splenocytes were counted and incubated with 1:10 mg TNL/mg splenocyte or PBS. T cells were fixed and permeabilized and stained for T cell markers CD3, CD4 and CD8 and intracellular cytokines IL-2, TNF, and IFN. Splenocytes were stained with B cell marker CD19 and activation markers including CD40, CD69, CD86 and majorhistocompatability complexes (MHCs). Additionally, splenocytes were stained for CD68 and CD11c and stained for costimulatory molecules.

Tetramer staining: Splenocytes were isolated as previously described and plated overnight. Cells were treated for 24 h with PBS, LPS, CpG, or 1:10 mg TNL/mg DC2.4 (TNL$_{Low}$) or 2:1 mg TNL/mg DC2.4 (TNL$_{High}$). Splenocytes were stained for extracellular marker CD3 and H-2Ld MuLV gp70 Tetramer.

Viability assay: Splenocytes were isolated as previously described and plated overnight. Cells were treated with PBS or 1:10 TNL/mg splenocytes and 4T1 cells were plated separately. After 24 h, splenocytes were added to 4T1 cells for 24 h at concentrations of 1:10, 1:5, or 2:5 mg splenocytes/mg 4T1 cells. A control of PBS treated-4T1 cells (no-splenocytes) was maintained. Cells were then lifted, centrifuged and resuspended in 150 µL HBSS -/-. 5 µL of annexin v (AV) and 5 µL of propidium iodide (PI) were added to each sample, and unstained and single stained controls were used. Cells were stained for 15 min in darkness and 100 µL HBSS was added to each sample before flow cytometry analysis. Statistical significance was determined using Two-way ANOVA.

Proteome profiler: DC2.4 cells were treated with plated overnight and treated with TNL at 1:10 mg TNL/mg DC2.4, equal volume of PBS as a vehicle control, or CpG. After 24 h, the supernatant was collected from cell suspensions and centrifuged at 4800 RPM at 4°C for 10 min. Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems) was used for

analysis and the manufacturer's instructions were followed for the procedure. An Odyssey CLx imager was used for 2 min chemiluminescence imaging. Membrane mean pixel density was quantified using Image Studio Lite software. Two replicates were completed for each treatment group.

Confocal microscopy: DCs were seeded onto glass coverslips previously coated with poly-l-lysine (PLL). After 24 h, DCs were treated with PBS, CpG or TNL. After 4 h or 24 h, cells were fixed with 4% PFA, washed and then permeabilized with 1% Triton. After washing, cells were blocked for 45 min with 5% BSA + 5% goat serum, and then stained with primary antibodies (LAMP2, Syntaxin18) at 1:100 in coating buffer for 1 h. Cells were then stained with a 4 μL Alexa 488 secondary antibody, DAPI nuclear stain and ActinRed 555 for 30 min. Vectashield was added to slides followed by coverslips, and cells were imaged using a Zeiss LSM 900 confocal microscope. Image analysis was performed using FIJI software.

In vivo studies: The studies were approved by IACUC protocol #M1700009-02. Eight-week-old female BALB/cj (Strain #: 000651) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were monitored by veterinary staff at the Division of Animal Care (DAC) at Vanderbilt University according to institutional guidelines. Mice experienced identical care regimens and housing conditions, and mice were euthanized at humane endpoints, determined by factors such as mobility and tumor size.

In vivo temporal analysis: Healthy BALB/c mice were injected via tail vein with one dose of TNL (10^5 sonicated 4T1 cells in 100 µL of PBS) or PBS as a vehicle control. After 1 d, 3 d, 7 d, and 10 d, n=4 mice from each group (PBS, TNL) were sacrificed and underwent cardiac puncture and spleen resection. Part of each collected blood sample was kept for in-lab analyses and part was given to the Vanderbilt Medical Center Translational Pathology Shared Resource (TPSR)

for a complete blood count (CBC). Splenocytes were isolated as described above, counted, and analyzed for extracellular immune cell markers and activation markers. Peripheral blood mononuclear cells (PBMCs) were isolated via differential centrifugation and Ficoll-Paque. Isolated cells were then analyzed for extracellular markers.

In vivo multi-dose study: Healthy BALB/c mice were injected with 3 doses of TNL (10^5 sonicated 4T1 cells in 100 µL of PBS) every 10 d, or 3 doses of 100 µL PBS. 10 d after the final dose, mice were inoculated in the anatomical right 4th mammary fat pad with 30,000 4T1-Luciferase cells in 50 µL PBS. Hair was removed from the area surrounding the fat pad prior to inoculation and mice were anesthetized for 5-10 min via continuous inhalation of isoflurane during the administration. Tumors were monitored twice per week via bioluminescent imaging (IVIS Lumina III, PerkinElmer Inc.) and caliper measurements. Subcutaneous injection of 100 µL of D-luciferin (150 mg/kg) was used for imaging. During imaging, mice were anesthetized for 5-10 min via continuous inhalation of isoflurane. Tumor volume was estimated as [length (l) x width (w)^2]/2. First signs of observed metastases were considered as bioluminescent signals in a location other than the primary tumor, and these days were recorded. Splenocytes were isolated as previously described and analyzed for immune cell populations and activation markers. n=2 spleens were collected for the PBS group.

Immunohistochemistry (IHC): After resection, tumors were immediately fixed in 10% neutral buffered formalin (NBF) for 24 h. A board-certified veterinary pathologist examined hematoxylin and eosin (H&E) stained tumor slides and did not find any obvious morphologic differences between treated and control tumors. The Vanderbilt TPSR core routinely processed, paraffin embedded, sectioned at 5 µ.Slides were stained the tumors for CD3m (T cells), CD45r (B cells), or Ki-67 (proliferation) using the Leica Bond-RX staining platform. Briefly, slides were deparaffinized and heat induced antigen retrieval was performed on the Bond Max using their

Epitope Retrieval 2 solution for 5-20 min depending on the primary antibody. Slides were incubated with either anti-CD3 (Cat.# ab16669, Abcam, Cambridge, UK) at 1:250, anti-CD45r (Cat.# 553086, BD Pharmingen, San Diego, CA) at 1:30,000 or anti-Ki-67 (Cat.# ab16667, Abcam, Cambridge, UK) at 1:100 for one hour. The Bond Refine (DS9800, Buffalo Grove, IL, USA) detection system was used for visualization. Slides were then dehydrated and cleared, and coverslips were placed on them. Slides were scanned and uploaded to SlideViewer (3DHistotech) to take snapshots for analysis. FIJI was used to quantify DAB positive staining expression with a Macro developed by A.R.F. Briefly, the color deconvolution function was applied to the snapshots to provide an H&E, DAB, and residual image, so that area fractions (percent positive) can be determined from the DAB image. Data are presented as the percent positive DAB stain ± standard error mean of two sections per tumor per IHC stain.

Statistics: Data are reported as mean and standard error of the mean. Unless otherwise indicated, statistics were determined using student's t test. Experiments included at least three independent replicates unless otherwise indicated. $*p<0.05$, $**p<0.01$, $***p<0.005$, and $****p<0.001$ for significance; otherwise, there is no significance. GraphPad Prism software was used to perform statistical analyses and produce figures for this paper.

RESULTS

Antigen-presenting cells are activated by TNL

Splenocytes were isolated and treated with TNL or vehicle control for 24 h. Populations were analyzed for activation via costimulatory molecule expression or intracellular cytokine staining. CD3+CD4+ and CD3+CD8+ T cells were observed to not be significantly activated by TNL (**Fig. 4.1A-B**). Similarly, CD68+ macrophages did not have a change in costimulatory molecule

expression following incubation with TNL when compared to PBS controls (**Fig. 4.1C**). B cells, however, were observed to have significant increases in CD80 and CD83 costimulatory molecule expression and major histocompatibility complexes (MHC) I and II (**Fig. 4.1D**). DCs were similarly activated by TNL, as observed by a significant increase in costimulory molecule expression (**Fig. 4.1E**). Given that antigen-presenting cells (APCs) are being activated by TNL, and that DCs are often used in successful anti-cancer vaccines, we sought to further explore their response to TNL ex vivo and in vivo.
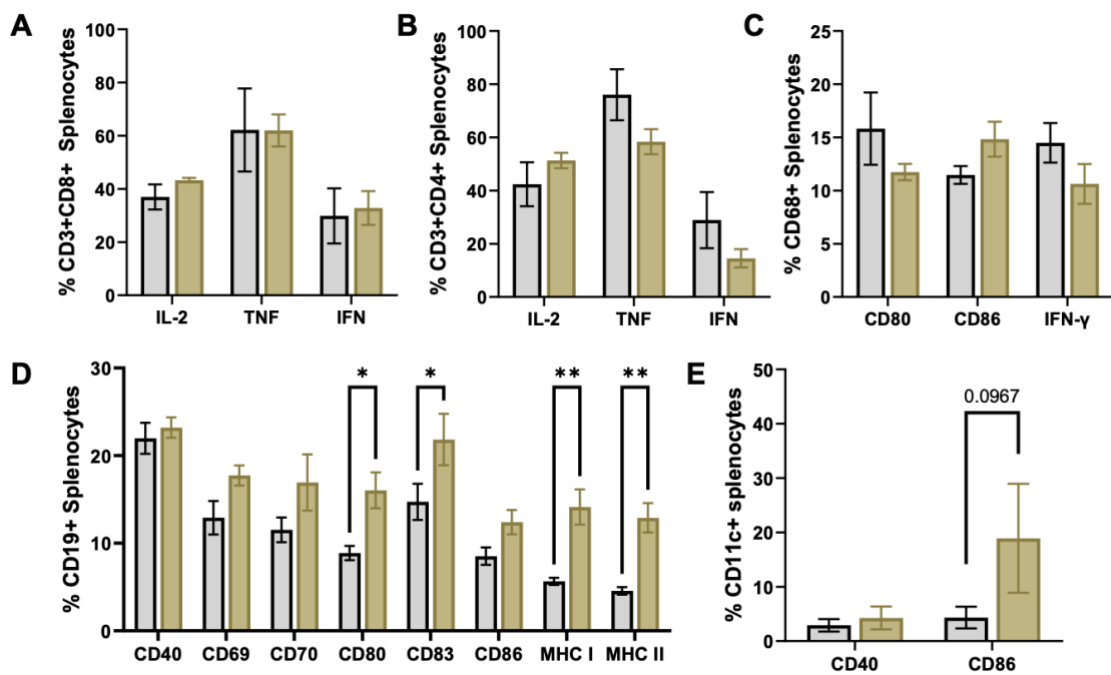
Figure 4.1. Cell activation by tumor nano-lysate ex vivo. Intracellular cytokine staining of (A) CD3CD8+ and (B) CD3CD4+ splenocytes. (C) Intracellular cytokine and costimulatory molecule staining of CD68+ splenocytes. (D) Costimulatory molecule and MHC staining of CD19+ splenocytes. (E) Costimulatory molecule staining of CD11c+ splenocytes. *p<0.05, **p<0.01.

DCs undergo morphological and metabolic changes in response to TNL

In order to explore the pathways by which DCs are being activated, DC2.4 immortalized cells were stained for intracellular and extracellular activation markers. NF-kB phosphorylation and c-Fos phosphorylation are regulated by calcium influx, and promote cell cycle progression, proliferation and cytokine release.[12–15] Ki-67 expression represents an increase in proliferation in a variety of cell types and is often associated with activation.[16–18] These activation markers were analyzed for DC2.4 cells treated with LPS, CpG and two concentrations of PBS and TNL at 4 h (**Fig. 4.2A**) and 24 h (**Fig. 4.2B**). Significant increases in phospho-NF-kB and -c-Fos were observed after 4 h for TNL-treated DCs compared to PBS vehicle controls. Changes in c-Fos phosphorylation was observed for up to 24 h.

Morphological changes in DCs are indicative of the successful uptake and presentation of an antigen, and this can be observed by changes in RAC1 expression.[19] DCs were treated with CpG and two different concentrations of PBS and TNL. After 24 h, a trend in enhanced RAC1 expression was observed for CpG- and TNL-treated cells (**Fig. 4.2C**).

Cytokine and chemokine release is necessary for activated DC trafficking and for T cell priming.[20] Using a cytokine array to analyze a panel of cytokines and chemokines, DC2.4 cells were treated with PBS, CpG or TNL. After 24 h, cytokine release was analyzed and a significant increase or trend in release was observed for TNL-treated cells (**Fig. 4.2D-E**).

The activation of above pathways is essential to DCs successfully activating T cells and initiating a preventative immune response. Interestingly, DCs responded similarly to or better than potent stimulators LPS and CpG.
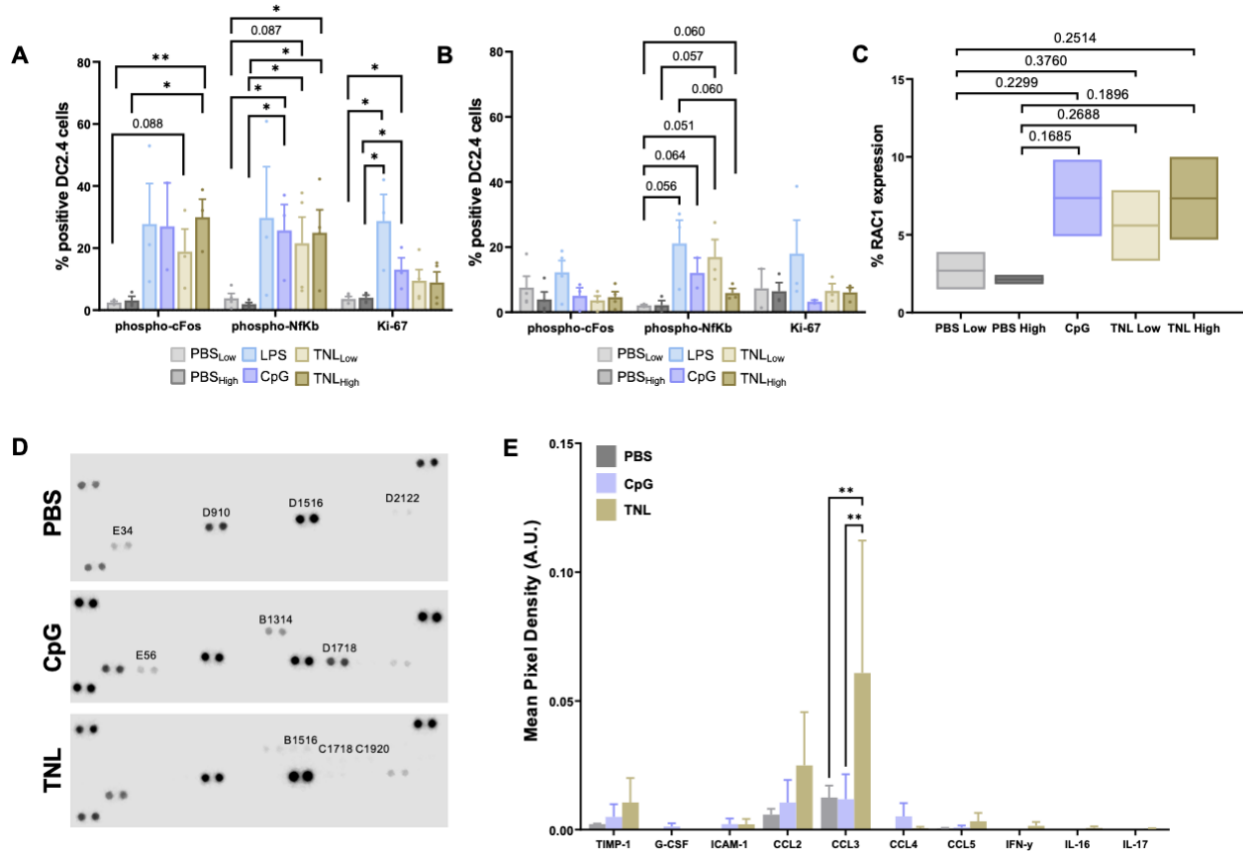
Figure 4.2. Analysis of DC activation markers. DC2.4 NF-kB phosphorylation, c-Fos phosphorylation and Ki-67 expression after (A) 4 h and (B) 24 h. (C) Percent RAC1 expression in DC2.4 cells after 24 h compared to costimulatory controls. (D) Representative proteome profiler arrays depicting cytokine release. (E) Cytokine and chemokine release in DC2.4 cells after 24 h. *p<0.05, **p<0.01.

DCs phagocytose TNL and a preventative response is elicited

DCs are activated when they successfully uptake an antigen, and LAMP2 and Syntaxin18 can play a role in this phagocytosis.[21] DC2.4 cells were treated with PBS or TNL, stained for phagocytosis markers and imaged using confocal microscopy (**Fig. 4.3A**). While no significant difference was observed in Syntaxin18 expression after 4 h (**Fig. 4.3B**), there was a trend in

increased expression after 24 h (**Fig. 4.3C**). Similarly, after 24 h, there was a significant increase in LAMP2 expression for TNL-treated DCs (**Fig. 4.3D**).

As 4T1 cells express the antigen gp70, a H-2Ld MuLV gp70 tetramer can be used to identify specific activated CD3+ T cells as indicated by CD3+Tetramer+ cells.[22–24] When CD3+ splenocytes were treated with LPS, CpG and two different concentrations of PBS and TNL for 24 h, a trend in increased tetramer expression was observed for CpG and both TNL conditions (**Fig. 4.4E**).

Splenocytes were treated for 24 h with PBS or TNL, and PBS- or TNL-treated splenocytes were used to treat 4T1 cells at three different concentrations. After 24 h, 4T1 cells were lifted, and an Annexin V viability assay was performed to analyze 4T1 cell viability. Percent viability was significantly reduced in 4T1s treated with TNL-treated splenocytes compared to PBS-treated 4T1 cells and 4T1s treated with PBS-treated splenocytes (**Fig. 4.3F**).
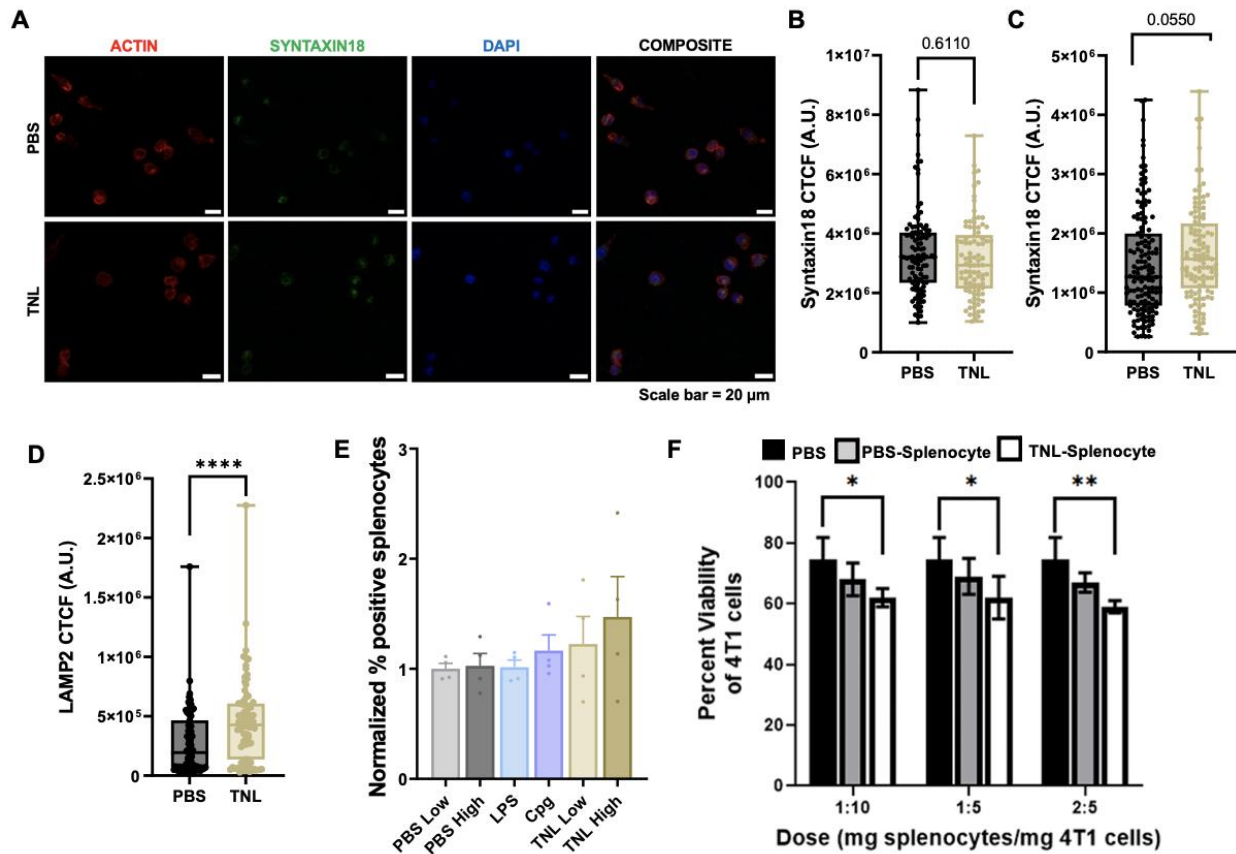
Figure 4.3. DC phagocytosis and functional analyses. (A) Syntaxin18 24 h representative

micrograph. Syntaxin18 expression after (B) 4 h and (C) 24 h. (D) LAMP2 expression after 24 h. (E)

CD3+ Tetramer staining. (F) Functional viability assay treating 4T1 breast cancer cells with TNL-

treated splenocytes. Scale bar = 50 μm. *p<0.05, **p<0.01, ****p<0.001.

Initial immune activation is sustained for up to one week

Healthy mice were treated with one dose of PBS or TNL and analyzed over time for a temporal

immune response. No significant differences were observed in RBC-related counts over time for

TNL-treated mice compared to mice that had received the PBS control (**Fig. 4.4A-G**). White

blood cell changes over time between PBS- and TNL-treated mice were largely unchanged

(**Fig. 4.4H, L-M**). Neutrophils, however, had higher counts for TNL-treated mice overall (**Fig.**

4.4I) and lymphocytes and monocytes had higher counts for TNL-treated mice at 7 d (**Fig. 4.4J-K**).

PBMCs isolated from whole blood had no significant changes in total PBMCs (**Fig. 4.4N**), but significant differences in CD3+ T cells between PBS- and TNL-treated mice at 3 d, and these counts were significantly reduced after 7 d (**Fig. 4.4O**). While there were no significant differences between CD3+CD69+ T cells between groups, there was a significant difference between CD3+CD25+ T cells between population, and these counts were reduced significantly at 7 d and then 10 d (**Fig. 4.4P-Q**).

Splenocyte composition analyses had no significant changes in DCs, B cells or macrophages (**Fig. 4.4R, T, V**). There was an increase in T cell counts for TNL-treated mice after 7 d and a trend in increased natural killer (NK) cell counts after 3 d and 7 d (**Fig. 4.4S, U**).
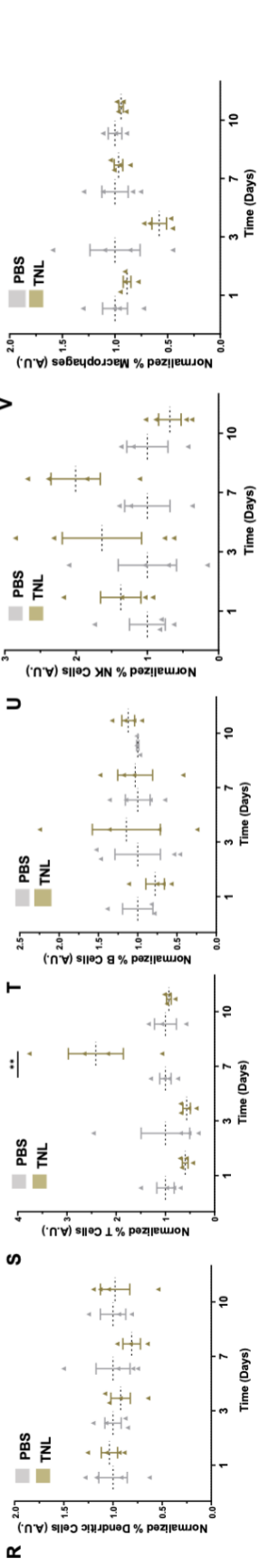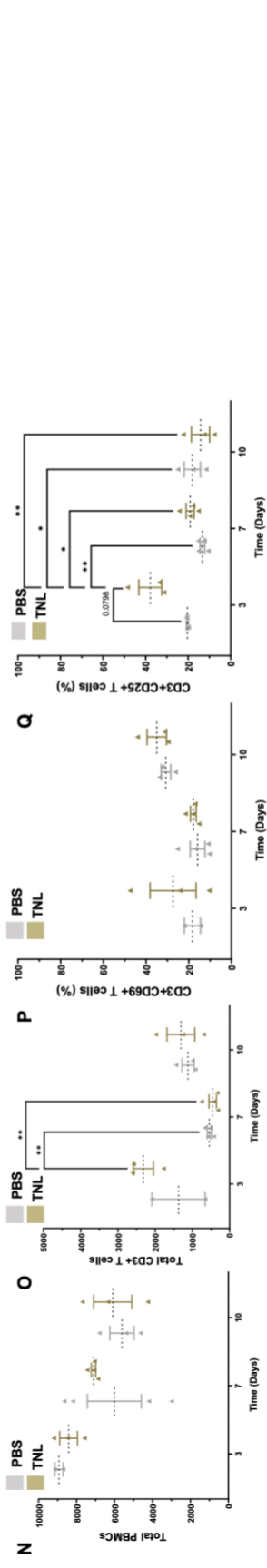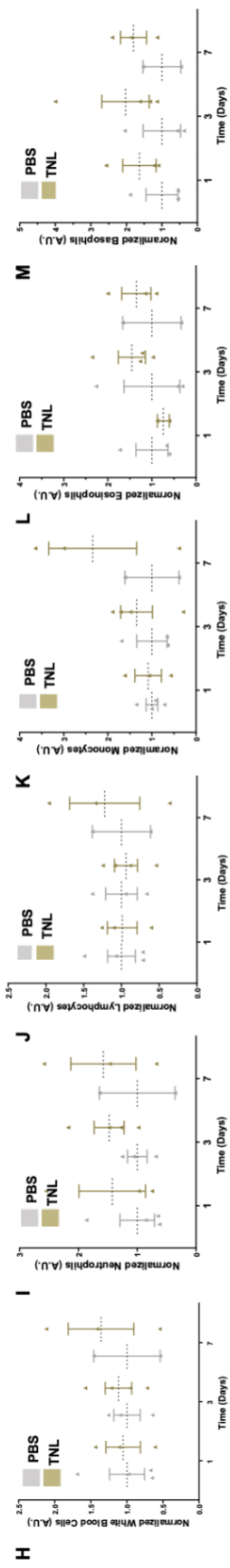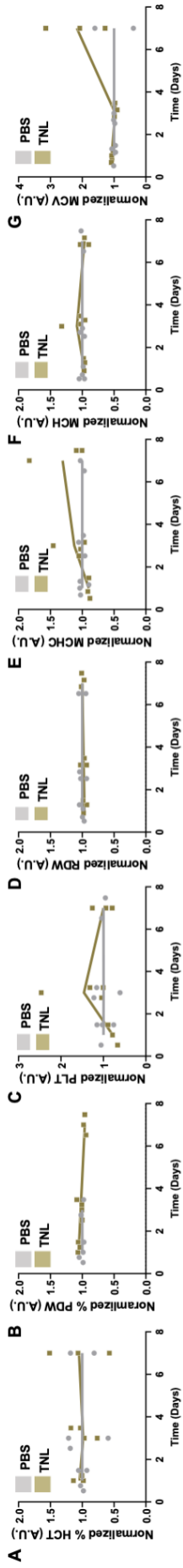
Figure 4.4. Temporal analysis of immune response to TNL. The CBC analyzed general red blood cell (RBC)-related counts including (A) HCT – Hematocrit, (B) %PDW – platelet distribution width, (C) PLT – platelet count, (D) RDW – RBC distribution width size/volume (E) MCHC – mean corpuscular hemoglobin concentration, (F) MCH – mean corpuscular hemoglobin, and (G) MCV – mean corpuscular volume. The CBC also analyzed various white blood cell (WBC) populations including (H) general WBC, (I) neutrophils, (J) lymphocytes, (K) monocytes, (L) eosinophils, and (M) basophils. PBMCs were analyzed for (N) total PBMCs, (O) total CD3+ T cells, and (P) CD69+ and (Q) CD25+ populations. Splenocyte composition was also determined via flow cytometry, marker for (R) DCs, (S) T cells, (T) B cells, (U) NK cells, and (V) macrophages. $*p < 0.05$, $**p < 0.01$.

Multiple dose study demonstrates potential for tumor nano-lysate as a preventative vaccine

Healthy mice were treated with 3 doses of TNL or PBS control every 10 d. 10 d after the final dose, mice were inoculated with 4T1 breast cancer (**Fig. 4.5A**). Survival for TNL-vaccinated mice was enhanced from an average of 44 to 49.5 d, and onset of metastasis was delayed an average of 10 d (**Fig. 4.5B-C**). Tumor volume overtime was slower for TNL-vaccinated mice than PBS-vaccinated mice, although the final tumor volumes and final tumor weights were not statistically significant (**Fig. 4.5D-G**). While there was not a significant difference in splenocyte composition post-mortem between the groups, there was a trend in enhanced percent positive CD68+CD40+ splenocytes for the TNL group (**Fig. 4.5H-J**). IHC was performed to analyze immune cell infiltration at the tumor site and proliferation of tumor cells (**Fig. 4.5K**). There was a significant increase in CD3+ T cells and CD45r+ B cells in the tumors of TNL-vaccinated mice compared to controls (**Fig. 4.5L-M**). Interestingly, despite the increased immune cell infiltration, the percent of tumor cell proliferation was also significantly enhanced for TNL-vaccinated mice compared to controls (**Fig. 4.5N**).
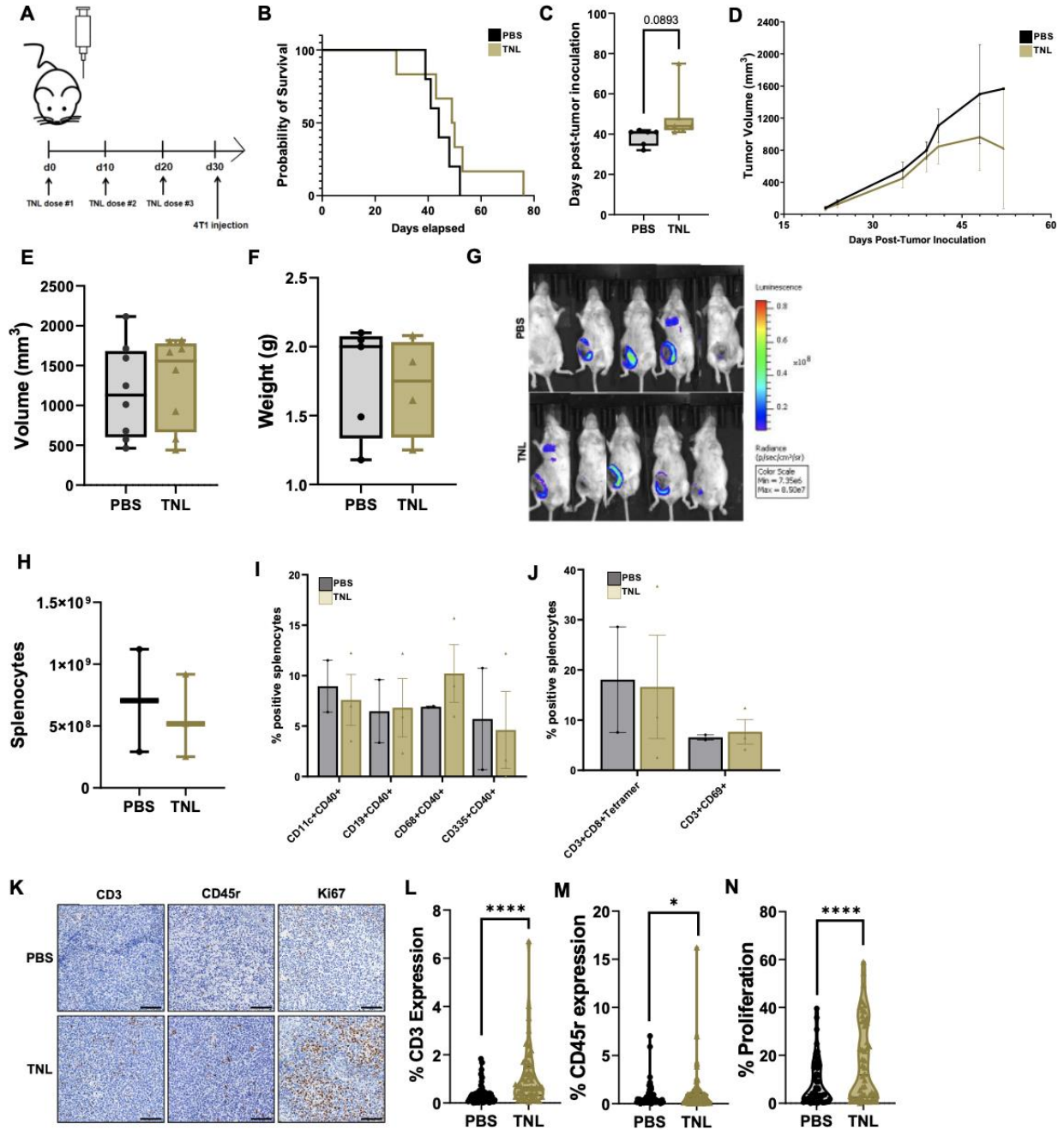
Figure 4.5. In vivo multi-dose TNL study. (A) Multi-dose mouse protocol. (B) Survival curves for PBS-
and TNL- treated mice. (C) Day of observed metastasis onset following tumor inoculation. (D)

Changes in tumor volume over time. (e) Final tumor volume of each mouse. (F) Final tumor weight

following resection. (G) Representative bioluminescent imaging. (H) Splenocyte population counts.

(I) Percent positive splenocyte activation markers and (J) percent positive splenocyte tetramers. (K)

Representative immunohistochemistry micrographs. (L) % CD3 expression (M) % CD45r expression and (N) % Proliferation. Scale bar = 100 µm. *p<0.05, ****p<0.001.

DISCUSSION

In this study, we were able to expand upon our previous research and analyze the immunological response to TNL vaccine both ex vivo and in vivo. Antigen-presenting cells such as DCs were determined to be activated by TNL and were further explored for specific pathways of activation. It was observed that the NF-kB and c-Fos pathways are being significantly activated by TNL, and subsequently cytokine release was enhanced. After 24 h, changes in morphology through RAC1 expression occurred, as well as specific markers of TNL uptake. Through functional assays, we were able to see specific activation of T cells via CD3+Tetramer+ cells, and through loss of 4T1 cell viability after treatment with TNL-treated splenocytes.

Temporal analyses revealed sustained activation for one week with increased counts of lymphocytes, monocytes, and neutrophils, and activation of T cells. The results of the study demonstrated a potential need for multiple "booster" doses. Overall, the multi-dose in vivo study showed a reduced tumor burden, delayed onset of metastasis, and increased survival for TNL-vaccinated mice compared to PBS-vaccinated mice. Tumor infiltration was also enhanced for TNL-vaccinated mice, indicating an increased immune response. Increased intertumoral immune cell infiltration has been associated with improved patient prognosis.[25] Further studies will need to be conducted to better understand the increase in proliferation despite the increase in immune cell infiltration. Although the mice in the TNL group ultimately developed tumors, the results from this study demonstrate the potential for TNL as a preventative vaccine. Understanding the immunogenicity of the TNL vaccine provides insight into the effectiveness of

our vaccine in eliciting a preventative response. With the knowledge gained from this study, we can develop future iterations of the TNL vaccine with enhanced targeting of specific immune cell populations. For instance, the addition of an adjuvant to the TNL formulation may aid in augmenting the immune response for improved performance, or delivery methods may be required for immune cell targeting.

AUTHOR CONTRIBUTIONS

Conceptualization: JAD, MRK

Methodology: JAD, ARF, MRK

Investigation: JAD, ARF, SVK, SJR, KNGC

Supervision: MRK

Writing: JAD, MRK

Editing: JAD, MRK

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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CHAPTER 5:

Fluid shear stress enhances dendritic cell activation

Dombroski, J.A., Rowland, S.J., Fabiano, A.R., Knoblauch, S.V., Hope, J.M., and King, M.R. Fluid shear stress enhances dendritic cell activation. In review, *Scientific Reports.*

ABSTRACT

The ex vivo activation of dendritic cells has been widely explored for targeted therapeutics, although these treatments remain relatively expensive. Finding ways to reduce treatment costs while enhancing cell activation could help to make immunotherapies more broadly accessible. Cells can be activated by both internal and external forces including fluid shear stress. Fluid shear stress due to blood flow activates immune cells in circulation via opening of mechanosensitive ion channels. In this study, dendritic cells were activated by exposure to circulatory levels of fluid shear stress using a cone-and-plate flow device and analyzed for activation markers. After 1 h of shear stress exposure, an increase in cytokine release was present in immortalized cells as well as phosphorylation of the proteins NF-κB and cFos in primary DCs. Changes in DC morphology, metabolism and proliferation were also observed. These compelling new findings point to the potential for using FSS to activate DCs FSS for ex vivo therapeutics.

INTRODUCTION

Therapeutics have been developed in which a patient's dendritic cells (DCs) are removed, activated ex vivo, and reintroduced into the body to enhance the efficacy of treatments.[1–3] The first FDA-approved therapeutic cancer vaccine, Sipuleucel-T (Provenge), was approved in 2010 for the treatment of metastatic castration-resistant prostate cancer (mCRPC).[4,5] For this therapy, DCs are activated with granulocyte macrophage-colony stimulating factor (GM-CSF) or prostatic acid phosphatase (PAP), facilitating T cell priming and enabling the targeting of prostate cancer

cells.[4,5] While Sipuleucel-T is associated with low treatment periods as well as low hospitalization rate due to adverse events, the cost of the therapeutic remains expensive to patients compared to traditional cancer treatments.[4] Additionally, although a novel concept and showing promise when compared to placebo, Sipuleucel-T has demonstrated limited effectiveness in recent studies.[6,7] Therefore, boosting the efficacy of Sipuleucel-T or finding alternative ex vivo therapeutics has emerged as a significant need.

Internal and external forces play roles in activating a variety of cell types, ranging from endothelial cells and immune cells to cancer cells.[8–10] Shear stress is a force which activates cells by initiating membrane deformation, resulting in the opening of mechanosensitive ion channels (MSCs) , which affect signal transduction through the influx of calcium ions.[11–16] Calcium is a ubiquitous secondary messenger responsible for a host of intracellular responses.[10,17–20] Fluid shear stress (FSS) is a force which immune cells experience due to blood flow in circulation.[21–23] Several studies have demonstrated that blood cells such as T cells and platelets are sensitive to shear stress, with increased proliferation and/or cytokine release.[13,24,25] Neutrophils have also been observed to have increased activation following FSS.[26] Specifically, shear stress promotes the maturation, growth, and progression of the cell cycle in DCs.[27] One study applied cyclic strain to DCs and observed a resulting increase in the expression of MHC II and costimulatory molecules, although there is a general dearth of research into DC activation via shear stress.[28]

FSS can be recreated in vitro within cone-and-plate or other flow devices.[29–31] Cone-and-plate flow devices are advantageous in that, based on their designs, they apply the same local shear rate to cells at every location within the device, which is not true in pressure-driven flow or Couette flow devices.[29] In this study, we stimulated DCs with FSS applied via a cone-and-plate flow device, analyzing various markers of activation. Both immortalized DCs and two primary cell lines were used in this study.

MATERIALS AND METHODS

Cell culture: DC2.4 murine DCs were purchased from Sigma-Aldrich (Catalog No. SCC142). DCs were grown in culture media consisting of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1X L-Glutamine, 1X non-essential amino acids, 1X HEPES buffer solution and 0.0054X β-Mercaptoethanol, as recommended by the manufacturer. Cells were maintained in an incubator at 37°C with 5% CO_2, and experiments performed at ~80% confluency.

Reagents: RPMI-1640 and FBS (Invitrogen), non-essential amino acids and HEPES buffer (Gibco), and β-Mercaptoethanol (Sigma-Aldrich) were obtained for preparation of the DC2.4 cell culture media. Hank's balanced salt solution (HBSS) with and without calcium and magnesium was purchased from Gibco. Lipopolysaccharide (LPS) solution (500X) was purchased from Thermo Scientific. 32% paraformaldehyde aqueous solution, electron microscopy grade was obtained from Electron Microscopy Sciences. Anti-mouse PE-MHC (Class I H-2Kk) was purchased from Antibodies-Online.com. Anti-mouse PE-CD40 (5C3), anti-human PE-CD40 (5C3), and PE anti-CD86 human monoclonal antibody were purchased from BioLegend. Anti-mouse PE-CD70 (FR70), PE-CD80 (16-10A1), PE-I A/I E (M5/114.15.2) (MHC II), PE-IL-6 (MP5 20F3), PE-IL-12 (p40/p70) (C15.6), and PE mouse anti-Ki-67 (567720) were used for extracellular and intracellular staining (BD). Anti-mouse PE-CD83 (Michel 17), PE-CD197 (CCR7) (4B12), PE-phospho-NFkB p65 (Ser529) (NFkBp65S529-H3), PE-phospho-c-Fos (AP-1) (Ser32) (cFosS32-BA9), and PE (MA5-36891) were obtained from Thermo Scientific. Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006) was used to analyze cytokine release from DCs (R&D Systems). VECTASHIELD® Antifade Mounting Medium (H-1000-10) and Anti-RAC1 (MBS9200589) were purchased from Vector Laboratories and MyBioSource, respectively, and used for confocal microscopy. 2-NBD-Glucose (186689-07-6) was used to measure glucose uptake (Abcam). DAPI (D9542-10MG), Tween® 20, viscous liquid, CAS 9005-64-5 (P1379)

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Ficoll-Paque PLUS (GE17-1440-03), Poly-L-lysine solution and Triton X-100 were acquired from Sigma-Aldrich. ActinRed 555 ReadyProbes Reagent and secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 goat anti-mouse IgG (H+L) were purchased from Invitrogen for confocal imaging. 10% Normal Goat Serum was also purchased for imaging (Life Technologies). For isolation of healthy patient dendritic cells, Blood Dendritic Cell Isolation Kit II, human (130-091-379) was purchased from Miltenyi Biotec.

Fluid shear stress application: Six Brookfield cone-and-plate viscometers, which consist of a stationary plate in near contact with a rotating cone, were used to apply FSS to DCs throughout this study. The protocol used for these experiments followed that described previously.[11,29] The advantage of using a cone-and-plate flow device for applying FSS is that the cells experience the same shear rate at all locations within the fluid volume. Flow is assumed to be laminar, and the fluid assumed to be Newtonian. A series of equations can thus be used to predict the local shear rate and stress. The equations are as follows:

$G = \frac{\omega}{\tan(\theta)}$, where G is shear rate, ω is angular velocity (rad/s), and θ is the angle of the cone (rad).

$\tau = \mu G$, where τ is the FSS and μ is the viscosity (cP). μ is expected to be 2.5 cP for these cell suspensions.

Prior to flow experiments, surfaces of the cone-and-plate device were incubated with 5% bovine serum albumin (BSA) for 1 h to block nonspecific binding. In mouse serum albumin (MSA) control experiments, surfaces are blocked with 0.1% MSA. DC2.4 cells were lifted with trypsin, washed, and resuspended in complete RMPI media at 200,000 cells/mL. The viscometers were equipped with Brookfield Cone Part No. CPA-41Z spindles, accommodating a total of 2 mL of each sample. Cells were placed on a rotator to produce "static" conditions (<0.05 dyn/cm^2). For "shear"

conditions, an FSS of 5 dyn/cm^2 was applied for 1h using the cone-and-plate flow device unless otherwise indicated. Static and shear conditions were either left untreated or treated with lipopolysaccharide (LPS), a potent stimulator, at 10 μg/mL. "0 dyn/cm^2" was also used as a control, where viscometers were set up identically to shear experiments, but motors were left in an "off" state to ensure that nothing in the viscometer device was causing activation in the absence of force.

Flow cytometry analysis and antibody staining: Following FSS stimulation, cells were plated overnight or immediately prepared for flow cytometry analysis. For intracellular proteins such as phospho-NF-κB and -cFos, cells were fixed with 4% paraformaldehyde for 10 min. Cells were washed with HBSS and permeabilized with 100% ice cold methanol. Cells were washed again and stained for 15 min in the dark with antibodies fluorescently tagged with PE fluorophore while suspended in 1% BSA. A Guava easyCyte 12HT flow cytometer (Millipore, Billerica, MA) was used to measure fluorescence intensity, with FlowJo software used for gating and analysis. For extracellular proteins analyzed after 24 h, cells were lifted with trypsin, washed, and incubated with primary antibodies pre-conjugated with fluorophores in 1% BSA and then washed once more prior to analysis. For RAC1 staining, cells were first stained with the primary antibody and then stained for 15 min with an Alexa 488 secondary antibody and washed prior to analysis via flow cytometry

Glucose uptake analysis: 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) is used as a fluorescent probe to analyze glucose uptake. 2-NBDG consists of a fluorescent d-glucose analog, which cells uptake in glucose-free media and is visualized using flow cytometry and confocal imaging.[32] 5 mg 2-NBDG was reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 25 mM. For this study, cells were exposed to 0 or 5 dyn/cm^2 FSS

for 1 h in glucose-free media, incubated with 35 μM 2-NBDG for 30 min in the dark, washed, and analyzed via flow cytometry.

pH measurements: Immediately following applied FSS, the pH of the media was measured using a Thermo Scientific Orion Star A211 Benchtop Meter and pH Electrode Set. Calibrations were performed using 4.0, 7.0 and 10.0 pH buffers, and measurements were taken following calibration.

Annexin V assay: Annexin V assay for apoptosis was performed to test the effects of FSS on cell viability, following previously established protocols.[30,33–35] 100,000 cells were collected following 1 h FSS at 5 dyn/cm^2, washed, and resuspended in 150 µL HBSS with calcium and magnesium, and incubated with 3 µL annexin V (AV) and 3 µL propidium iodide (PI). Controls were prepared for unstained, AV only and PI only samples. Cells were incubated at RT for 15 min in the absence of light exposure. 100 µL of HBSS was added to each of the samples, which were then run through the flow cytometer. Cells that were negative for both AV and PI were identified as viable, as they lacked markers for apoptosis (AV) and necrosis (PI).

Cytokine staining: For intracellular cytokine staining (ICS), cells were plated overnight following FSS stimulation. After 24 h, cells were treated with 1:1000 GolgiPlug Transport Inhibitor to block cytokine secretion. After 4 h, cells were lifted and stained following the previously described protocol for staining intracellular proteins. IL-6, IL-12 and chemokine receptor type 7 (CCR7) were investigated in this study. A two-way ANOVA test was used to determine statistical significance.

Proteome profiler: DC2.4 cells were prepared as described above and brought to a final concentration of 1 x 10^6 cells/mL. FSS of 0 or 5 dyn/cm^2 was applied to cells for 1 h and then cells were plated for 24-48 h to analyze cytokine expression using Proteome Profiler Mouse Cytokine

Array Kit, Panel A (R&D Systems). The supernatant was collected from the cell suspensions and centrifuged at 4800 RPM for 10 min at 4°C. The manufacturer's instructions were followed for the array procedure, and an Odyssey CLx imager was used for chemiluminescence imaging for 2 min exposure. Image Studio Lite software was used to quantify membrane fluorescence intensities.

Confocal microscopy: Following the application of FSS, DCs were seeded onto glass coverslips previously coated with poly-l-lysine (PLL). After 4 h, cells were fixed with 4% paraformaldehyde and then permeabilized with 1% Triton. Cells were blocked with 5% BSA and 5% goat serum for 45 min, and later stained with DAPI nuclear stain and ActinRed 555 for 30 min. Coverslips were added to slides using Vectashield and cells were subsequently imaged using a Zeiss LSM 710 confocal microscope. Image analysis was performed using FIJI software. DCs with dendrite formation were defined as cells with 3 or more evident protrusions.

Isolation of bone marrow dendritic cells (BMDCs): BMDCs were isolated following the protocol by Madaan, et al.[36] Femurs were harvested from healthy BALB/c mice and bone marrow was flushed out using HBSS and a 29G syringe. Bone marrow cells were treated with 20 ng/mL GM-CSF on Day 0, and on Day 3 10 mL fresh supplemented RPMI was added to the dish with 20 ng/mL GM-CSF. On Day 6, macrophages remined adhered and DCs were in suspension. DCs were collected and used for FSS experiments as previously described.

Human dendritic cell isolation: Whole blood was collected from healthy human volunteers after informed consent (Vanderbilt University IRB Protocol #170222). Blood was collected in BD Vacutainer collection tubes with sodium citrate, with peripheral blood mononuclear cells (PBMCs) subsequently isolated using Ficoll-Paque gradient centrifugation. DCs were then isolated using Miltenyi Biotec's Blood Dendritic Cell Isolation Kit II according to the manufacturer's instructions.

Cells were labeled and non-DCs were depleted, followed by labeling and positive selection of DCs. DCs resuspended in RPMI at 5 x 10^4 cells/mL and FSS was applied as described previously.

Statistics: All data are reported as mean and standard error of the mean. Statistics were determined using student's t test unless otherwise indicated. Each experiment included a minimum of three independent replicates unless otherwise indicated. Significance is indicated by *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. GraphPad Prism software was used to perform statistical comparisons and produce figures for this article.

RESULTS

FSS from cone-and-plate flow device does not significantly affect cell viability of DCs

DCs were suspended in complete media and stimulated with FSS in a cone-and-plate flow viscometer at 5 dyn/cm² for 1 h. Tubes were left on a rotator for "static" conditions (<0.05 dyn/cm²). The cone-and-plate viscometer creates an environment where the same shear rate is applied to all DCs regardless of their position within the volume **(Fig. 5.1A)**. Following FSS exposure, cells were analyzed for viability using an Annexin V assay. DC2.4 cells under static conditions showed an average viability of 90.9% and cells under shear conditions showed an average viability of 83.8%. There was not a significant difference in viability in DCs under static conditions compared

shear conditions **(Fig. 5.1B-C)**. These results indicate that FSS was not causing significant apoptosis or necrosis in DCs and that experiments for ex vivo activation was justified.
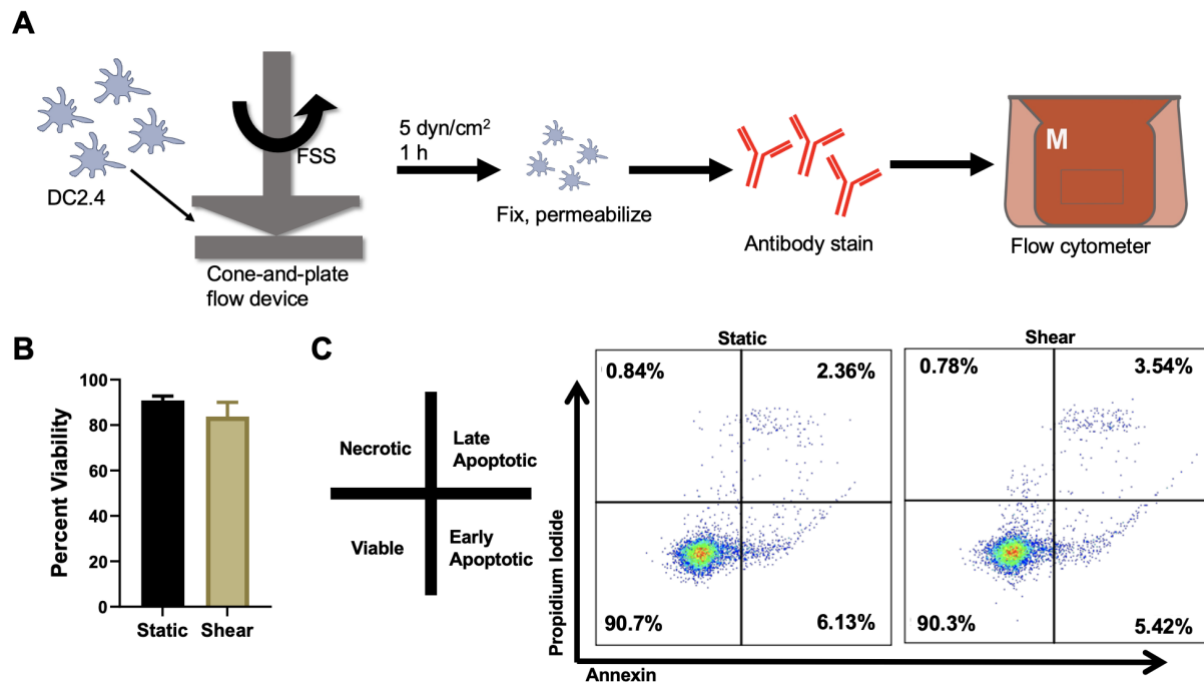
Figure 5.1. Cone-and-plate viscometer setup and cell viability assay. (A) Experimental design for FSS application. (B) Summary data for percent viability as determined by Annexin V assay. The average percent cell viability for DC2.4 cells under static conditions was 90.9 ± 1.9%, and 83.8 ± 6.2% for shear. There was no significant difference in these averages. (C) Graphical representation for interpreting Annexin V flow cytometry plots. Cells negative for propidium iodide (PI) and Annexin V (AV) were considered viable. Representative flow cytometry plots correspond to static and shear conditions.

Potent stimulator LPS plays a role in early MHC and costimulatory molecule expression on DCs following FSS

Following 1 h stimulation in a cone-and-plate flow device at 5 dyn/cm^2 FSS, DC2.4 cells were plated and incubated at 37°C for 24 h to measure expression of later markers of activation and compared to static conditions. Expression of major histocompatibility complex (MHC) and costimulatory molecules including CD40, CD70, CD80, and CD86 are indicative of DCs

functioning as antigen-presenting cells (APCs) to facilitate T cell priming.[37–39] After 24 h, cells were analyzed for the presence of MHCs and costimulatory molecules. No significant increase in MHC expression with DC stimulation via FSS was observed **(Fig. 5.2A)**. Costimulatory molecule expression was found to be enhanced in the presence of LPS, regardless of whether shear stress was present **(Fig. 5.2B)**. This trend was observed for CD86, and significant for CD40 and CD80. The mean percentage expression of CD40 under static conditions was 3.8% without LPS, and
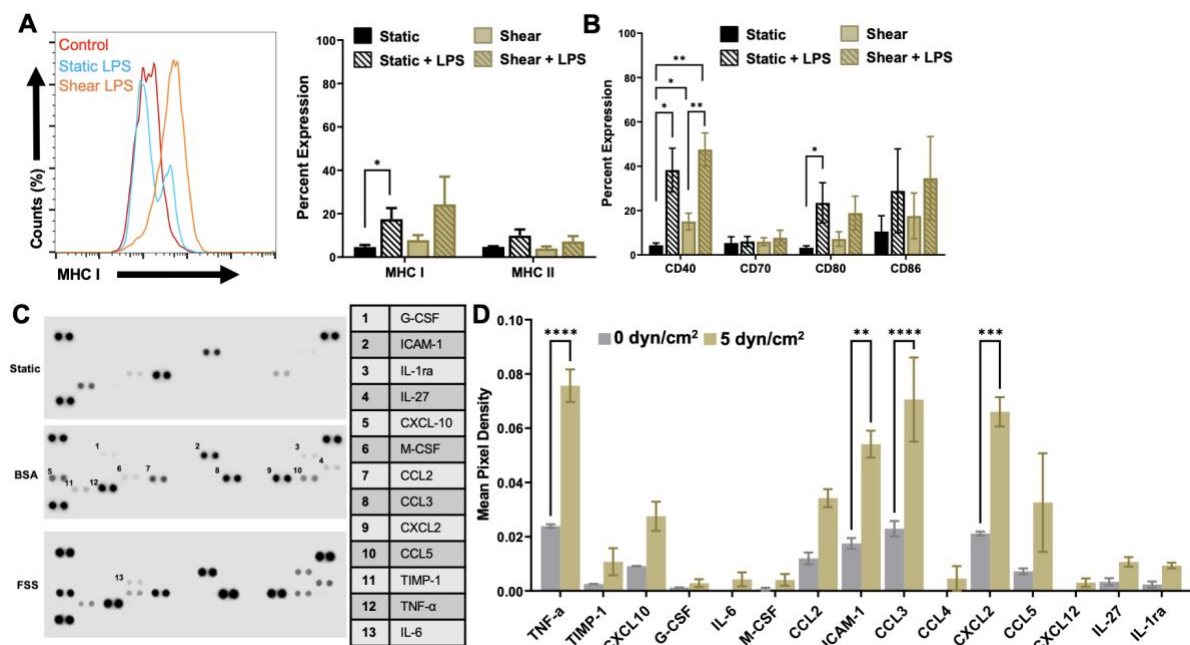
Figure 5.2. Costimulatory molecule and cytokine analysis of FSS-activated DCs. (A) Histogram reflecting representative data for MHC I expression following FSS and summary data for percentage of MHC I and MHC II expression. (B) Summary data for costimulatory molecule expression analyzed after 24 h. A trend was evident in increased CD80 expression in the presence of LPS and a significant increase in CD40 expression in the presence of LPS. (C) Proteome profiler representative cytokine array results and corresponding cytokines. (D) Summary of cytokine release analyzed after 24 h. A significant increase in cytokine release was observed after DCs were stimulated via FSS compared to DCs under static conditions. *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001.

47% with LPS. Under shear conditions, a similar pattern was observed, with the mean percentage expression 14% without LPS and 45% with LPS.

Increase in cytokine release was observed after FSS stimulation

DC2.4 cells were plated for 24 h following stimulation via 5 dyn/cm^2 FSS for 1 h, while control samples were maintained at 0 dyn/cm^2. Cytokines and chemokines are a direct measure of immune cell activation, especially for DCs, where they aid in trafficking and driving specific T cell activation.[40] Performing intracellular staining for a few key DC cytokines, a significant increase in release of IL-6, IL-12 and CCR7 was observed for FSS-activated DCs compared to static controls (**Supplemental Figure 5.1**). Interestingly, no significant difference was observed between FSS and FSS with LPS. A more complete cytokine array was then performed using Mouse Cytokine Array Panel A, a proteome profiler from R&D Systems. 40 different cytokines were analyzed using the panel, comparing the supernatant of FSS-stimulated DC2.4 cells. There was an increase in cytokine release for TIMP-1, CXCL10, G-CSF, IL-6, M-CSF, CCL2, CCL4, CCL5, CXCL12, IL-27 and IL-1ra, and a significant increase for TNF-α, ICAM-1, CCL3 and CXCL2 **(Fig. 5.2C-D)**. An analysis was performed to validate that BSA pre-blocking of the viscometers was not responsible for the activation seen during FSS application by blocking with MSA (**Supplemental Figure 5.2A-B**).

DCs stimulated by FSS maintain activation state for an extended period

Interestingly, while there were small differences observed in costimulatory molecule expression after 24 h, these differences were more prominent after 48 h. DCs 48 h after FSS treatment showed a trend of increased CD40, CD80 and CD86 expression compared to unstimulated cells (**Fig. 5.3A**). After 72 h, these differences were less obvious (**Fig. 5.3B**). After 48 h, cytokine

release, as detected via proteome profiler, was reduced from 24 h but still higher for most cytokines for FSS-stimulated cells (**Fig. 5.3C**).
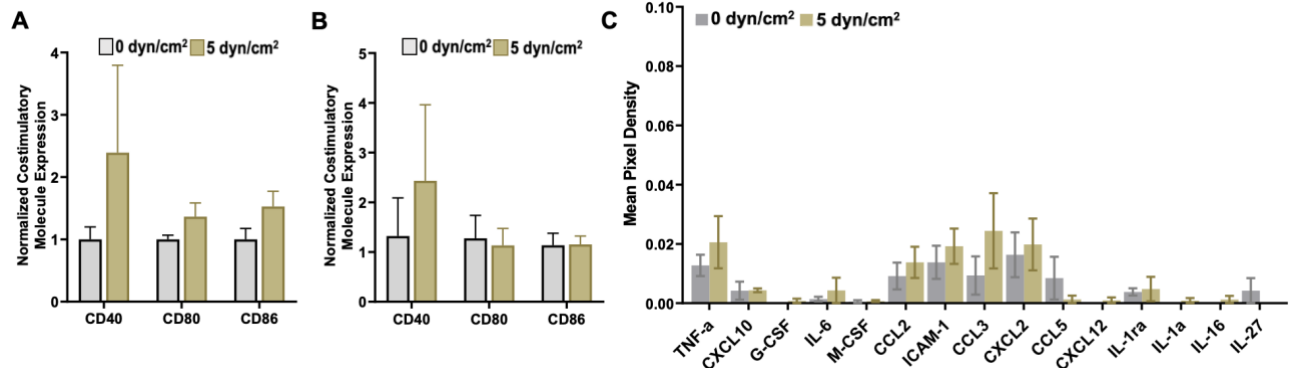
Figure 5.3. DC activation for extended time points. (A) A trend of increased costimulatory molecule expression was observed for up to 48 h following FSS. (B) Differences in costimulatory molecule expression between FSS-stimulated cells and unstimulated cells were reduced after 72 h. (C) Summary data for cytokine release as observed after 48 h using proteome profiler.

Changes in DC metabolism were evident following FSS

Increased uptake of 2-NBDG was detected for cells exposed to 5 dyn/cm^2 of FSS for 1 h, compared to cells exposed to 0 dyn/cm^2 **(Fig. 5.4A).** As 2-NBDG consists of a d-glucose analog, higher levels of 2-NBDG are indicative of increased glucose uptake by cells.[32] Following FSS, notable visual changes in cell culture media were observed when compared to cells exposed to 0 dyn/cm^2 (**Supplemental Figure 5.3**). Therefore, using a Thermo Scientific Orion Star A211 Benchtop Meter and pH Electrode Set, changes in pH were analyzed following FSS treatment. There was a significant increase in pH immediately following 1 h of FSS (**Fig. 5.4B).** Percent positive DCs with cell proliferation marker Ki67 was significantly increased immediately following FSS (**Fig. 5.4C-D).** These changes were still observed up to 24 h when compared to cells under static conditions **(Fig. 5.4E-F).**

DC morphology was significantly affected by FSS with enhanced dendrite formation
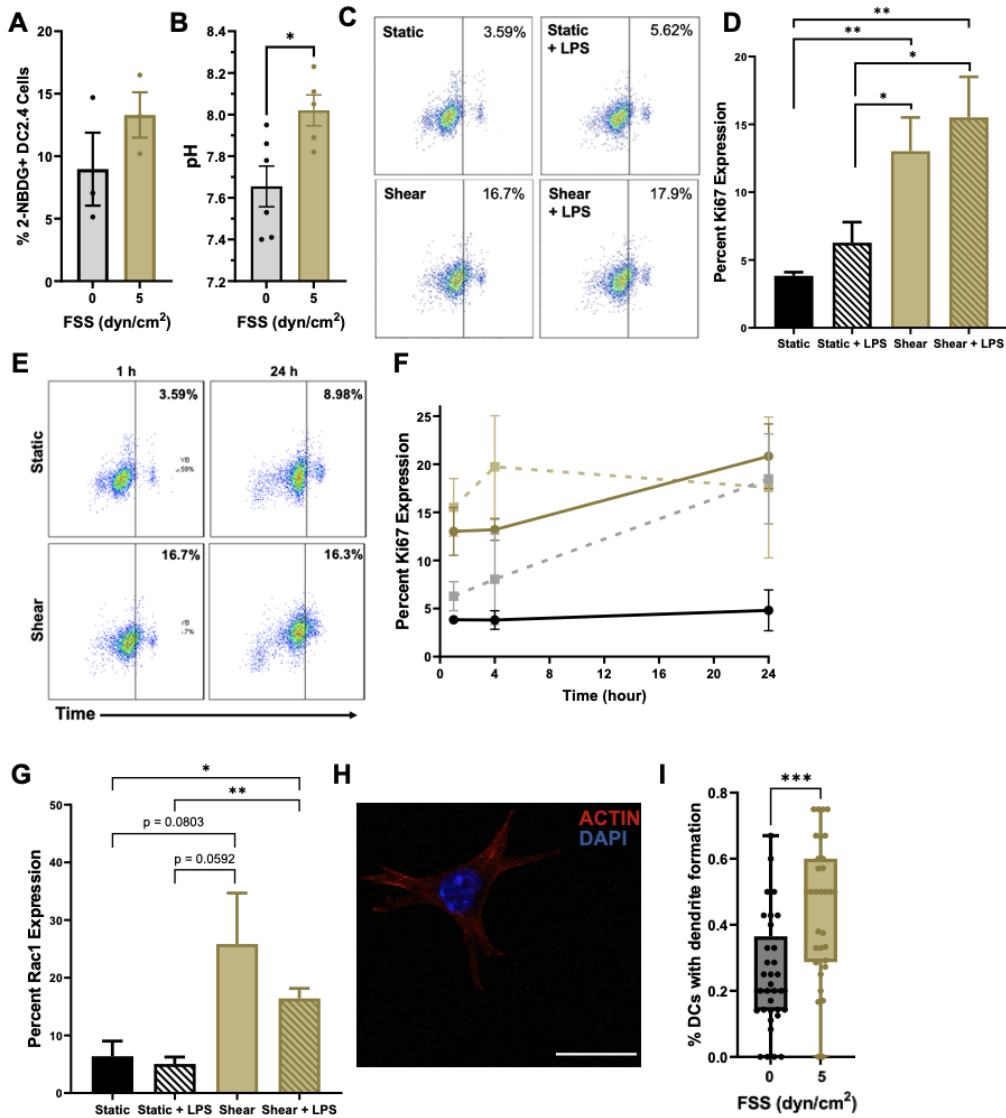
Figure 5.4. Metabolic and morphological changes to DCs following FSS treatment. (A) Glucose uptake by DC2.4 cells was increased following 5 dyn/cm^2 of FSS exposure, as observed by the increase in 2-NBDG uptake into cells. (B) The pH of the media containing cells exposed to 5 dyn/cm^2 FSS for 1 h became significantly higher compared to cells exposed to 0 dyn/cm^2. (C) Representative flow cytometry plots of percentage of Ki67 expression following FSS. (D) Combined percentage Ki67 expression comparing cells under four conditions: static, static + LPS, shear, and shear + LPS. (E) Representative flow cytometry plots for percentage Ki67 expression over time in cells following FSS. (F) Percentage Ki67 expression over time in DCs following FSS. (G) RAC1 expression in DC2.4 cells following 1 h FSS. (H) Representative micrograph of dendrite formation in DCs stimulated with FSS. (I) Percent of DCs with dendrite formation following FSS stimulation. *p<0.05, **p<0.01, and ***p<0.005. Scale bar = 20 μm.

Enhanced RAC1 is indicative of dendrite formation in DCs.[41] Percent positive RAC1 expression in DC2.4 cells was significantly increased following FSS, compared to cells under static conditions **(Fig. 5.4G).** Additionally, the percentage of DCs with dendrite formation was significantly increased following stimulation via FSS, as observed via confocal microscopy **(Fig. 5.4H-I)**. Actin levels were decreased following FSS treatment (**Supplemental Figure 5.4**).

Primary DCs become activated by FSS ex vivo

Primary cells were then investigated for activation via FSS. Nuclear factor-kappa B (NF-κB) phosphorylation (phospho) was measured in primary DCs to analyze activation. NF-κB transcription factor is regulated by calcium influx, and leads to increased cytokine expression, maturation, survival, and cell cycle progression in a variety of cell types including DCs.[42–46] Activator protein 1 (AP-1) expression was measured by analyzing activation of the AP-1 transcription factor complex using an antibody for phospho-c-Fos. AP-1 is another transcription factor that is regulated by calcium ions and responsible for controlling cellular processes such as proliferation and regulation of cytokine release.[47,48] BMDCs were isolated from healthy mice and stimulated with 0 or 5 dyn/cm^2 FSS for 1 h, as in experiments with immortalized cells. A trend in increased costimulatory molecule expression of BMDCs was observed (**Fig. 5.5A**). There was a significant increase in NF-κB and cFos phosphorylation (**Fig. 5.5B**). DCs isolated from healthy human volunteers were stimulated with 0 or 5 dyn/cm^2 FSS for 1 h. Following FSS, a significant increase in NF- κB phosphorylation was observed, as well as a trend in enhanced CD86 (**Fig. 5.5C-D**).
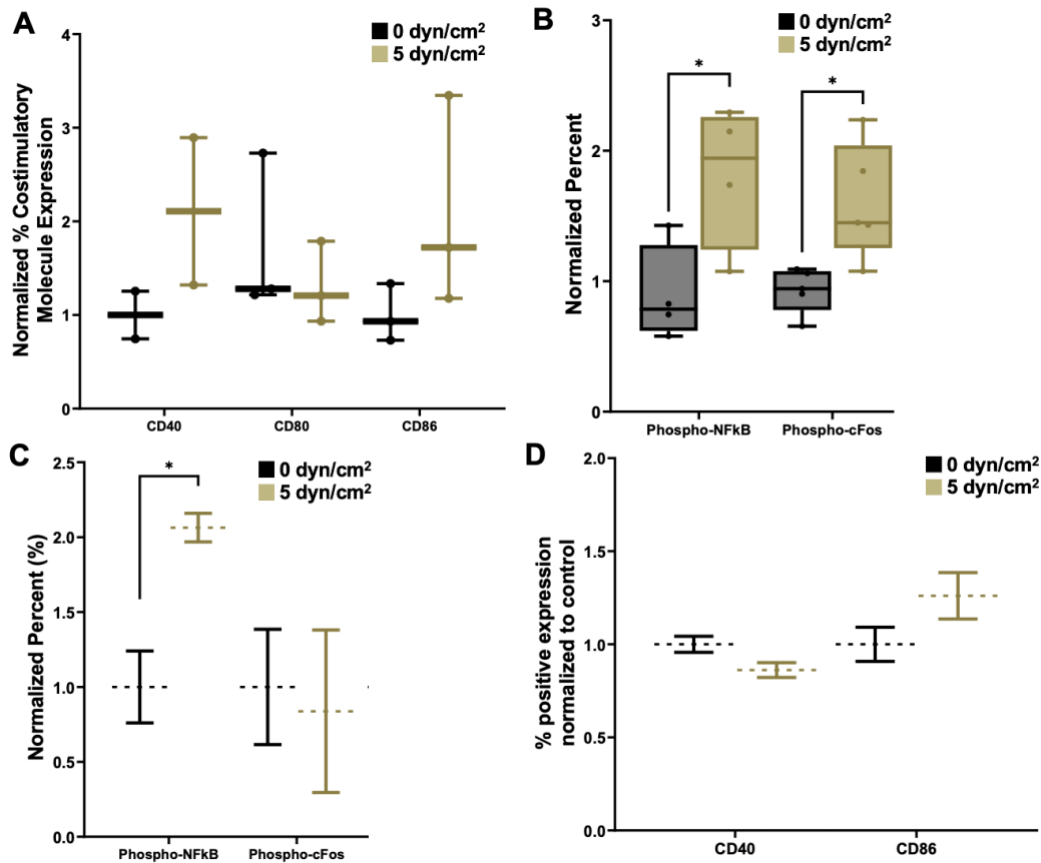
Figure 5.5. Primary DC response to stimulation via FSS. (A) Trend in increased costimulatory molecule expression of murine BMDCs exposed to 5 dyn/cm^2 of FSS for 1 h, compared to cells exposed to 0 dyn/cm^2. (B) Significant increase in phosphorylation of NF-κB and cFos for BMDCs stimulated with FSS for 1 h. (C) Increased NF-κB phosphorylation for primary human DCs following FSS stimulation for 1 h at 5 dyn/cm^2, compared to cells exposed to 0 dyn/cm^2. (D) Trend in increased CD86 costimulatory molecule in human DCs following FSS stimulation. *$p < 0.05$.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate that FSS activates DCs. It was first established that shear stress minimally reduces cell viability in DCs, suggesting that it is a

safe mechanism for ex vivo activation of immune cells. With just 1 h of applied FSS, significant cellular changes were recorded, indicative of DC activation.

Cytokine release and costimulatory molecule expression, two essential components of the immune synapse for T cell activation by APCs, were enhanced following FSS. DC cytokine release is important for recruiting immune cells and driving specific T cell responses[40]. While there was not a significant difference in MHC and costimulatory molecule expression in DCs following 24 h of FSS stimulation, there was a significant enhancement in the presence of the potent stimulator LPS, particularly for CD40. The results suggest that, if enhanced costimulatory molecule expression is desired at such an early time point, a stimulator may be necessary in addition to the FSS treatments. Incubation with a tumor-specific antigen (TSA) or tumor-associated antigen (TAA) could aid in achieving this specific response; for instance, PAP could be applied to mCRP therapies[49]. Nevertheless, at later time points, enhanced costimulatory molecule expression due to FSS alone was observed.

Exploring metabolic and morphological changes to DCs following FSS stimulation revealed interesting findings compared to non-stimulated cells. Cells showed increased glucose uptake and Ki67 expression and pH levels were enhanced, indicating metabolic processes occurring at a cellular level. It would be interesting to further explore these aspects of DC function following FSS activation to gain further insight into the processes taking place. Additionally, changes in morphology with RAC1 and dendrite formation were observed, indicating DC activation as an APC.

In primary cells, NF-κB and cFos phosphorylation were enhanced with stimulation via FSS. Increased expression of these transcription factors gives insight into the pathways involved in DC

activation via FSS. These transcription factors play a major role in DC function and maturation, particularly through the release of cytokines to drive T cell priming.

Despite the major advances in ex vivo therapeutics, the costs of these treatments remain high for patients. Identifying a way to reduce these costs while further enhancing activation will be advantageous for developing improved therapies. The results from this study suggest that FSS could be used in the production of DC-based immunotherapies to drive down the costs of these treatments. Through the ex vivo stimulation of DCs via FSS in combination with an additional activating factor, the necessary transcription factors can be activated, cytokines released, and costimulatory molecules enhanced, to yield successful therapeutics.

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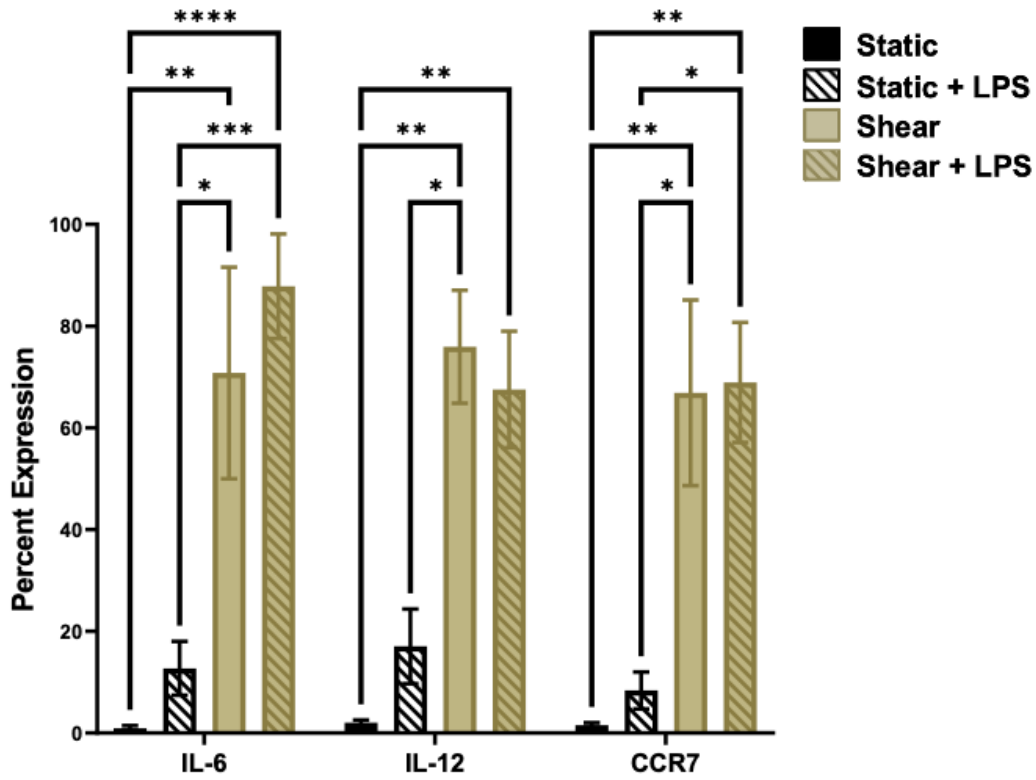
Supplemental Figure 5.1. Intracellular cytokine staining of DC2.4 cells in static (rotator) and shear (FSS) conditions. No significant difference was observed between FSS and FSS with LPS. The mean percent expression of cytokine release was 0.92% (static) and 71% (shear) for IL-6, 2.0% (static) and 76% (shear) for IL-12, and 1.5% (static) and 67% (shear) for CCR7. *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$.
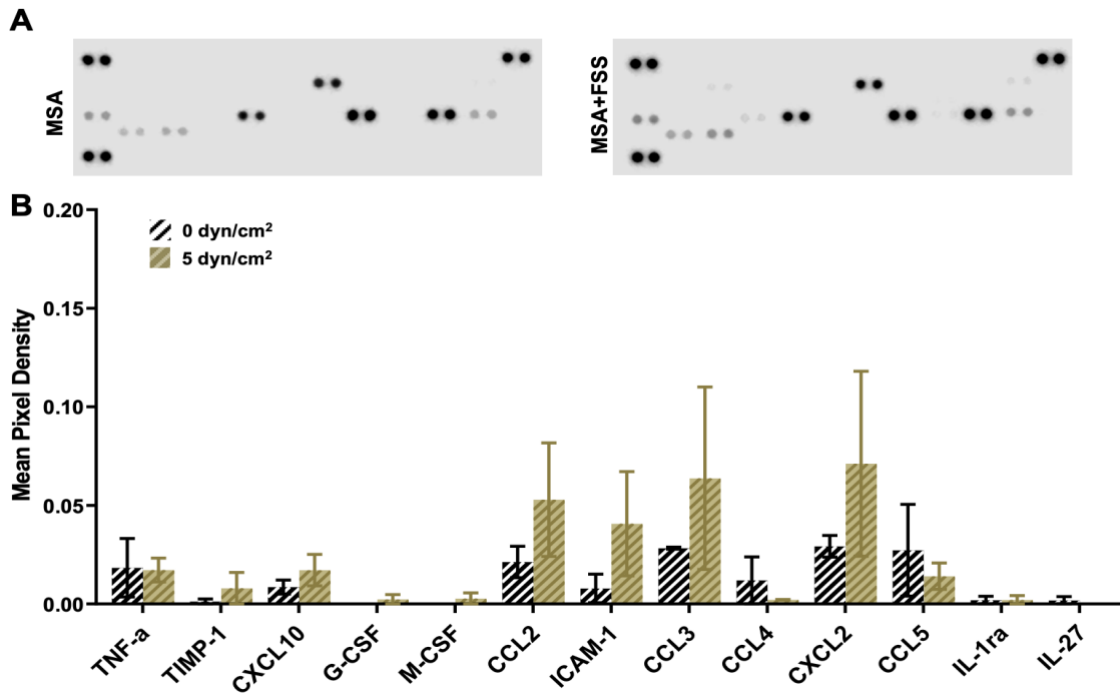
Supplemental Figure 5.2. Analysis of the effect of pre-blocking cone and plate flow devices with MSA instead of BSA. (A) Representative proteome profiler arrays for Mouse Cytokine Array, Panel A. (B) Summarized results for cytokine release following FSS of cone and plate flow device following pre-blocking with MSA.

Supplemental Figure 5.3. Visual changes in media color comparing cells exposed to 0 and 5 dyn/cm^2.
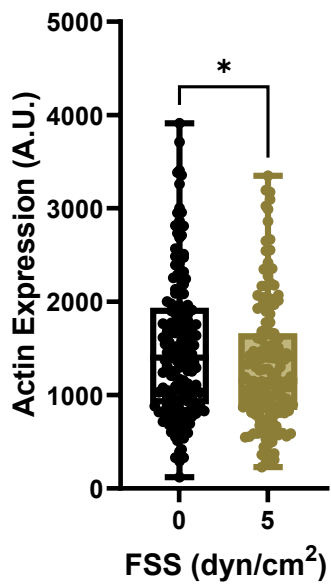
Supplemental Figure 5.4. Actin expression following FSS stimulation. *p<0.05.

Chapter 6:

Dendritic cells isolated from the blood of prostate cancer patients respond to ex vivo fluid shear stress stimulation

Jenna A. Dombroski, Monika Antunovic, Kerry R. Schaffer, Paula J. Hurley, and Michael R. King. Dendritic cells isolated from the blood of prostate cancer patients respond to ex vivo fluid shear stress stimulation.

ABSTRACT

Prostate cancer is the most common cancer among men in the United States, and one of the leading causes of cancer-related deaths in men. Patients diagnosed with prostate cancer can receive a variety of treatment regimens including chemotherapy, radiation, hormone therapy, and immunotherapy. Cancer immunotherapies often boast the advantage of being patient-specific and some current therapies involve removing patient immune cells and activating them ex vivo. Various cell types can be activated by external forces such as fluid shear stress. We have hypothesized that cancer patient dendritic cells, antigen-presenting cells essential to initiating an effective T cell response, can be activated by fluid shear stress more efficiently than existing methods. In this paper, we report a new protocol for activating cancer patient dendritic cells with fluid shear stress ex vivo and analyze the resulting response.

INTRODUCTION

1 in 8 men in the United States will be diagnosed with prostate cancer in his lifetime.[1] As with other cancers, 5-year survival rates vary based on stage, dropping from 99% for early stages to 32% for late-stage prostate cancer.[2] Mortality was heavily reduced after 1993 with early

detection through prostate specific antigen (PSA) screening, but this decline has severely slowed down due to a reduction in screening for PSA.[1] Current therapies for prostate cancer include surgical removal, chemotherapy, radiation, hormone therapy, and immunotherapy.[3–6] Androgen deprivation therapies, which commonly improve disease prognosis and reduce patient symptoms, are successful for 18-36 months before the cancer can ultimately become castration-resistant.[3] Despite many advances in prostate cancer therapy, finding ways to increase the effectiveness of current treatments maintains a need in the field, particularly for patients with metastasis.

Provenge (Sipuleucel-T) was the first FDA-approved cancer immunotherapy, approved for metastatic castration-resistant prostate cancer (mCRPC) in 2010.[7] Provenge activates a patient's immune cells with granulocyte macrophage-colony stimulating factor (GM-CSF) and prostatic acid peptide (PAP) ex vivo and reintroduces the cells back into the patient for enhanced lymphoid homing and T cell priming.[7,8] While Provenge increases the overall survival rate of mCRPC patients, its effectiveness remains limited.[9–11] This motivates the development of a more effective protocol for the ex vivo activation of a cancer patient's immune cells.

Pluvicto (^{177}Lu-PSMA-617) has recently been approved by the FDA as the first radiotherapeutic for prostate cancer.[12] Pluvicto functions as a two-part therapeutic consisting of PSMA-617, which directly targets PSMA+ prostate cancer cells and allows for the therapeutic to be endocytosed, where the radionucleotide second component, Lutetium, causes DNA breaks within the cell and surrounding cells.[12] Despite the promise of Pluvicto as a therapy, some major limitations remain, such as high costs at around $50,000 per cycle and supply chain issues and shortages that can have detrimental consequences for patients.[13–15]

It is understood that cells can be activated by forces, and this has been demonstrated in our lab with immune cells such as T cells responding to fluid shear stress (FSS).[16–18] Cells sense and respond to forces via the opening of mechanosensitive ion channels to allow an influx of ions, enabling a host of responses.[19] In this study, we aim to determine if dendritic cells (DCs) isolated from cancer patients can be activated ex vivo within a cone-and-plate flow device.

MATERIALS AND METHODS

Cell culture: Dendritic cells (DCs) were suspended and/or grown in culture media consisting of RPMI-1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (P/S). Cells were maintained at 37°C with 5% CO_2.

Human dendritic cell isolation: All experimental protocols involving humans or human tissue samples were approved by Vanderbilt University IRB Protocol #170222. All methods were carried out in accordance with relevant guidelines and regulations, and patient samples remained deidentified throughout the entire study. The cohort consisted of prostate cancer patients undergoing laboratory testing at the Vanderbilt-Ingram Cancer Center (VICC) in Nashville, Tennessee.

Following informed consent from patients, whole blood was collected in BD Vacutainer collection tubes containing sodium citrate (BD 366643). Peripheral blood mononuclear cells (PBMCs) were subsequently isolated via gradient centrifugation using Ficoll-Paque. DCs were isolated from PBMCs according to the manufacturer's directions for magnetic bead isolation (Miltenyi Biotec's Blood Dendritic Cell Isolation Kit II). Cells were negatively selected for non-DCs and then positively selected for DCs, and the resulting DCs were resuspended in RPMI at 1×10^5 cells/mL.

Fluid shear stress application: FSS was applied to DCs via an array of Brookfield Cone-and-Plate Viscometers, which consist of a stationary plate and rotating cone in near contact. The protocol for applying FSS to cells has been previously described.[16,20–22] It is important to note that the cells in the flow device receive the same shear rate at all locations within the fluid, the fluid is assumed to be Newtonian, and flow is assumed to be laminar. As such, the equation used to determine the local shear rate is: $G = \frac{\omega}{\tan(\theta)}$, where G is shear rate, ω is angular velocity (rad/s), and θ is the cone angle (rad).

Once the shear rate is set, the shear stress can be determined by: $\tau = \mu G$, where τ is the FSS and μ is the viscosity (cP). Note that μ is approximated as 2.5 cP for the cell suspensions.

The flow devices were first calibrated according to the manufacturer's directions and the surfaces subsequently blocked for 1 h with 5% bovine serum albumin (BSA) to prevent nonspecific binding. Brookfield Cone Part No. CPA-41Z spindles were used with the flow devices to accommodate a total of 2 mL of each sample. Following isolation, DCs were immediately resuspended at a concentration of 100,000 cells/mL in complete RPMI media. Cells were stimulated with 5 dyn/cm^2 of FSS or 0 dyn/cm^2 for static control samples loaded into the device with zero rotation.

Reagents: RPMI-1640 and fetal bovine serum (FBS) for cell culture were purchased from Invitrogen. Hank's balanced salt solution (HBSS) without calcium and magnesium was purchased from Gibco. Anti-human PE-CD40 (5C3), Anti-human PE-CD80 (305207) and PE anti-CD86 human monoclonal antibody were purchased from BioLegend. PE-phospho-NFkB p65 (Ser529) (NFkBp65S529-H3) and PE-phospho-c-Fos (AP-1) (Ser32) (cFosS32-BA9)

antibodies were purchased from Thermo Scientific. Blood Dendritic Cell Isolation Kit II, human (130-091-379) was purchased from Miltenyi Biotec. CAS 9005-64-5 (P1379) Ficoll-Paque PLUS (GE17-1440-03) was obtained from Sigma-Aldrich. Blood tubes were purchased from BD (366643). 32% paraformaldehyde aqueous solution (electron microscopy grade) was obtained from Electron Microscopy Sciences.

Flow cytometry analysis and antibody staining: Following FSS stimulation, cells were plated overnight or immediately prepared for analysis. For intracellular proteins phospho-NF-κB and phospho-c-Fos, cells were first fixed for 10 min with 4% paraformaldehyde (PFA). Following HBSS washing, cells were permeabilized with 100% methanol (4ºC), washed, and stained for 15 min in the absence of light with fluorescent antibodies in 1% bovine serum albumin (BSA). For staining extracellular markers such as costimulatory molecules, cells were lifted, washed and incubated for 15 min with fluorescently-expressing antibodies in 1% BSA. Cells were washed again prior to analysis. Fluorescence intensity was measured using a Guava easyCyte 12HT flow cytometer (Millipore, Billerica, MA) and FlowJo software was used for gating and analysis.

Statistics: All data are reported as mean and standard error of the mean. Statistical differences were tested using a paired t test unless otherwise indicated. Significance is indicated by *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$, or ns if not reported. GraphPad Prism software was used to perform statistical comparisons and produce figures for this article.

RESULTS

Prostate cancer patient DC stimulation via FSS

Deidentified prostate cancer patient information was collected in the Department of Medicine at Vanderbilt University Medical Center, from September 2021-January 2023. The information collected includes age of diagnosis, race, ethnicity, time in treatment sample received, number of circulating tumor cells (CTCs)/mL in blood, Gleason score, and disease state (**Table 6.1**). The Gleason score corresponds to the grading system used for the prostate cancer patients and is roughly correlated with prognosis.[23] Similarly, the number of CTCs/mL in blood can be used as a biomarker for disease progression and therapeutic efficacy.[24] The disease state indicates the location of metastases from the primary site.

Table 6.1. Patient breakdown.

Patient	Age of diagnosis	Race	Ethnicity	Time in treatment sample received	CTCs/mL in blood	Gleason	Disease State
K1	58	CA	NH	Post-leuprolide	NR	4+4=8	Bone, liver
K2	58	CA	NH	Post-leuprolide, docetaxel, carboplatin	NR	4+3=7	Bone, LN
K3	67	CA	NH	Post-leuprolide, docetaxel	NR	4+3=7	Bone, LN
K5	76	CA	NH	Post-denosumab, leuprolide, docetaxel	NR	5+4=9	Bone
K7	56	CA	NH	Post-carboplatin, etoposide, leuprolide	NR	5+4=9	Lung, LN, liver, brain
K14	NR	CA	NH	Pluvicto pre-C2	NR	4+5=9	LN
K15	74	CA	NH	Pluvicto pre-C2	NR	3+4=7	Bone, LN
K17	60	CA	NH	Pluvicto pre-C3	6,667	3+4=7	Bone, LN
K18	76	CA	NH	Pluvicto pre-C2, pre-C3	26,000	5+4=9	Bone

CA, Caucasian; NH, Non-Hispanic; NR, Not Reported; CTCs, Circulating Tumor Cells; LN, lymph node; C2, Treatment in Course 2; C3, Treatment in Course 3.

Whole blood was collected from patients undergoing lab analysis at VICC following informed consent. PBMCs were isolated by combining blood with Ficoll-Paque and carrying out gradient centrifugation. Using a Blood Dendritic Cell Isolation Kit II from Miltenyi Biotec, PBMCs were passed through columns and DCs isolated according to the manufacturer's instructions. Following isolation, DCs were resuspended in complete RPMI media at 1 x 10^5 cells/mL and stimulated with 0 dyn/cm^2 ("static") or 5 dyn/cm^2 of FSS ("shear"). Cells were analyzed immediately afterward using fluorescently-labeled antibodies or plated overnight (**Fig. 6.1**).
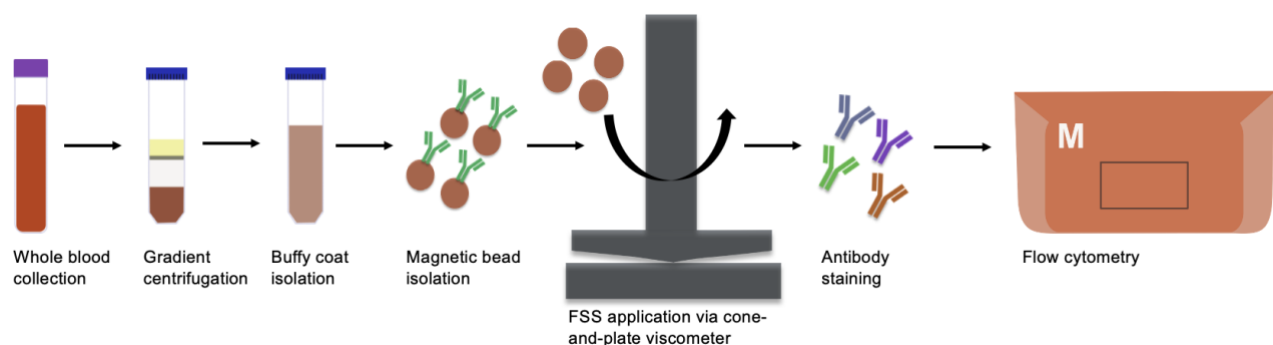
Figure 6.1. FSS stimulation procedure. Dendritic cells were isolated from the whole blood of patients via magnetic bead isolation and then stimulated via FSS.

Cancer patient DC subtypes consist of plasmacytoid and conventional DCs

PBMCs consist of multiple cell types, including different populations of DCs. Plasmacytoid dendritic cells (pDCs) are a subset of DCs that secrete large amounts of type I interferons (IFN-I) in response to antigen exposure, particularly via toll-like receptor (TLR) -7 and -9.[25,26] Another type of DCs are conventional dendritic cells (cDCs), which are professional antigen-presenting cells (APCs) and are further divided into subtypes 1 and 2, where cDC1 perform cross-presentation and cDC2 activate innate lymphoid cells.[27,28]

DCs under static conditions were analyzed for the presence of the following markers: CD303 vs. pDCs, CD1c vs. cDC2, or CD141 vs. cDC1 (**Fig. 6.2A-B**). The subpopulations were determined via flow cytometry and gating and analyses were performed with FlowJo software. The total number of DCs from the subpopulations was determined for static conditions for each patient and compared to the whole population of DCs (**Fig. 6.2C-D**).
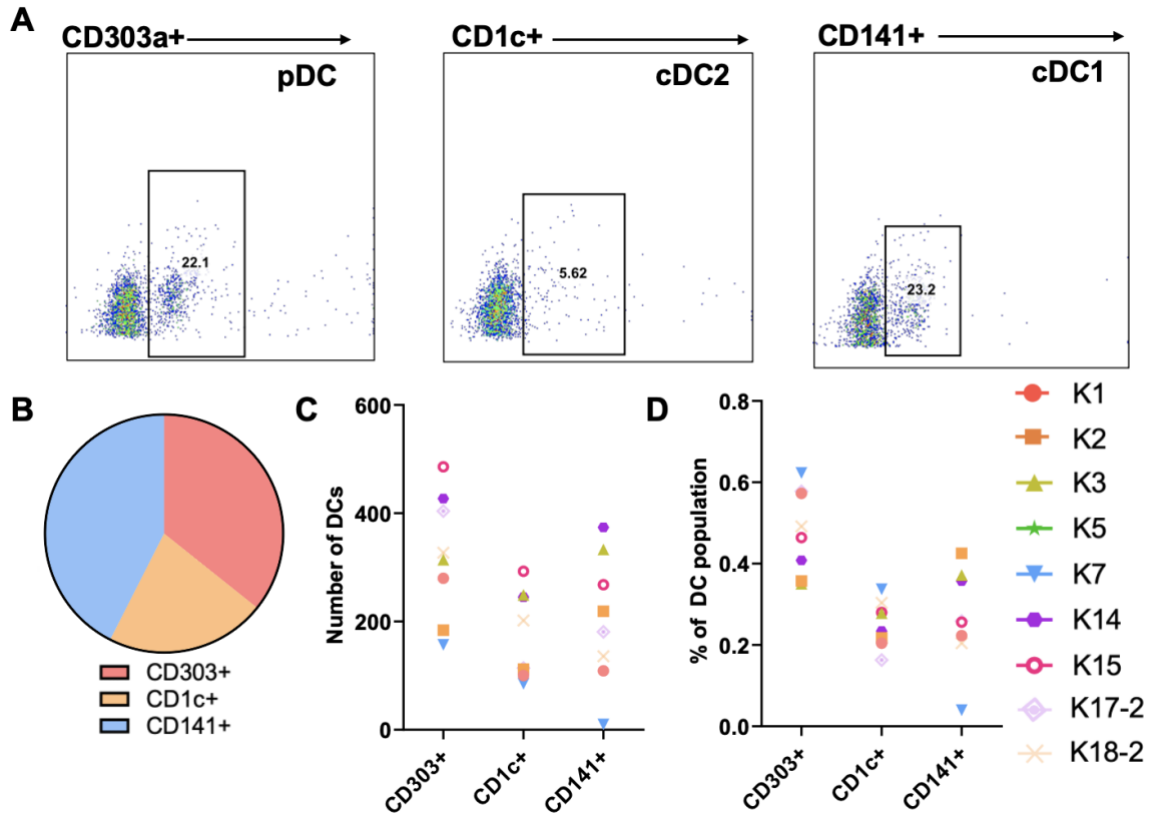
Figure 6.2. DC subpopulations. (A) Representative flow plots for CD303a+, CD1c+ and CD141+ populations. (B) Representative breakdown of three DC subpopulations. (C) Number of DCs per subtype after 0 dyn/cm^2 of FSS, broken up by subtype. (D) Percent of each subtype from DC population.

Increase in activation markers following FSS

Transcription factor NF-kB is regulated by an influx of calcium ions, and its phosphorylation ultimately leads to enhanced cytokine release as well as DC maturation and progression of the cell cycle.[29–33] NF-kb phosphorylation (phospho-NF-kB) of DCs was analyzed via flow cytometry following FSS stimulation. Prostate cancer patient DCs were observed to have an overall
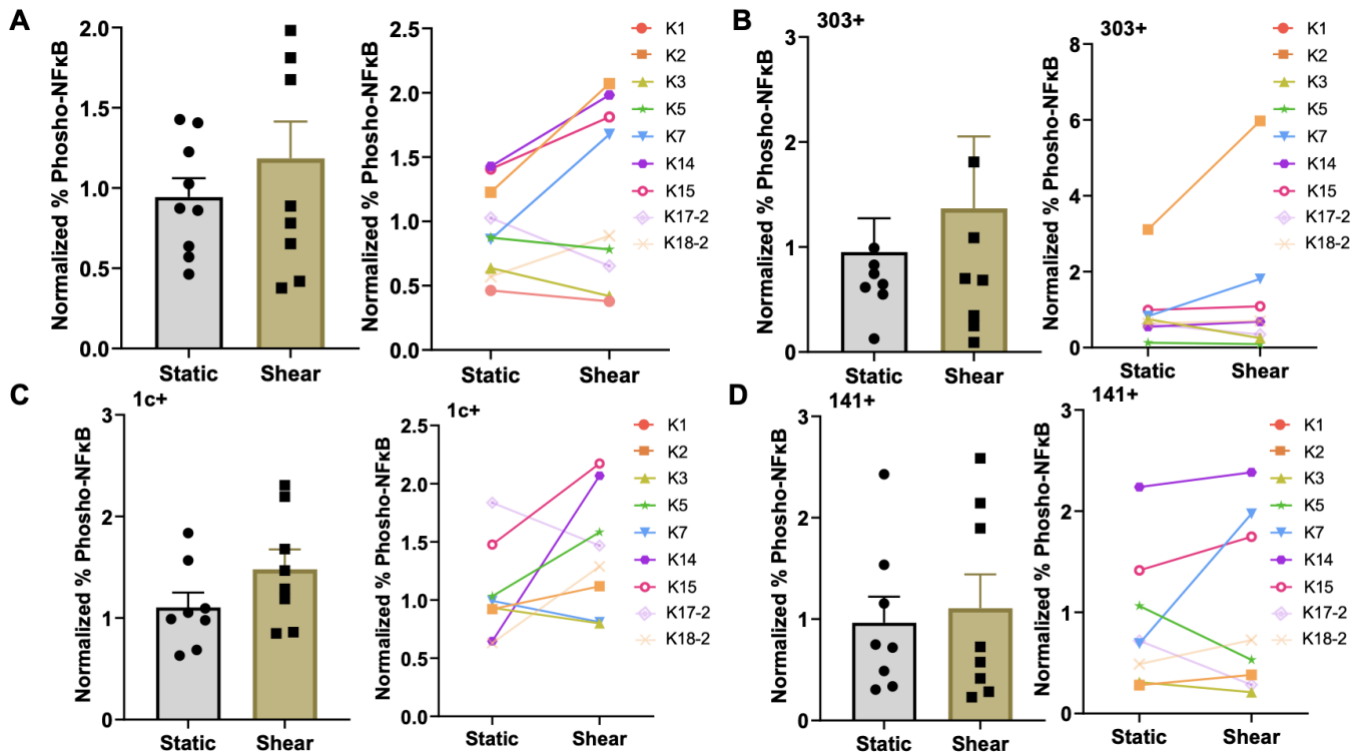
Figure 6.3. NF-kB phosphorylation. (A) Average phosphorylated NF-kB for static and shear conditions and broken down by patient. Average phospho-NF-kB and phospho-NF-kB broken down by population for (B) CD303+ DCs, (C) CD1c+ DCs and (D) CD141+ DCs. Data are normalized to static controls.

increase in phospho-NF-KB, and this trend was observed with most patients (**Fig. 6.3A**). This trend was observed in the different DC subtypes – pDC, cDC1 and cDC2 (**Fig. 6.3B-D**).

Similar to NF-kB, transcription factor AP-1 is regulated by a calcium influx and controls cytokine release, costimulatory molecule expression and proliferation in DCs. Its activation was analyzed using an antibody for phospho-c-Fos. Interestingly, AP-1 and NF-kB are simultaneously activated in DCs, and the balance of this activation is important to DC function.[33–35] Following

FSS stimulation, patient DCs were analyzed for phosphorylation of c-Fos (phospho-c-Fos). There was not a clear trend overall in phospho-c-Fos (**Fig. 6.4A**). However, when broken down into pDC, cDC1, and cDC2 subtypes, more clear trends were observed in an increase in phosphorylation (**Fig. 6.4B-D**).
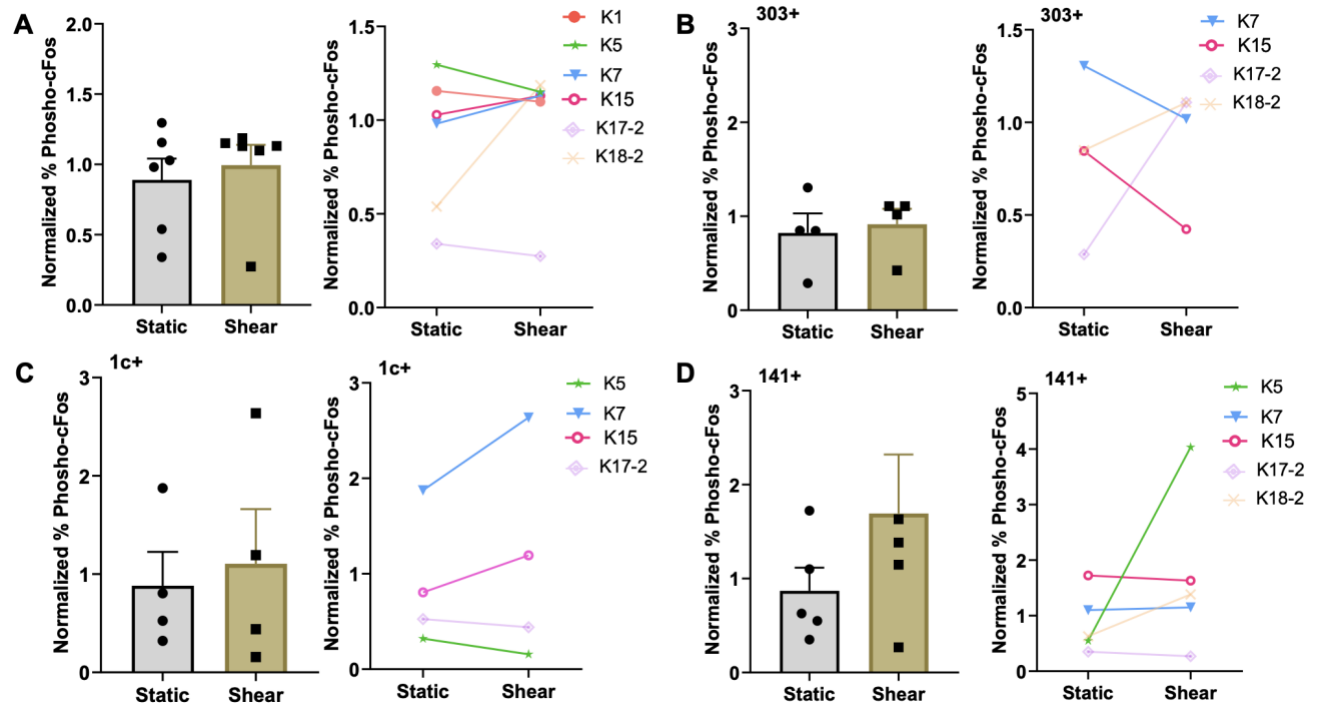
Figure 6.4. cFos phosphorylation. (A) Average phosphorylated cFos for static and shear conditions and broken down by patient. Average phospho-cFos and phospho-cFos broken down by population for (B) CD303+ DCs, (C) CD1c+ DCs and (D) CD141+ DCs. Data are normalized to static controls.

Varied responses in costimulatory molecule expression following FSS

Costimulatory molecules like CD40, CD80 and CD86 are necessary for DCs to effectively form an immune synapse with T cells and initiate a response.[36–38] Following FSS stimulation, DCs were plated overnight. They were then analyzed for extracellular markers via flow cytometry and static and shear conditions were compared (**Fig. 6.5A-C**). There was a wide range of responses

for patients, indicating that if enhanced costimulatory molecule expression was desired for the study outcomes, an additional stimulating factor would be necessary.
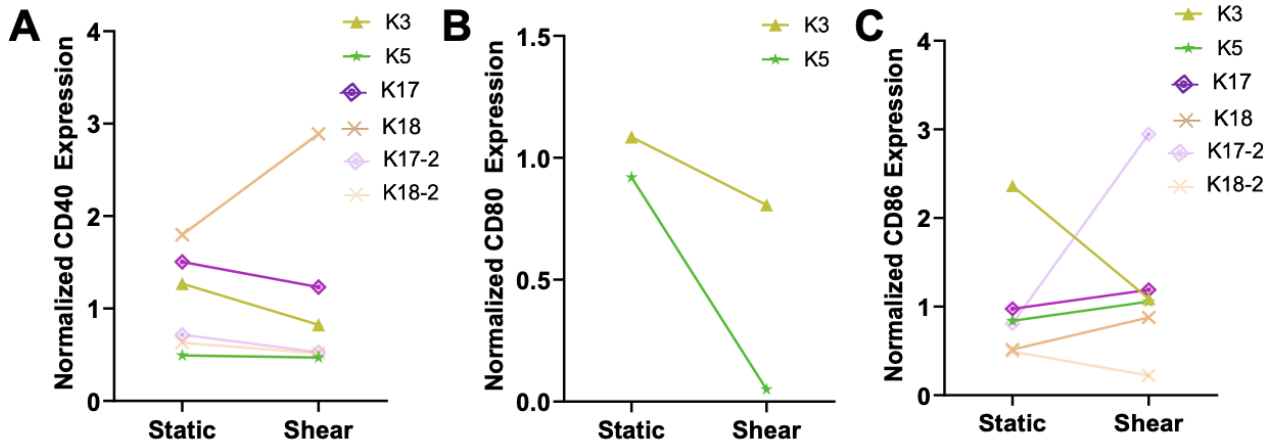
Figure 6.5. Costimulatory molecule expression. Following FSS, an analysis was performed for the following markers: (A) CD40, (B) CD80, and (C) CD86. (D) Data is normalized to static

DISCUSSION

To the best of our knowledge, this is the first study of its kind, where the DCs of a prostate cancer patient cohort have been investigated for population subtype and stimulated with FSS. In this study, we were able to gain insight into the different DC populations from these cancer patients and determine that we can activate these cells ex vivo using FSS. With just 1 h of stimulation, we saw increases in phospho-NF-kB and phospho-c-Fos and were able to see this on a patient-by-patient basis, as well as for particular subsets of DCs. The variance observed in costimulatory molecule expression following FSS indicates that an additional stimulating factor may be necessary for initiating robust T cell priming if this methodology is to be used in prostate cancer therapy.

This study was unique in that it was able to encompass a relatively wide age range of patients. The cohort included patients from 56-76 years old, providing a broader spectrum and eliminating potential age-related bias that could occur due to differing immune systems between age groups. However, a drawback is the only patients that consented to this study were only Caucasian (CA) and Non-Hispanic (NH), limiting our population diversity. Further studies are thus required with broader participant race and ethnicity.

AUTHOR CONTRIBUTIONS

Conceptualization: JAD, MRK

Methodology: JAD, MRK

Investigation: JAD

Materials: MA, KRS, PJH

Supervision: KRS, PJH, MRK

Writing: JAD, MRK

Editing: JAD, MA, KRS, PJH, MRK

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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Chapter 7:

Conclusions

CONCLUSIONS

Dendritic cells (DCs) can be useful tools for effective anti-cancer vaccines. In this thesis, we have developed vaccines that activate DCs in order to initiate an immune response with the goal to improve current therapies.

Fabricated and characterized a preventative vaccine for triple-negative breast cancer.

In Chapter 3, we developed a formulation for a vaccine comprised of cancer cell fragments on the nanometer scale and coined the term "tumor nano-lysate" (TNL) to describe the vaccine. We were able to effectively characterize the resulting formulation via size, polydispersity index, protein contents and morphology, and compared these to cell fragments resulting from TRAIL and FSS treatments. Interestingly, we confirmed that we had closely recapitulated the natural process.

An in vivo study was performed in which naïve mice were given tail vein injections of one dose of PBS vehicle control or one dose of TNL, and inoculated 10 days later with 4T1 breast cancer. After monitoring via caliper measurements and bioluminescent imaging, tumor size was determined to be reduced in TNL-vaccinated mice, onset of metastasis was delayed, and survival was increased. Although the vaccinated mice ultimately grew tumors, the results of the study revealed the potential of TNL as a preventative triple-negative breast cancer vaccine.

Determined dendritic cell-based in vivo response to tumor nano-lysate vaccine.

In Chapter 4, we studied the immune response to TNL ex vivo and in vivo. Exploring the TNL vaccine, we aimed to determine the immune response elicited by the vaccine, which is

important for developing an understanding as well as making improvements to the formulation. Intracellular and extracellular activation markers were analyzed via flow cytometry, cytokine production was quantified via Proteome Profiler, and phagocytosis was analyzed via confocal microscopy. Various immune cells and particularly DCs were activated by TNL, with changes in morphology, metabolism and cytokine release observed for DCs. The response to TNL was often comparable to other stimulators, and in some cases, significantly greater.

A study was conducted to allow for a temporal analysis of the immune response in vivo, providing a clearer picture of what is happening once the vaccine is introduced. We were able to determine that significant changes were occurring within 3 days, and that activation was reduced at 10 days. The results indicated that additional TNL doses may be necessary after 10 days to elicit a robust preventative response in vivo. The multi-dose in vivo study showed a trend in reduced tumor volume, increased survival, and delayed metastasis, although we hypothesize that more significant differences may be observed with the addition of an adjuvant in future studies.

Effectively stimulated dendritic cells ex vivo via FSS.
In Chapter 5, we addressed a complementary DC-based project, where we used DCs for the development of an anti-cancer therapeutic vaccine. We hypothesized that the mechanical forces of fluid shear stress (FSS) would cause the opening of mechanosensitive ion channels (MSCs) and a subsequent influx of calcium, thus activating DCs ex vivo. We applied FSS to DCs via a cone-and-plate device for 1 hour and included static controls. Activation was analyzed via flow cytometry and Proteome Profiler, as well as metabolic analyses such as pH testing and glucose uptake. Changes in morphology were quantified via confocal imaging. We observed that DCs experienced changes in metabolism, morphology, and cytokine release as a result of applied FSS. Activation was also observed in primary mouse and human cells. This methodology is

easily scalable and could be used alone or in combination with current therapeutics to boost their efficacy and enhance T cell priming.

In Chapter 6, we specifically activate DCs isolated from the whole blood of prostate cancer patients. Again, we used flow cytometry to analyze activation markers in DCs and were able to observe a trend in activation following FSS. Interestingly, these differences are augmented with patients receiving Pluvicto treatments. The success of our methodology with cancer patient DCs demonstrates its potential as an ex vivo therapeutic.

The development and characterization of TNL demonstrates our ability to closely recapitulate the phenomenon of cell fragmentation resulting from TRAIL and FSS treatments. Previously, there had been little to no characterization of cell lysate, and so this research represents an advance in the field in gaining a better understanding of the biophysical traits of cell lysate. The ex vivo and in vivo immunological studies elucidate the largely DC-based mechanism behind TNL and resulting temporal response. Eliciting a response for up to one week, the data depict the necessity of a "booster" dose, and the in vivo multi-dose study demonstrates the effectiveness of TNL as a preventative treatment. While the addition of an adjuvant may be necessary to enhance the efficacy of TNL, the results of the study demonstrate the potential of TNL as a preventative vaccine for TNBC. This research represents a hope for improved prevention of breast cancer and development of metastasis.

The ex vivo FSS stimulation of immune cells also focused on DC-based anti-cancer strategies. I have developed an easily repeatable and scalable methodology for activating DCs ex vivo for cancer therapeutics and explored the mechanisms of cellular activation. The results of the study demonstrate the exciting finding that FSS can activate DCs without any other stimulating factors, representing an efficient and inexpensive solution that can be applied to benefit cancer

patients in the future. With these therapeutic advances, we may reduce tumor burden and

potentially increase life expectancy of metastatic cancer patients. Preliminary data show the

potential of future studies combining FSS stimulation with TNL treatment (**Fig. 7**). Activating

DCs ex vivo with both FSS and TNL and then reintroducing DCs in vivo could enhance the

efficacy of TNL at preventing TNBC formation. Taken together, DC-based vaccines, alone or in

combination, hold potential for advancing the field of cancer immunotherapy.
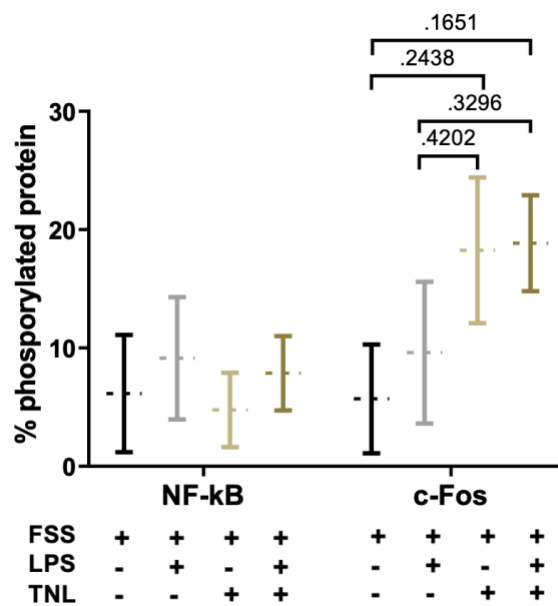
Figure 7. FSS combination with TNL. c-Fos phosphorylation of DC2.4 cells is enhanced with

FSS in combination with TNL with and without LPS a potent stimulator. *$p<0.05$.

FUTURE WORK

Refining TNL formula via size filtration

In our characterization study, we observed that the TNL had a large PDI and a wide size range,

as measured via Zetasizer and NanoSight, respectively. The diverse size range is a product of

the randomness of the sonication process generating cell fragments of varying diameters. It is

possible that specific size ranges will be more impactful in eliciting an immune response and for improved biodistribution.[1–3] In order to refine the size range and remove larger cell fragments, TNL could be filtered via extrusion.[4] TNL would then be characterized and the smaller and larger filtered fractions could be used in immunological assays to determine if a similar response occurs.

TNL vaccine combination therapy

Currently, the TNL vaccine boasts the advantage of being a very simple, scalable formula. In our studies it was proven to evoke an immune response comparable to common stimulators or more significant, however, adding a stimulator to the TNL formulation may ultimately prove more effective. One proposed formulation could involve the addition of an adjuvant, which is known to enhance the immunogenicity of an antigen.[5–7] In preclinical and clinical studies, adjuvants initiate a variety of functions including an increase in immune cell memory, increase in the speed of the first response, and initiation of immune cell activation and specificity.[6,8] Adjuvants function by activating an immune cell pathway, such as a Pattern Recognition Receptor (PRR), which indicates the presence of a foreign body.[6] Commonly used adjuvants in clinical practice and research include aluminum, virosomes, poly (I:C), CpG, and imiquimods.[6,9,10] Co-encapsulation with the toll-like receptor (TLR) PRR agonists has demonstrated enhanced receptor-based recognition as well as activation of immune cells.[11,12] A TLR-activating adjuvant with the ability to enhance immunogenicity via antigen-presenting cell (APC)-targeting while minimizing toxicity, CpG could be a successful adjuvant to combine with the TNL vaccine.[13–15] CpG leads to an immune cascade by initiating DC interferon (IFN) secretion and enhancing release of co-stimulatory signals, ultimately leading to an increase in cytokines and chemokines and promoting a cellular T_H1 response.[16] CpG also promotes a humoral response, enhancing B cell differentiation into plasma cells.[16] The addition of the CpG adjuvant could enhance the

success of the TNL vaccine, and future studies could explore its co-incubation with TNL or injection as a smart, complex particle fused with TNL.

Development of TNL from alternative cell types

Throughout these studies, TNL were fabricated from one cell type, 4T1 breast cancer cells. It would be interesting to explore the fabrication of a tumor nano-lysate vaccine made from an alternative cell line such as colorectal cancer (MC38), prostate cancer (PC3, LNCaP) or melanoma (B16F10). Different cell lines have different tumor specific antigens, which can elicit a specific response.[17] These cell types could similarly be characterized by size, PDI, morphology and protein contents and compared to 4T1-derived TNL. Additionally, ex vivo and in vivo assays to understand the immunological response could be performed. It would be interesting to see if specific cell types induced a more robust immune response.

In addition to changing the TNL formulation to a different cell type, it would be interesting to change the cancer type in which the cells are inoculated with, and this can be tested with the alternative TNL formulations as well. While the fragments derived from the 4T1 breast cancer cell line initiates a response to specific tumor specific antigens, testing the TNL vaccine against other cancer types such as melanoma would be prove interesting future directions.

Further investigate the role of mechanosensitive ion channels in DC activation

While we were able to effectively determine that FSS activates DCs ex vivo in immortalized and primary cells, future directions could involve further probing the mechanism behind this activation. We were able to determine that the proposed pathway behind activation is through phosphorylation of NF-kB, thus resulting in enhanced cytokine release. MSCs, which open to a mechanical stimulus to allow for an influx of calcium, are the suggested mechanism behind phenomenon, based on previous studies investigating other immune cells like T cells.[18] To

determine the impact of MSCs on this phenomenon, calcium influx could be inhibited using calcium free buffers and EGTA as a calcium chelator.[19] If activation is unable to occur without the presence of calcium, we can gain a better understanding of its role in this process. We can further probe the role by treating DCs with GsMTx4, an inhibitor isolated from spider venom, to inhibit MSCs in vitro.[20] In order to specifically analyze MSC Piezo1, which has been shown to play a role in a variety of cell types, a Piezo1 knockout could be performed via CRISPR/Cas9. Activation markers and cytokine release could be analyzed following these experiments via flow cytometry and Proteome Profiler, respectively.

In vivo mouse prostate cancer model

Having demonstrated that FSS stimulates DCs ex vivo, future directions could include the application of in vivo mouse models. One potential study would be to isolate immune cells from naïve mice, active DCs using FSS ex vivo, and reintroduce the cells. Various studies could be performed at different endpoints in the study including toxicity and immune analyses (splenocyte, peripheral blood mononuclear cell and complete blood counts). A study like this could provide much information about the complete immune response and the potential for this methodology as a therapeutic.

Additionally, a therapeutic in vivo study could be performed in which mice are inoculated with a cancer cell line (i.e. prostate cancer, breast cancer), and then isolate immune cells, activate DCs via FSS, and reintroduce cells. Mice would then be monitored for tumor growth over time using caliper measurements and bioluminescent imaging. The anticipated results of the study would be reduced tumor growth and increased survival for mice with receiving ex vivo FSS therapeutics.

Comparison of prostate cancer patient DC activation to cells isolated from healthy patients

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Currently, the study activating DCs of prostate cancer patients is limited to male patients between the ages of 56 and 76 years old. Studies have shown that the immune system has age-related differences.[21] In the future, it would be impactful to expand the cohort of the healthy population to include a larger age group, as it is currently a limited age group comprised of a combination of males and females. It would be interesting to see if there are any age-related differences in the study, and this could be further explored.

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Appendix A:

Engineering of exosomes to target cancer metastasis

This chapter is adapted from *Engineering of exosomes to target cancer metastasis* published in *Cellular and Molecular Bioengineering* in 2020. The work has been reproduced with the permission of the publisher and co-authors Zhenjiang Zhang and Michael R. King.

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ABSTRACT

As a nanoscale subset of extracellular vehicles, exosomes represent a new pathway of intercellular communication by delivering cargos such as proteins and nucleic acids to recipient cells. Importantly, it has been well documented that exosome-mediated delivery of such cargo is involved in many pathological processes such as tumor progression, cancer metastasis, and development of drug resistance. Innately biocompatible and possessing ideal structural properties, exosomes offer distinct advantages for drug delivery over artificial nanoscale drug carriers. In this review, we summarize recent progress in methods for engineering exosomes including isolation techniques and exogenous cargo encapsulation, with a focus on applications of engineered exosomes to target cancer metastasis.

1. **INTRODUCTION**

Extracellular vesicles (EVs) were first described by Trams et al. in 1981 as cell-secreted particles that carried membrane-bound enzymes, and could be taken up by recipient cells [1]. The authors keenly predicted that EVs could represent an important pathway to transfer information between cells and might be developed to package and deliver therapeutic molecules like structurally similar liposomes. However, initially, EVs were more widely regarded as "garbage bags" for disposal of undesired cellular components [2]. A subset of extracellular vesicles in the 30 - 150 nm, which are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body (MVB), with the plasma membrane, were later defined as exosomes [3]. Exosomes were subsequently found to be specialized for intercellular signaling by carrying proteins, nucleic acids, lipids and metabolic cargos from source cells to neighboring recipient cells or even to distant organs [4].

Exosomes facilitate effective intercellular communication that can regulate cellular functions such as proliferation, apoptosis and migration [5]. Mounting studies support the understanding of exosomes as a key player in tumor growth [5, 6]. In fact, cancer cells have been found to secrete more exosomes than noncancerous cells [7]. Over the last decade, exosomes shed by cancer cells have been found to facilitate metastasis, which accounts for over 90% of cancer-related deaths [8-12]. Metastasis occurs when a cancer cell derived from a primary tumor intravasates into the bloodstream in the form of a circulating tumor cell, which has the potential to form a secondary tumor following extravasation [13]. Evidence has supported that exosomes play a critical role in several steps in the metastatic process [10]. As a result, exosomes have become an increasingly important research target for the prevention of metastasis [12]. Anti-metastatic treatments that have attracted intensive research efforts include immunotherapy such as chimeric antigen receptor T (CAR T) cells or TRAIL-coated leukocytes as well as stem cell and virotherapy [14-17].

Exosomes have been pursued as a delivery vehicle for a variety of therapeutics for targeted treatment [18-23]. Compared to artificial nanoscale vehicles, exosomes possess a number of advantages that can be exploited. For one, exosomes naturally deliver their membrane and cytoplasm components by fusing with the target cell membrane [23]. Exogenous therapeutics can thus be encapsulated in exosomes and delivered in a hitchhiking manner. In addition, exosomes, particularly those collected from patient tissues or blood, possess low immunogenicity and thus intrinsic long-term circulatory capability, and excellent biocompatibility [24]. Several studies also suggest that exosomes secreted by specific cell types exhibit a very specific cell tropism, supporting highly targeted cargo delivery [25, 26].

Our growing understanding of the biology of exosomes and experience in engineering exosomes for diagnostic or therapeutic purposes have provided promising potential for the treatment of tumor metastases [27-29]. In this review, we discuss the recent advances concerning the engineering of exosomes to target metastasis, with a focus on the methods of exosome isolation and engineering, and therapeutic effects of engineered exosomes for antimetastatic therapy. We will only briefly introduce the biogenesis, structure, and contents of exosomes, and their roles in cancer, as several existing review articles published have covered these topics [2-5, 19-24].

1.1 Biogenesis

Exosomes are defined as extracellular vesicles originating from the exocytosis of multivesicular endosomes (MVEs) from the plasma membrane of a cell [30]. This exosome biogenesis was discovered by two groups of researchers in the 1980s, with papers published within a week of each other [31, 32]. The process, applied to maturing reticulocytes at the time, was eventually found to be applicable across all cell types [31]. During this biogenesis process, the plasma membrane invaginates to form an early endosome. Upon maturation into MVE containing proteins, endosome will either be degraded by the lysosome or fused back into the plasma membrane [32]. Its exocytosis from the membrane results in the release of the exosome into circulation [33] (**Fig. A.1**).
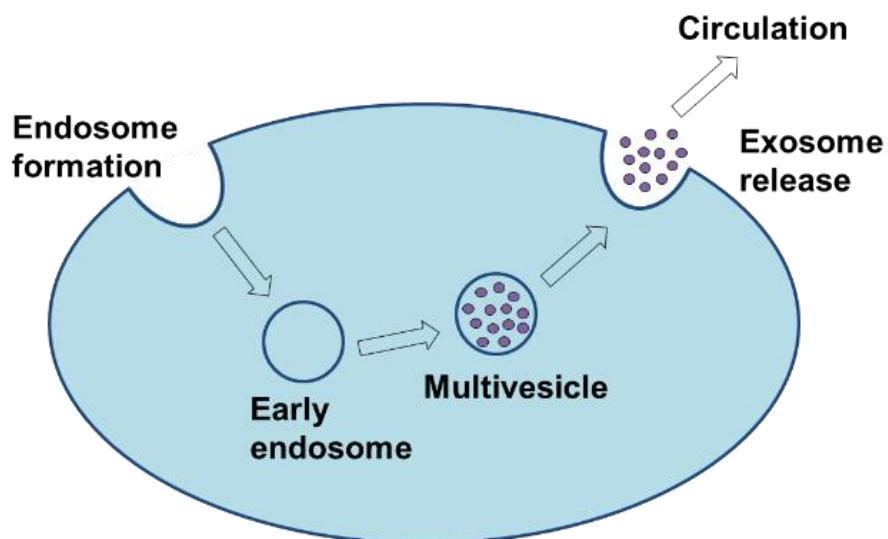
Figure A.1. Exosome biogenesis. Formation of exosomes begins with membrane invagination in the form of an endosome, leading to the development of the early endosome. Upon maturation, the endosome becomes a multivesicular endosome, which releases its contents in the form of exosomes.

1.2 Structure and Contents

While exosomes possess similar morphological qualities to other EVs in their flat, round, "saucer-like" shape and a shelf of lipid bilayer membrane, they have unique features such as size, density,

and composition [34]. Exosomes can be distinguished from other EVs by size, with a diameter ranging from 30 to 150 nm [34]. In addition, the density of exosomes is between 1.15 and 1.19 g/mL, which allow them to float on a continuous sucrose gradient [35].

The composition of exosomes includes proteins, nucleic acids, lipids and metabolic cargos [36]. Proteins found in exosomes are limited in range, derived primarily from the cytosol [36]. Proteins include those related to the endocytic pathway, as well as adhesion and targeting proteins. Many of these are membrane bound proteins, originating from the invagination of the membrane that produces the exosomes.

Given that exosomes stem from the invagination of the plasma membrane, exosomes are composed of lipids consistent with the lipid bilayer of their parental cells. Consequently, they possess a similar fraction of the membrane phospholipid PE (Phosphatidylethanolamine) in exosomes as their parent cells [37].

1.3 Characterization Techniques

The characterization of exosomes can be performed visually, by using various dynamic light scattering (DLS) and microscopy techniques, and flow cytometry. Due to the small size range of exosomes, scanning electron microscope (SEM) and transmission electron microscopy (TEM) are frequently used to visualize exosomes via negative staining. Size distribution and concentration, as well as sample purity, can be measured with DLS using a ZetaSizer instrument (Malvern) and nanoparticle tracking analysis (NTA, Malvern) [38]. Labelling exosomes with lipophilic membrane dyes such as, PKH-26 or PKH-67, enables analysis via flow cytometry [39]. Fluorescent visualization is aided by the metabolic labeling of exosomes, using fatty acid analogues [40]. In conjunction with this labeling, Western Blot can be used to analyze specific proteins such as heat shock protein 90 (HSP90), ALG-2-interacting protein X (Alix), Tumor susceptibility gene 101 (TSG101), and the EV related tetraspanin protein (CD63) [40].

2.   ROLE OF EXOSOMES IN METASTASIS

It has been shown that cancer cells secrete more exosomes than their non-cancerous counterparts [41]. Cancer-derived exosomes have been reported to promote metastasis in a variety of ways including altering the immune system, promoting epithelial to mesenchymal transition (EMT), organotropism, and angiogenesis. These phenomena were observed particularly in exosomes derived from metastatic cancer cells, which were found to transfer their host cell's invasive properties to non-metastatic cancer cells [42].

Altering the Immune System

Exosomes have been found to exhibit the ability to induce immune suppression [43]. For instance, tumor-derived exosomes upregulate specific immunosuppressive factors such as GM-CSF and TNF-α [44]. They also impair the ability of natural killer (NK) cells to carry out cytotoxic functions by secreting TGF-1β or blocking IL-2 mediated activation [45]. Exosomes can contain FAS and TRAIL ligands to induce the death-receptor activated killing of lymphocytes, extending their immune-modulating effects [46]. The effect of tumor exosomes on cellular immunity has also been studied. Tumor exosomes have been found to reduce cytotoxic T cell (CD8$^+$) counts [47]. Additionally, exosomes can promote the conversion of helper T cells (CD4$^+$) into regulatory T cells [48]. The upregulation of T_{regs} aids in the ability of the tumor microenvironment to suppress and evade an immune system response. T cell activation has also been inhibited by exosomes [49]. The mechanism in which they are able to achieve this inhibition is via targeting TGF-β [50]. Similarly, exosomes released from metastatic melanomas have been found to carry surface programmed death-ligand 1 (PD-L1), which aids in tumor growth by inhibiting CD8 T cell function [51].

Promoting EMT

The process of EMT occurs when tissue epithelial cells possess altered biochemical factors that leave them with a more mesenchymal phenotype, aiding in functions such as migration and invasion [52]. Exosomes released from cancer cells have been found to directly promote the EMT process by their delivery contents. For instance, cancer cell-derived exosomes can contain high amounts of TGF-β, caveolin-1, HIF-1α, β-catenin, LMP1 and H1H1α, which results in more invasive phenotype for receiving cells [53]. miR-21 and matrix metalloproteinase-13 can also be enriched in these exosomes, which enhance mesenchymal markers like vimentin and suppresses epithelial markers like E-cadherin [54]. EMT can also be triggered indirectly when cancer-associated fibroblasts (CAFs) release exosomes that convert mesenchymal stem cells to fibrous-associated fibroblasts in the pericellular microenvironment [55].

Metastatic Organotropism

Organotropism is defined as the non-random process which distributes distant metastasis to specific organs [28]. Exosomes derived from tumors present integrins that helps drive organotropic properties [56]. This had been observed from breast cancer exosomes moving to lung tissue in an orthotopic mouse model, as well as in pancreatic ductal adenocarcinoma [29]. Specifically, MDA-MB-231 breast cancer cell-derived exosomes were shown to have metastasis homing to the lungs and brain regardless of whether they were injected via tail vein, the intracardial or retro-orbital route [56].

Angiogenesis

Angiogenesis and vascular permeability are activated and upregulated by exosomes derived from cancer cells. These exosomes carry pro-angiogenic factors such as VEGF, TIMP-1, IL-6, and FGF and cause their upregulation in recipient cells [57]. In addition, they carry paracrine signaling factors and mRNAs to alter the genetics and drive genetic expression toward angiogenesis [58].

Angiogenesis- and metastasis-promoting microRNAs such as miR-9, miR-23a, and miR-210 have been found as exosome cargo [27]. Tumor-derived exosomes, as well as exosomes derived from cancer associated fibroblasts (CAFs), release factors that recruit and activate endothelial progenitor cells [58].

3. EXOSOME ISOLATION TECHNIQUES

One crucial step in the study of exosomes is to isolate exosomes from a complex mixture of cell culture medium, tissues or bodily fluids that contains cells, cell debris, other particulate components and macromolecules. An optimal method for exosome isolation is expected to exhibit high recovery yield and high purity of exosomes, and high efficiency as well. Several isolation techniques have been utilized in published exosome studies, each exploiting particular properties of exosomes, such as their density, shape, size, and unique surface proteins to aid their isolation [**Table A.1**].

Table A.1. Summary of exosome isolation techniques.

Isolation techniques	Isolation Principles	Advantages	Disadvantages	Ref.
Centrifugation	Density, size and shape	High yield, low cost and scalability	Time-consuming, subject to equipment variability	59-63
Size-based Techniques	Size	Fast, high purity, moderate scalability	Moderate yield, subject to clogging and loss due to column or filter attachment	62, 66, 70
Immunoaffinity	Specific interaction between antibody and antigen	Fast, high purity	Low yield, high possibility of subtyping	76-78
Polymer precipitation	Solubility and dispersity	Easy to use, high scalability	Subject to protein contamination	80-83
Microfluidic separation	Various properties incorporated into microfluidic channels	Fast, easy integration with other techniques	Limited to small sample volume, requires in house made microfluidic devices	84-89

3.1 Differential Ultracentrifugation and Density Gradient Centrifugation

Ultracentrifugation is considered the "gold standard" for exosome isolation and makes up >50% of all exosome isolation techniques utilized in reported exosome research [59]. Sequential centrifugation is performed in a typical isolation experiment to remove other components until only the exosomes remain. First, a low speed centrifugation step (~ 400× g) is performed to remove cells and large cellular debris from an exosome containing sample, e.g. conditioned cell culture media or biological fluid. Next to be removed are the smaller debris and intact organelles at 10,000-20,000× g. The final step is ultracentrifugation (100,000-150,000× g) of the supernatant to form a pellet of exosomes [60]. Due to the heterogeneity of exosomes and considerable overlap in size with other EVs, exosomes isolated by differential ultracentrifugation are often found to be contaminated by other EVs, protein aggregates or even large individual proteins [61]. One resolution to this challenge is to remove the contaminants from the isolated exosomes with gradient density centrifugation which uses a continuous sucrose gradient to separate exosomes from contaminants based on their difference in densities [62]. This technique has been known to improve the purity of exosomes isolate. However, gradient centrifugation is notorious for being time consuming, ~80 h in the case of exosome purification [63]. Also, this method is subject to equipment-dependent variability because minimal differences of centrifugation factors such as rotor type, angle and radius can result in variations in the type, purity and yield of exosomes isolated [64].

3.2 Size-Based Filtration, Chromatography and Fractionation

Ultrafiltration, including syringe-driven filtration, might be the most straightforward method for exosome isolation [65]. Exosomes can be separated from other components in the sample using membrane filters with defined pore size or molecular weight (MW) limits. Due to the size heterogeneity of the components in exosome-containing samples, sequential filtration is needed to remove other components that are significantly larger or smaller than exosomes [66]. The most common pore sizes for filtration membranes are 0.8, 0.45 or 0.22 µm [38].

A commonly used protein concentrator has been reported to concentrate exosomes from urine samples with high yield and efficiency [67]. The samples were first centrifuged at 17,000 *g* to remove particles much larger than exosomes before being concentrated with a concentrator with a uniform pore size of 13 nm or a ~100 kDa MW cutoff (MWCO) [67].

For exosome isolation from cell culture supernatants, a 3-step sequential filtration has been reported. The first step is to remove floating cells and large cell debris using a100-nm polyethersulfone (PES) filter [63]. To remove components smaller than exosomes, the filtrate is then subjected to tangential flow filtration with 500 kDa MWCO hollow fiber PES filters. The retentate collected is then dialyzed to further remove contaminants as completely as possible before finally being filtered with a 100 nm polycarbonate track-etched filter [63].

Exosome isolation by ultrafiltration is much faster than that by ultracentrifugation and can be completed without using any special equipment. However, Exosomes may partly get deformed or broken when they are forced through nanoscale filters [63].

Size exclusion chromatography (SEC) is a promising method for exosome isolation because of its capability to separate nanoscale particles based on their hydrodynamic size [62]. The SEC column is packed with porous beads so that components with a smaller size have to go through many small pores before being eluted out of the column while larger components can pass the beads faster by avoiding entering the pores [68]. Exosomes in mesenchymal stem cell (MSC)-conditioned medium were reported to be successfully isolated by SEC [69]. As examined by TEM, the isolated exosomes are structurally intact [69]. The major advantage of using SEC for exosome isolation is that the technique preserves the structural integrity and biological activity of exosomes while other components are removed. Moreover, SEC is a very sensitive method for exosome

isolation and exhibits high reproducibility [23, 63]. While SEC is commonly driven by gravity flow, which is a time-consuming process, it can be sped up by incorporation of a SEC column with HPLC or Fast Protein Liquid Chromatography (FPLC) [68]. In addition, SEC can be coupled with ultracentrifugation to concentrate the final exosome dispersion [70].

Another size-based separation technique that has been applied to exosome isolation is Asymmetric Flow Field-Flow Fractionation (AF4) [71]. AF4 is a fractionation method that is commonly used for the separation/analysis of polymers, proteins and nanoparticles [72]. Fractionation in AF4 takes place in a thin chamber, in which a laminar flow carries a sample through the chamber and crossflow separation field pushes the particulate components towards the accumulation wall of the chamber. Brownian motion of the flowing particles in the sample are thus interfered by the crossflow against the accumulation wall in a diffusivity dependent manner. Smaller particles that diffuse faster are bounced back into the faster flowing center from the accumulation wall and are eluted earlier than larger ones. Successful isolation using AF4 has been reported to isolate exosomes from B16-F10 mouse melanoma cell culture into vesicle subpopulations by size [73]. In another AF4 study of exosomes, two major factors on fractionation quality of exosomes were identified to be cross-flow conditions and the channel thickness, while the focusing time has less significant impact. Also, the exosomes were found to be eluted together with a population of smaller vesicle-like particles, as revealed by on-line UV and multi-angle light scattering (MALS), and the subsequent DLS analysis [74]. While possessing the potential to greatly facilitate exosome research and application, AF4 does require specialized facilities and operation expertise.

3.3 Immunoaffinity

Surface proteins and other molecules that are unique to or highly concentrated on exosomes in exosome containing samples offer opportunities for specific isolation by designing antibody-

mediated immunoaffinity interaction [75]. Such surface molecules that have been identified include tetraspanin, TSG101, Alix, annexin, EpCAM and Rab5 [76]. In the immunoaffinity methods, antibodies against the surface markers are immobilized on the surface of beads, filters or other matrices to allow exosomes bind to the matrices specifically. After washing off the unbound fraction, the bound fraction will then be collected by detaching the exosomes from the stationary phase [60]. Because this technique is based on highly specific antibody recognition, the exosomes obtained are often found to be more pure than those isolated by other methods which are based on their less unique physical properties [77]. However, there are a few drawbacks of note to this isolation method. First, only a subset of all exosomes express the surface markers and can thus be captured, resulting a low yield [23]. In addition, recovering fully intact exosomes can be difficult after antibody binding in immunoffinaity isolation [78].

Zarovni et al. evaluated several commercially available kits for immunoaffinity-based isolation and modified their protocols to increase the purity of exosomes obtained [79]. Antibodies specific to several distinct exosome surface proteins have been evaluated to identify desirable molecular targets for total exosome capture. Furthermore, the authors incorporated downstream steps allowing on-line quantification and analysis of bound exosomes, which enabled rapid quantification and validation of subpopulations of exosomes with manifold yield [79]. It has been reported that the optimized assays exhibited high sensitivity which can downscale working plasma volumes to as little as 100 μL.

The efficiency of immunoaffinity based isolation may be further improved by using antibody modified magnetic beads [80]. After incubation with exosome containing samples, magnetic beads is subjected to a magnetic field to separate the beads out of the sample. Zarovni et al. evaluated the feasibility of magnetic beads for isolating exosomes. As little as 1.0 mL of cell

culture supernatant can be handled with a similar capture efficiency to that of ultracentrifugation [80].

Compared to ultracentrifugation for exosome isolation from cell culture medium, immunoaffinity based isolation exhibits comparable yield with higher purity and advantages of ease of operation and much higher efficiency [63]. For exosome isolation from a large volume of plasma sample, the yield achieved by magneto-immunocapture capture was found to be an order of magnitude higher than that obtained by ultracentrifugation [81].

3.4 Polymer precipitation

Exosomes can also be isolated via a so-called polymer-based precipitation method, which is a widely used method to precipiate viruses and other macromolecules [82]. Typically, exosome-containing samples are incubated with a precipitation solution containing polyethylene glycol (PEG) with a (MW) of 8000 Da at 4 °C overnight to sequester water molecules and force less soluble components out of solution. The mixture is then subjected to centrifugation at a low speed to pellet the precipitated exosomes [83].

Compared to other methods of isolation, polymer-based precipitation is easy to use, scalable for large sample volumes and does not require any specialized equipment or a lengthy run time [84]. One weakness of this method is that the exosomes obtained are often found to be contaminated by proteins, subcellular particles and polymer materials [85]. Additional steps before or after isolation may be used to address this issue. Subcellular particles such as lipoproteins may be removed by centrifugation before isolation, while the polymer might be removed by using SEC [86].

3.5 Microfluidic separation

As a rapidly-growing engineering field, microfluidics has been widely used for the separation of particles ranging from nanoscale to microscale such as cells and nanoparticles [87]. It represents a promising solution that can be incorporated with various up-to-date separation and sensing mechanisms for exosome isolation and analysis. Although still at an early-stage of development, microfluidics-based isolation methods hold great promise for translation into the clinic as they typically require a very small volume of samples and yield highly pure exosomes with minimal processing time [88]. Microfluidics-based technologies for exosome isolation are typically used for diagnostic purposes due to their high sensitivity but limitation in processed sample volume [89].

Techniques that have been incorporated in microdevices for exosome isolation include immunoaffinity, sieving, and trapping exosomes on porous structures [87]. Similar to the macroscale immunoaffinity-based method for exosome isolation, antibodies can also be immobilized to the surface of the channels in microfluidic devices for microscale isolation of exosomes [90]. Multiple groups have described approaches that incorporate microfluidics and immunoaffinity to isolate exosomes and microvesicles, highlighting quantitative and high-throughput analyses of exosome contents [87]. Membrane filters can also be incorporated in microfluidic devices to filter exosomes and other EVs. Vesicles in a blood samples were collected by sieving driven either by pressure or electrophoresis [91]. The latter was applied to separate proteins from vesicles based on their distinctly different surface charges. Trapping of exosomes and other vesicles with similar size in microfluidic channels can be achieved based mainly on the difference in size of the components in a sample. By incorporating a porous ciliated silicon microstructure, Wang et al. demonstrated a microchip that selectively trapsexosome-like lipid vesicles 40 - 100 nm, while sieving out proteins and cell debris [89]. The trapped vesicles were released and collected by simply dispersing the porous structure into PBS buffer [92].

As mentioned above, the exosome isolation techniques are based on particular properties of exosomes, each with its advantages and disadvantages. Combining two or more technique could further improve the isolation of exosomes [63] [**Table A.1**].

4.   ENGINEERING EXOSOMES AS A THERAPEUTIC DELIVERY SYSTEM

Exosomes play significant and diverse roles in intercellular communications, particularly in long-distance intercellular signaling. This mechanism of communication is highly robust and efficient in exchanging information between cells [93]. As such, intact exosomes derived from certain cells possess desirable therapeutic activities [24]. For example, tumor-derived exosomes that carry specific antigens have been explored for the promotion of specific immune responses against tumors [94, 95]. However, it was later found that tumor-derived exosomes can also suppress the immune response and promote metastasis and drug resistance development, shifting the research focus in using exosomes for cancer vaccination towards activating antigen presenting cells [96, 97]. For example, exosomes derived from DC, which include peptide-MHC complexes that can be transferred to recipient cells, have been extensively tested for tumor vaccination [12, 98]. Intact exosomes derived from human NK cells have been demonstrated to cause tumor cell lysis [99].

Inspired by their physiochemical properties and natural cargo delivering capability, researchers have also explored the potential of exosomes to deliver various exogenous therapeutics [21, 23]. Naturally derived from the body, exosomes are able to avoid phagocytosis, fuse with the cell membrane, and bypass the engulfment by lysosomes while causing only limited immune response [22, 100]. Exosomes can be engineered to present various targeting/therapeutic molecules on their surface, incorporating hydrophobic compounds in the lipid bilayer membrane and, encapsulating hydrophilic compounds or macromolecules inside their aqueous core.

Engineering of exosomes can either be done on the parental cells which will secrete exosomes carrying the desirable therapeutics or directly on the exosomes after they are isolated [73, 101] [**Fig. A.2.**].
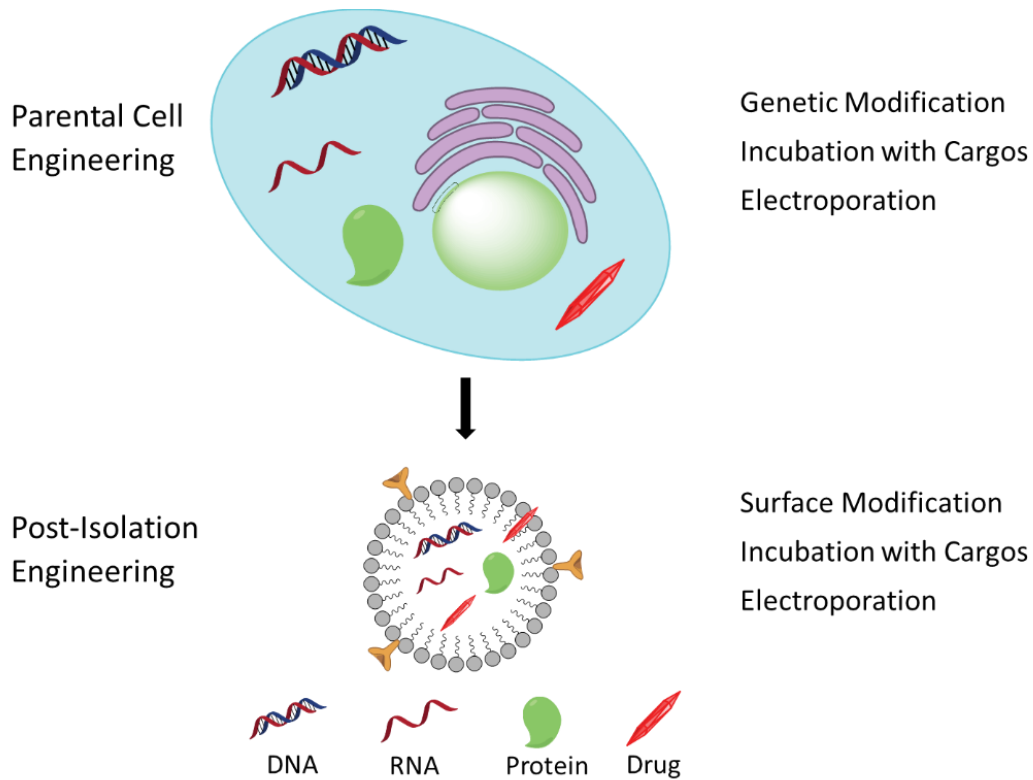
Figure A.2. Illustration of exosome engineering through parental cells (upper) and post isolation (lower). Parental cells can be genetically modified to express desirable therapeutic protein or nucleic acids. Drugs can be encapsulated inside parental cells simply by coincubation or electroporation. Exosome surface can be modified with imaging or therapeutic molecules by chemistry or other conjugation methods. Hydrophilic drugs can be encapsulated inside exosomes via simple co-incubation or electroporation while hydrophobic drugs are inserted into the hydrophobic core of the lipid bilayer membrane of exosomes.

4.1 Engineering of Parental Cells

Due to the availability of various cellular engineering methods, most modifications of exosomes have been performed on parental cells which are then cultured to secrete modified exosomes [18, 19]. Approaches to engineer parental cells include: (A) transfecting/infecting parental cells with DNA encoding therapeutically active compounds which are then released via exosomes or (B) loading parental cells with a drug, which is then released in exosomes [23].

4.1.1 Transfection and activation

Protein sequences, along with different types of RNA, are frequently used as synthetic oligonucleotides to be used for cell transfection, ultimately altering the phenotype of the released exosomes [75]. Genetic engineering strategies may require the use of the calcium phosphate or lipid method (i.e. via Lipofectamine) to load cargo or result in the desired genetic expression [75]. By altering the synthesis of the exosomes, one can control the therapeutic cargo that they will carry [75].

Protein expression

TRAIL is a therapeutic that has been loaded into cells via transfection [102, 103]. TRAIL, or TNF-related apoptosis inducing ligand, is a cancer therapeutic that targets death receptors 4 and 5 on cancer cells, ultimately inducing apoptosis [15, 16]. In one study, TRAIL-containing exosomes were created by transducing k562 leukemic cells with TRAIL [102]. The resulting exosomes enhanced apoptosis for melanoma and lymphoma cells. Additionally, MSCs were engineered to create exosomes with TRAIL, which also resulted in the apoptosis of various cancer cell lines, including lung, mesothelioma, breast and renal [103].

Transfection has been employed to alter exosomes derived from murine immature dendritic cells (DCs). For pre-isolation, immature DCS were transfected with a viral vector to express Lysosome-associated membrane protein 2 (Lamp2b) fused to the αγ integrin-specific iRGD peptide, a

membrane protein of exosomal origin, to enhance targeting efficiency to the tumor site [104]. Post-isolation, exosomes were loaded via electroporation with doxorubicin (Dox) and injected intravenously into the murine model, which resulted in reduced breast cancer tumor growth [104].

DNA/RNA

One way that exosome transfection has been accomplished is through miRNA expression vectors, which can result in exosomes carrying miRNA [75]. In the past, miRNA has been added to exosomes by transfecting miR-143 into THP-1 macrophages [105]. This modified form of miRNA is then overexpressed in parental cells, resulting in passive loading of the miRNA into exosomes.

For harnessing exosomes for cancer treatments, a study was performed by modifying an invasive triple negative breast cancer cell line (Hs578T) to overexpress miR-134 [106]. The exosomes containing the miRNA were isolated and then used to decrease expression of Hsp90. The miR-134 delivery reduced cell migration and increased the therapeutic efficacy of anti-Hsp90 treatments to the cells [106].

Similarly, in an effort to analyze the efficacy of exosomes as carriers for anti-tumor microRNAs, Katakowski, et al, used electroporation to transfect marrow stromal cells with a miR-146b expression plasmid [107]. Male Fischer rats were injected intratumorally with the marrow stromal cell-derived exosomes 5 days after glioma injection. Glioma growth was significantly reduced in the rats treated with exosomes containing miR-146b.

To heighten the sensitivity of Hepatocellular carcinoma (HCC) cells to chemotherapeutic agents, it was explored whether exosomes could be delivered to enhance expression of miR-122, a microRNA found to increase the chemosensitivity of HCCs [105]. Adipose tissue-derived MSCs

(AMSCs) were transfected with plasmids containing has-miR-122 using Lipofectamine 2000, or a control plasmid of cel-miR-67, which for human cells contains no mRNA-binding targets. Exosomes were isolated from the supernatant of the AMSCs, and classified as either 122-Exo or 67-Exo depending on their origin and contents. In vitro, HepG2 and Huh7 hepatoma cells were exposed to 122-Exo and chemotherapeutic drugs, revealing a decrease in cell viability when compared to controls. *In vivo*, intratumoral injections of 122-Exo increased the sensitivity of HCCs to sorafenib, as observed by reduced tumor size in the mouse model when compared to controls [108].

Exosomal delivery was also used to deliver microRNA to breast cancer cells expressing epidermal growth factor receptor (EGFR) [10]. Donor cells were transfected with a plasmid to express a fusion of the transmembrane domain of platelet-derived growth factor receptor (PDGF-R) and the GE11 peptide, which binds to EGFR. GE11-positive cells were transfected via lipofection with miRNA let-7a, a microRNA reduced in various cancers including breast cancer, whose expression suppresses tumor growth. Exosomes isolated from these cells were intravenously injected into mice with HCC70 breast cancer, successfully delivering the microRNA to the tumor site, as evidenced by reduced tumor growth when compared to the control [109].

Viral packaging can result in exosomes loaded with nucleic acids. This method employs Adeno-associated vectors (AAV) to load exosomes with a viral vector, and has been termed as "vexosomes" (vector-exosomes) in a study by Maguire et al. [110]. Human 293T cells were transfected with an AAV plasmid for 48h, with the resulting vexosomes isolated and collected. The exosomes contained AAV capsids for both strains of the AAV (AAV1 and AAV2), and were subsequently used to deliver DNA to a human glioblastoma cell line U87 [110].

A major benefit of exo-AAV is that they are able to evade neutralizing antibodies, compared to wild type AAV vectors that have the potential to evoke an immune response that can block their delivery [111]. With difficult to target cancer-types, such as glioblastoma, this benefit can be especially useful. Viral exosome delivery was used in the treatment of the GL261 mouse glioblastoma (GBM) model to determine its efficacy as a potential treatment for GBM [112]. As described previously, exo-AAV were made from transfecting human 293T cells with an AAV plasmid for 48 h [110]. The role of the vectors was to genetically modify the target GBM cells, causing GFP expression to specifically target Tumor-Associated Macrophages/Microglia (TAMs) and reactive astrocytes. The resulting pathway expressed interferon beta, a cytokine destructive to the brain tumor stromal cells, and was found to increase survival when compared to controls [112]. The ability of exo-AAV to evade neutralizing antibodies is also beneficial in that it allows for reduced dosing for gene therapy [113].

4.1.2 Loading of Exogenous Cargo

Exosomes can be preloaded with a protein or drug of choice when parental cells are loaded with exogenous cargo. The biogenesis process results in the preloading of the exosomes released from the cells, and is especially beneficial for oligonucleotides [75]. For instance, the parental cells can be incubated along with a specific drug [18]. The advantage of this strategy is in its simplicity, although there can be issues such as low efficiency of loading and a concern for cytotoxicity of the drug to the cells [75].

Protein

Extracellular vesicles, characterized as exosomes, were conditioned to bear heat shock proteins (HSPs) and isolated from resistant hepatocellular carcinoma cells (HepG2) that had been previously treated with resistant or sensitive anticancer cells [114]. HepG2 cells were incubated at 43 °C, with the HSP-bearing exosomes collected from the media. These exosomes

stimulated the cytotoxicity of NK cells and production of granzyme B, and upregulated inhibitory receptors while downregulating activation receptors. Exosomes derived from resistant anticancer drugs were greater in number, with more bearing HSPs, thus increasing the cytotoxic response to NK cells [114].

Liposomes formed the basis of a study involving exosome delivery of proteins to cancer cells, where they were co-incubated with a murine melanoma cell line (B16BL6) [115, 116]. The liposomes used for co-incubation were either fluid DOPE-based (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) or solid HSPC-based (hydrogenated soy phosphatidylcholine). Exosomes were collected and isolated from the culture media, their protein contents analyzed and broken up into categories (tetraspanins, heat shock proteins, enzymes and others). The resulting exosomes were then delivered to the B16BL6 melanoma cell line, as well as a murine colorectal cancer cell line (C26), and analyses were performed for studying how exosomal expression of the different proteins impacted their uptake by cancer cells [115]. The ability to incubate liposomes with cancer cells to produce exosomes with specific proteins could be highly useful for therapeutic delivery.

Hydrophilic drugs

Drug preloading for exosomal delivery of cancer treatments can be accomplished via "liposome-based cellular engineering", which attempts to engineer parental cells via membrane fusogenic liposomes (MFLs) [117]. The MFLs have been used to deliver hydro- and lipophilic agents into the membrane and cell cytosol, their resulting extracellular vesicles, which included exosomes and microvesicles, were functionalized to have specific contents without compromising their internal properties. These were able to successfully reduce cancer cell viability compared to control [117].

Hydrophobic drugs

Macrovesicles (MVs) and exosomes secreted from these MSCs have been frequently studied for therapeutic benefits in regenerative medicine due to their paracrine secretions, in the form of extracellular vesicles such as MVs and exosomes [118]. In a study of MSCs for use in drug delivery for cancer therapeutics, MSCs were engineered to deliver MVs, and specifically exosomes, with encapsulated paclitaxel (PTX). In this study, PTX priming of the murine MSC cell line SR4987 occurred with a high dosage (2000 ng/mL) of the drug. Researchers found that the MVs released by the MSCs were largely composed of exosomes, which contained PTX. Their delivery in vitro and in vivo to the human pancreatic adenocarcinoma cell line (CFPAC-1) inhibited cancer cell growth and proliferation both *in vitro* and *in vivo* [119].

As in the case of hydrophilic drug delivery, membrane fusogenic liposomes can also be used for the delivery of hydrophobic drugs. This method of parental cell engineering was used for anti-tumor drug loading when EVs containing the chemotherapeutics PTX and tirapazamine were co-incubated in Transwell experiments with B16F10 (melanoma) or MDA-MB-231 (late-stage breast cancer) cells. MFLs were able to reduce cell viability when compared to controls, demonstrating the efficacy of preloaded anti-tumor exosomes via liposome delivery to parent cells [117].

4.2 Post-isolation engineering

In situations where engineering of exosomes at cellular level is not feasible, exosomes derived from various origins can be engineered to carry functional molecules after being isolated [18, 66]. The liposome-like structure of exosomes provides different modification strategies that have been used for liposome modification. The type of cargos to be encapsulated often dictates their loading methods [22]. Therapeutic cargos that can be loaded into isolated exosomes include small molecules, nucleic acids and proteins [24]. Macromolecules for targeting, imaging or therapeutic

purposes can be conjugated with exosome surface molecules via valence bond or other specific conjugation methods [120, 121]. Hydrophobic compounds or hydrophilic compounds with a lipid-like hydrophobic tail can be inserted into the hydrophobic core of the lipid bilayer membrane [100]. Post-isolation modification of one or more exosome structural components falls well in the scope of nanotechnology which have demonstrated as promising applications in biomedicine, particularly in cancer therapy [20, 101].

4.2.1 Surface modification

A few reports have demonstrated the feasibility of exosome surface modification via chemical methods [120-122]. Post-isolation modification of exosome surface structures allows for exosome imaging and tracking *in vivo* [123, 124].

To label exosomes for an imaging modality, Smyth et al. conjugated fluorescent molecules to the surface of exosomes derived from mouse 4T1 breast cancer cells using click chemistry, a highly efficient and widely-used bioconjugation method [120]. In the study, the amine groups of exosome surface proteins were first functionalized with terminal alkyne groups which were then reacted with a model azide dye, azide-fluor 545. The mild conjugation didn't change the size distribution of the exosomes or their adherence/internalization property with recipient cells [120]. Instead of using chemical conjugation, an alternative way that has proven to be effective is take advantage of specific and tight avidin-biotin interactions. Lai *et al.* transfected human embryonic kidney 293T exosomes to express a surface luciferase with a fused biotin domain which was then coupled with fluorescent Alex Fluor® 680-Streptavidin [125]. Compared to Cell Tracker insertion labeling, the conjugation labeling increased spatial and temporal imaging resolution of exosomes and enabled the tracking of exosome delivery to tumor sites *in vivo* and analysis their blood circulation life [125].

Genetic engineering has also been reported as a method that can result in the surface modification of exosomes [19]. In one study, exosomes were engineered to express iRGD-Lamp2b to target human breast cancer cell lines to be used for the delivery of chemotherapeutic agents [104].

4.2.2 Loading of Exogenous Cargos

Exosome membranes can be loaded with hydrophobic therapeutics to increase drug solubility and stability while hydrophilic therapeutics such as RNA can be encapsulated in exosomes to improve cellular delivery.

Hydrophobic Drugs

Similar to their passive encapsulation into parental cells, hydrophobic drugs can be inserted into the membrane simply by incubation with exosomes. One of the earliest studies of this kind is the exosomal delivery of curcumin, an anti-inflammatory agent [126]. In this study, curcumin was mixed with mouse tumor cell line EL-4-derived exosomes at room temperature before the exosomes were purified via sucrose gradient centrifugation. Characterization of curcumin-loaded exosomes revealed higher solubility, stability, and bioavailability than free curcumin. In another study, the same group loaded JSI-124, a potent inhibitor of JAK/STAT3 signaling pathway with anti-tumor activity, into EL-4 exosomes, suggesting the potential of exosomes as a general delivery vehicle for hydrophobic compounds [126]. This incubation method was also used to encapsulate PTX, a hydrophobic chemotherapy drug, and rhodamine 123, a hydrophobic fluorescent compounds into exosomes derived from brain tumor cells and endothelial bEND.3 cells for their intranasal delivery across the blood-brain barrier [119, 127, 128]. To combine multiple therapeutic modules in one single nanoscale construct, gold nanorod (GNR)-conjugated Dox was also incorporated into the exosome membrane by simple incubation [129]. As revealed by TEM, the multiple GNRs were successfully incorporated into the lipid bilayer of the exosome

membrane. However, no separation of exosomes and non-encapsulated GNRs was described in the study [129].

To develop exosome-encapsulated PTX to overcome multiple drug resistance (MDR) in cancer cells, Batrakova's group compared three different encapsulation methods, incubation at room temperature, electroporation, and sonication to encapsulate PTX into exosomes derived from mouse macrophage RAW 264.7 cells [130]. Excess free drug was removed by SEC. As measured by HPLC, reformation of the exosomal membrane upon sonication resulted in the highest loading efficiency [130].

Hydrophilic drugs

Electroporation is more commonly used to load small hydrophilic compounds or nucleic acids into isolated exosomes [18]. To demonstrate targeted drug delivery for cancer therapy with low immunogenicity and toxicity, Tian et al. dispersed purified exosomes derived from immature a mouse DCs line in a Dox solution before electroporation was applied [104]. The mixture was then incubated at 37 °C for 30 min to allow the plasma membrane of the exosomes to recover. After purification by ultracentrifugation to remove unincorporated drug, the encapsulation efficiency reached 20% and the Dox-encapsulated exosomes were able to target and accumulate in breast tumors in mice and inhibit their growth [104].

RNA

As exosomes naturally deliver nucleic acids to recipient cells, exosomes have been expected to deliver exogenous siRNA in an efficient and targeted manner. Similar to their loading into cells, loading of siRNA into exosomes can be achieved by electroporation. Alvarez-Erviti *et al.* reported the first siRNA delivery by isolated exosomes in 2011 [131]. To target the exosomes to the brain of mice, DCs were transfected to express Lamp2b (an exosomal membrane protein) fused to the

neuron-specific RVG peptide3. Electroporation was performed on purified exosomes to load exogenous siRNA. Intravenously injected RVG-targeted exosomes delivered the siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown [131]. Exosomes were also used to deliver siRNA target Parkinson's disease [132]. Exosomes derived from mouse DCs were loaded with α-synuclein siRNA to target α-synuclein aggregates in the brain in mice with Parkinson's disease. Brain-specific uptake was observed following intravenous administration of the α-synuclein siRNA-loaded exosomes. Protein aggregates were found reduced in one week after injection. This study further supports the feasibility of using exosomes as nanocarriers for transporting cargo across the blood-brain barrier [132]. To demonstrate that nucleic acids can be delivered across cell's plasma membrane, the same group of authors tested different methods to load RNA into human exosomes of various origins and identified electroporation as the best methods for RNA loading. The siRNA loaded exosomes effectively delivered siRNA into lymphocytes and monocytes, and silenced mitogen-activated protein kinase 1 selectively [133]. Momen-Heravi et al. demonstrated that B cell-derived exosomes can deliver an exogenous miRNA-155 mimic into hepatocytes or macrophages to inhibit malignant growth. Unlike in parental B cells, baseline level of miRNA-155 was found to be very low in B cell-derived exosomes. The authors optimized the loading efficiency of miRNA-155 by electroporation at various RNA to exosome ratios. Exosomes loaded with miRNA-155 mimic significantly increased miRNA-155 levels in primary mouse hepatocytes and the liver of miRNA-155 knockout mice [134].

5. ANTI-METASTATIC APPLICATIONS

As mentioned above, previous reviews have summarized exosome engineering to target various diseases, in particular cancer [10-12, 18-23]. In this section, we introduce the most recent reports of engineered exosomes for metastasis targeting, and their potential for clinical translation.

Phase I clinical trials have demonstrated the feasibility of large-scale production of DC-derived exosomes and the safety of the exosomes in patients with metastatic colorectal, lung cancer, and melanoma [135-137]. In the lung cancer trial, T cell immune responses were detected in a third of exosome-treated patients and increased NK lytic activity in half of the treatment group. Some patients exhibited long term stability of disease and activation of immune effectors. Mouse studies also revealed that the DC-derived exosomes promoted an IL-15Rα- and NKG2D-dependent NK cell proliferation and activation respectively, resulting in anti-metastatic effects mediated by NK1.1 (+) cells. In humans, DC-derived exosomes were found to express functional IL-15α which allows proliferation and NK cell secretion of IFN-γ, a cytokine that is critical for innate and adaptive immunity [137].

Encouraging results were also obtained in a Phase II trial testing DC-derived exosomes as maintenance immunotherapy after induction chemotherapy in patients with metastatic lung cancer [138]. The exosomes used in this trial were derived from IFN-γ maturated DCs because such exosomes induce greater T cell stimulation compared to those from immature DCs. The exosome treatment increased NKp30-dependent NK cell functions in treated patients, and 32% of patients experienced stabilization for at least 4 months [138]. This Phase II trial confirmed that DC-derived exosomes could boost the NK cell arm of antitumor immunity in patients with metastatic lung cancer.

A promising option in cancer immunotherapy is active vaccination with autologous DCs loaded with tumor-associated peptides. However, the immune response of pancreatic cancer (PaCa) by this strategy is often found suppressed. To overcome this issue, Xiao et al. combined vaccination with tumor exosome-loaded DCs (DC-TEX) with drugs affecting myeloid-derived suppressor cells (MDSC). In the study, autologous DCs were loaded with PaCa cell-derived exosomes to vaccinate for PaCa in xenograft mice together with drugs such as Gemcitabine (GEM), all-transretinoic acid

(ATRA) and sunitinib [139]. A reduction of MDSC including tumor-infiltrating MDSC and a decrease in migrating and metastasizing tumor cells was observed in the groups treated with Sun, ATRA and most efficiently GEM. Vaccination by DC-TEX with any of the three drugs increased the number of activated T cells in the tumor and subsequently the survival time in mice compared those vaccinated only by DC-TEX. A reduction in metastatic spread was observed with the combination of (DC-TEX) with sunitinib compared to the group treated with sunitinib alone [139].

The mutant form of the GTPase KRAS is a key driver of PaCa, which controls macropinocytosis in PaCa cells and increases exosomes uptake. This inspired Kamerkar et al. to develop exosomes derived from normal fibroblast-like mesenchymal cells to carry siRNA specific to oncogenic KRAS. Compared to control liposomes, the engineered exosomes exhibited an enhanced retention in circulation and a subsequent enhanced efficacy that is dependent on CD47. It was confirmed that the enhanced retention of exosomes is due to CD47 mediated protection of exosomes from phagocytosis by monocytes and macrophages. The engineered exosomes suppressed cancer in multiple mouse models of PaCa and significantly increased their overall survival. To push these exciting discoveries into human translation, the same lab also reported their bioreactor-based procedures employing good manufacturing practice (GMP) standards to generate large scale of siRNA-loaded clinical-grade exosomes. The clinical-grade GMP exosomes were tested in multiple *in vitro* and *in vivo* studies to confirm suppression of oncogenic *Kras* and an increase in the survival of several mouse models with PaCa [140]. The authors also demonstrated that the treatment efficacy could be further improved when combined with the chemotherapy drug gemcitabine [141].

To overcome MDR in cancer cells, PTX-loaded exosomes were used to treat both drug resistant cancer cell lines, $MDCK_{MDR1}$ and their sensitive counterpart $MDCK_{WT}$ [130]. In both cell lines, the loading of PTX into exosomes significantly increased drug cytotoxicity as compared to PTX alone,

with a greater increase in resistant cell lines than sensitive ones. The same researchers also developed macrophage-derived exosomes for targeted PTX delivery to pulmonary metastases [130]. The drug-loaded exosomes were modified with an aminoethylanisamide-polyethylene glycol vector moiety to target the sigma receptor, which is overexpressed by lung cancer cells. The exosome formulations showed a dramatic ability to accumulate in cancer cells following systemic administration in a C57BL/6 mouse lung cancer model, improved therapeutic outcomes.

6. CONCLUSIONS

Exosomes are specialized intercellular messengers that alter the functional state of their target cells by delivering cargos such as proteins and nucleic acids from their parental cells. Exosome's role in cancer including metastasis has been intensively investigated. Our understanding of their properties and activities have provided solid foundation to engineer exosomes for the targeting of metastasis, which could significantly increase survival among cancer patients. After a decade of research, many engineered exosome engineering methods including those for isolation and cargo incorporation have proven to be successful for modifying exosomes with desirable diagnostic and therapeutic functionalities. Application of engineered exosomes to target metastasis have yielded encouraging results that would support further development toward clinical practice.

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