Improving Anticancer Therapy with ELP-based Drug Delivery Systems

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

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ABSTRACT

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Abstract

Cytotoxic chemotherapy is a mainstay of cancer treatment, administered to patients who require systemic treatment based on the stage of their disease. Due to its dose-limiting side-effects, chemotherapy is commonly administered to avoid lethal toxicity rather than to maximize efficacy. Therefore, a great deal of effort has been focused on packaging drugs into nanoparticles, promoting its accumulation in tumors due to the presence of leaky neo-vasculature and re-directing the drug away from critical organs. While these drug-delivery technologies consistently improve treatment efficacy compared to conventional therapies in primary tumors of preclinical models, new treatment modalities are often not assessed for their ability to interfere with the metastatic process. Therefore, we studied the efficacy of our genetically encoded polypeptide nanoparticle for doxorubicin delivery (CP-Dox) in syngeneic metastatic murine models 4T1 and Lewis lung carcinoma. When our nanoparticle drug treatment was combined with primary tumor resection, greater than 60% of the mice were cured in both the 4T1 and Lewis lung carcinoma models as opposed to a 20% survival rate when treated with free drug. Mechanistic studies suggest that metastasis inhibition and survival increase were achieved by preventing the dissemination of viable tumor cells from the primary tumor.
While targeting metastatic disease directly is the standard goal of systemic chemotherapy, drugs that improve the treatment of primary tumors still hold promise for treating disseminated disease if they can stimulate a systemic host antitumoral immune response. Interestingly, certain cytotoxic drugs like doxorubicin can alter the host immune response to tumors by causing immunogenic cell death, revealing tumor-associated antigens and recruiting antitumoral leukocytes that can be further activated by co-treatment with immunostimulatory agents. However, the drug delivery field currently lacks an understanding of how packaging cytotoxic drugs into nanoparticles alters or potentially enhances these immunomodulatory phenomena. Previous studies have often administered doxorubicin intratumorally or studied the phenomenon in immunogenic tumor models. Whether the immunomodulatory properties of doxorubicin can be observed with systemic administration against a poorly immunogenic tumor model is unclear. Therefore, we have performed extensive studies of CP-Dox while interfering with aspects of the immune system to determine the role of the host antitumoral immune response in its efficacy. In this project, we show that a single intravenous (IV) administration of CP-Dox enhances the host anti-tumor immune response, enabling CD8+ cells to contribute to the prevention of metastasis in 4T1 mammary carcinoma. We show that IV CP-Dox increases the ratio of Th1 to Th2 cytokines in the tumor, and that IFN-γ depletion reduces the efficacy of CP-Dox. We observed that the myeloid cell infiltrate was re-polarized to express markers associated
with an anti-tumor phenotype. Importantly, these effects were not seen in mice treated with free doxorubicin. Our studies provide evidence that formulating cytotoxic chemotherapies as nanoparticles can better enable their in vivo immunomodulatory capabilities, demonstrating the potential of combining nanoparticle delivery strategies with immunotherapy to improve the treatment of cancer.

Cytokine treatment was the first successful immunotherapy for cancer. Systemic administration of IL-2 leads to treatment responses in about 20% of patients with melanoma and renal cell carcinoma. However, the treatments have significant, life-threatening side-effects, leading to interest in local administration of IL-2. We have developed a bioactive ELP/IL-2 fusion which forms an insoluble coacervate upon in vivo injection. Intratumoral treatment of the fusion delayed the growth of B16 melanoma, and showed improved efficacy when combined with CP-Dox treatment. We also developed a bioactive ELP/GM-CSF fusion which was shown to recruit leukocytes to the site of subcutaneous injections. We show that the immunostimulatory activity of CpG is enhanced by condensation an ELP containing a lysine trailer. Thus we have developed several key ingredients for an in situ cancer vaccine which can generate antigen (CP-Dox), stimulate antigen presentation (GM-CSF and CpG), and stimulate T cells (IL-2).
Dedication

Dad for inspiring me to be an engineer, Mom for inspiring me to be a doctor, and Dr. Eniola-Adefeso for helping me realize I could be both.
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Chapter 1: Introduction

Cancer epidemiology

Cancer is the second leading cause of death in the United States, accounting for nearly 600,000 deaths in 2014. Based on the most recent estimates, approximately 40% of people will develop cancer at some point in their lifetimes. The toll that cancer has taken on human life has inspired massive research efforts to better understand, diagnose, and treat the disease. This undertaking appears to be having a tangible impact, as the five-year survival rate for cancer has slowly but steadily risen from 49% in 1975 to 69% in 2008. Nonetheless, metastatic cancer remains a daunting diagnosis with a poor prognosis. For example, the survival rate for patients with Stage III (lymph node involvement and/or large primary tumor but no distant metastasis) and Stage IV breast cancer (distant metastasis regardless of tumor size or lymph node involvement) are 72% and 22% respectively. Thus, there remains a great need for improving metastatic cancer treatment, which accounts for approximately 90% of deaths for breast cancer patients.

Current standard of care for cancer

Cancer has traditionally been treated with a combination of surgery, radiation, and chemotherapy. Immunotherapy, an emerging treatment approach which may gain widespread clinical adoption and become a fourth pillar of cancer treatment, will be discussed in a later section. The selection of treatment modality depends on the tissue of
origin and the stage of disease at diagnosis, and treatment approach may evolve if
disease progresses.

Surgery is an effective local treatment therapy, with the potential to be curative if
all disease can be removed. However, complete removal of disease may not be possible
due to anatomic location, tumor size, or pre-existing spread to distant organs. Although
surgery may still be administered in these settings (metastatic colon cancer limited to
one lobe of the liver for example), it will need to be supplemented with other treatment
approaches, including but not limited to radiation and chemotherapy. These approaches
are labeled relative to the time of surgery, with “neoadjuvant” occurring before surgery
and “adjuvant” after surgery.

Radiation therapy was first applied to treat cancer in the late 19th century, and
the field has evolved greatly since, improving in safety and efficacy5. Radiation kills cells
through a variety of mechanisms, including the induction of apoptosis via DNA and
membrane damage, which is increased by the presence of oxygen6. Certain tissues,
including skin and mucosal linings are particularly sensitive and lead to dose limiting
side effects of radiation therapy7. Although radiation also damages normal tissues,
radiation has relative selectivity for cancer cells because oncogenic transformation is
associated with increased sensitivity to apoptotic stimuli5. This relative sensitivity can be
increased further by delivery radiation in fractionated doses, giving time for normal
tissues to be repaired and for hypoxic regions of tumor to become re-oxygenated after
de-bulking of the mass from cell death. The two major means of administering radiation are external beam and brachytherapy, where the radioactive source is placed near or inside the area requiring treatment.

Radiation therapy is used in a variety of clinical settings. Radiation can be administered to the site of surgical resection to minimize the likelihood of residual disease, and expands the number of candidates for organ-preserving operations in place of radical resections. It can also be used in the palliative setting to reduce pain and other symptoms due to the presence of the tumor, even when cure is not a viable goal.

Radiation therapy can be administered concurrently with chemotherapy, and the potential for synergy between drugs which freeze cells in stages of the cell cycle most sensitive to radiation (cisplatin, 5-FU) has been the subject of much investigation. Radiation therapy also has the potential to re-model the tumor microenvironment, release damage associated patterns that stimulate the innate immune system, and re-instate tumor immunosurveillance. Combination therapies may also allow lower doses of each component treatment, achieving similar efficacy with reduced side effects. Thus, there is great potential for future combinations of chemotherapy, immunotherapy, and radiation therapy.

Anticancer chemotherapy refers to the use of pharmacological agents administered to treat cancer. Chemotherapy is the major systemic treatment modality for cancer. In the adjuvant setting, it is administered to reduce local or distant
recurrence of microscopic disease. Increasingly, chemotherapy is being administered in the neoadjuvant setting, which allows doctors to assess the response of the tumor to treatment and adjust drug regimens if necessary\textsuperscript{13}. Neoadjuvant chemotherapy may also permit organ-sparing surgical techniques, or allow a patient with a previously inoperable tumor to become a surgical candidate\textsuperscript{14}.

There are multiple classes of chemotherapy, including cytotoxic agents, anti-vascular agents, and small molecule inhibitors. The focus of this project will be on cytotoxic chemotherapy, which itself contains multiple classes of drugs categorized by their mechanism of action. Alkylating agents react with guanine and form cross-links across DNA strands, leading to DNA damage. Antimetabolites are generally nucleotide analogs which are incorporated into DNA and disrupt normal base-pairing. Mitotic inhibitors function by interfering with tubulin assembly or disassembly, which is critical for proper alignment and segregation of chromosomes during metaphase. Cells therefore cannot proceed through the cell cycle and replicate. Intercalating agents sit between base pairs of DNA, disrupting the normal shape of DNA, inhibiting DNA and RNA polymerase and therefore inhibiting DNA synthesis and transcription\textsuperscript{15}. Doxorubicin is a widely used intercalating agent which will be an important pharmacological agent for this project, and will therefore be discussed further in the next section.
In general, cytotoxic agents target cells with rapid division rates, and thus have slightly selective effects on cancerous cells due to their high proliferation rate and predisposition to apoptosis\textsuperscript{14}. However, since the mechanism of cell killing is not inherently specific to cancerous cells, cytotoxic agents have serious side effects and relatively narrow therapeutic windows. Tissues with rapid proliferation rates (mucous membranes, skin, hair, bone marrow, GI tract) are especially prone to toxicity. Thus dosing is often determined by avoiding irreversible toxicity rather than for maximizing therapeutic efficacy. Patients must be monitored for known side effects and may need to skip treatments when toxicity develops. Furthermore, many cytotoxic agents are poorly water-soluble and must be administered with vehicles which themselves can induce toxicity.

Chemotherapy is often administered in combination regimens. By using drugs with different mechanisms of action that affect cells at different stages of the cell cycle, more cell killing may be achieved. Tumors are heterogeneous mixtures of cells with varying levels of sensitivity to a given agent, so administering more than one drug decreases the likelihood of a cell being resistant to both and persisting despite treatment. A more practical reason for combination treatment is to allow for larger total doses of cytotoxic agents by using drugs with non-overlapping side-effect profiles, such that each agent can be used near its maximal tolerated dose. As the standard of care for a variety
of clinical settings, chemotherapy will also be used in combination with emerging therapies such as molecularly targeted therapies and immunotherapy.

Despite the fact that cytotoxic drugs have demonstrated efficacy over decades of use, they suffer from suboptimal biodistribution and pharmacokinetics which could be addressed by improved delivery strategies. A major strategy in this regard is packaging these drugs as nanoparticles, which will be discussed in depth in subsequent sections.

Doxorubicin

Doxorubicin is the cytotoxic agent used throughout this project, and therefore deserves particular attention here. Doxorubicin is an anthracycline antibiotic, first isolated from *Streptomyces peucetius*\(^\text{15}\). It has demonstrated activity in a wide variety of cancers, including acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms' tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, Hodgkin's disease, malignant lymphoma and bronchogenic carcinoma\(^\text{16}\).

Doxorubicin has several mechanisms of action: (1) DNA intercalation, (2) Topoisomerase II inhibition, and (3) the generation of free radicals. Intercalation between base pairs of DNA interferes with DNA replication and RNA synthesis. Topoisomerase II normally serves the function of relieving strain in DNA due to supercoiling, a process which involves breaking and resolving the DNA backbone. Doxorubicin induces the formation of a stable intermediate between the enzyme and the
5’ end of the DNA strand, preventing re-ligation of the strands which leads to double strand breaks\textsuperscript{17}. Doxorubicin also produces free radicals when it is reduced by an enzyme such as NADPH cytochrome P450 reductase. The unstable intermediate is oxidized, and can act as an alkylating or DNA crosslinking agent\textsuperscript{15}. When doxorubicin interacts with molecular oxygen it can form peroxides which cause cell death through damage of cell membranes via lipid peroxidation\textsuperscript{16}. The final mechanism of cell death due to doxorubicin may be necrosis or apoptosis\textsuperscript{18-20}. Importantly, regardless of the cell death modality, doxorubicin induces what is known as immunogenic cell death, whereby damage associated signals are released or displayed by dying cancer cells which activate the innate immune system\textsuperscript{20-22}.

The membrane damage secondary to free radical formation is also thought to contribute to the major dose-limiting toxicity of doxorubicin: cardiotoxicity. Damage to the sarcolemma and mitochondrial membrane within cardiomyocytes disrupts their function and can lead to cardiomyopathy even years after administration of the drug\textsuperscript{15}. Thus there is a cumulative life-time dose limit for doxorubicin\textsuperscript{21}. Another potentially dose-limiting side effect of doxorubicin is acute bone marrow suppression, so patients must be monitored for neutropenia\textsuperscript{22}. Doxorubicin also causes nephrotoxicity, stomatitis, nausea and vomiting, and alopecia\textsuperscript{16,24}.

The side effects of doxorubicin are not solely due its mechanism of action, but also to its suboptimal pharmacokinetics and biodistribution. Doxorubicin is rapidly
distributed throughout the tissues of the body, including the heart, with a distribution half-life of 5 minutes and a large volume of distribution \((700-1100 \text{ L/m}^2)\)\(^{16}\). The plasma half-life is relatively short (0.2-0.6 hours), but the elimination half-life is extended (~30 hours) due to high tissue extravasation\(^{16}\). Resistance to doxorubicin is largely mediated through the expression of drug efflux pumps such as p-glycoprotein, the product of the \textit{MDR1} gene\(^{15}\). Liposomal doxorubicin attempts to address some of the shortcomings of conventional doxorubicin, and will be discussed in the nanoparticles subsection.

**Nanoparticle drug delivery to tumors**

In cancer patients at high risk for recurrence, cytotoxic chemotherapy is administered to reduce the incidence of metastasis and improve survival\(^{25, 26}\). Nonetheless, patients may succumb to metastatic disease for a variety of reasons, including, but not limited to: insufficient concentrations of drug delivered to malignant cells, development of resistance during therapy, or severe side effects of the chemotherapy leading to inability to complete the regimen\(^{27}\). To improve cytotoxic chemotherapy, a great deal of effort has been focused on re-packaging these drugs into nanoparticles\(^{28}\). The basis for this approach is that tumors have leaky vasculature and a relative lack of lymphatics, so nanoparticles of the appropriate size (generally 10-100nm) passively accumulate in the tumor, a phenomenon known as the enhanced permeability and retention (EPR) effect\(^{29}\). Nanoparticle delivery also reduces side effects by limiting the drug’s extravasation into off-target organs, thereby permitting larger doses to be
administered\textsuperscript{27,30}. Nonetheless, the EPR effect is limited by several factors\textsuperscript{31}. High intratumoral interstitial fluid pressure, variability in porosity and spatial distribution of vasculature, and the large size of nanoparticles all limit the delivery of chemotherapy. Furthermore, the EPR effect is likely more pronounced in pre-clinical models compared to human tumors\textsuperscript{31}.

The nanoparticle formulation to first gain clinical adoption was Doxil\textsuperscript{32}. Doxil is a liposomal formulation of doxorubicin which achieves drastically enhanced circulation times due to its size (80-100nm) and its pegylation which slows opsonization and phagocytosis by the reticuloendothelial system (RES). Doxil also changes doxorubicin biodistribution, re-directing it away from the heart and improving its accumulation in tumors\textsuperscript{33}. Doxil has demonstrated clear improvements in efficacy over free doxorubicin in preclinical models, and in patients with Kaposi sarcoma, a highly vascular tumor, and multiple myeloma, a tumor of the bone marrow which lacks high IFP\textsuperscript{31}. It is also approved for the treatment of recurrent ovarian cancer and metastatic breast cancer\textsuperscript{32}, although the approval in these solid tumors largely due to reduced cardiotoxicity rather than greatly improved efficacy\textsuperscript{31}. Furthermore, Doxil suffers from dose-limiting Palmar Plantar Erythrodysthesia (PPE) or foot and hand syndrome which is a desquamating dermatitis\textsuperscript{32}, which has limited its widespread clinical adoption. Thus there is a need to develop newer generations of drug delivery vehicles. Thermosensitive liposomes, which protect the drug payload from normal tissues and achieve intravascular drug release
upon mild hyperthermia (40-42°C), and thus do not rely on the EPR effect for enhanced delivery, have shown great promise. This project will use Chimeric-polypeptide doxorubicin (CP-Dox), and micelle formulation of doxorubicin based on an elastin-like polypeptide (ELP) backbone, which will be described further in the next chapter.

**Tumor immunology and immunotherapy**

The immune system holds great potential to treat cancer, as a coordinated adaptive anticancer immune response can achieve unmatched selectivity and cure of disseminated disease. This is in contrast to the current standard of care for systemic treatment of cancer, chemotherapy, which only prolongs survival on the order of months and can only achieve cure in a specific subset of cancers. The potential of the immune system has been recognized from as early as the late 19th century when William Coley established the field of cancer immunotherapy. He observed that some cancer patients that developed and recovered from skin infections with *Streptococcus pyogenes* subsequently experienced regressions in their tumors. To confirm the causal nature of this phenomenon, he treated a cancer patient with a subcutaneous injection of *Streptococcus pyogenes*. The patient experienced severe illness and a high fever, as well as regression in the tumor. Coley went on to develop extracts from *S pyogenes* and *S marcescens* which became known as “Coley’s toxins” in order to avoid injecting live pathogens into ill patients. Despite sporadic success with the approach, it was difficult to consistently reproduce. Surgery, chemotherapy, and radiation became the standard
of care for cancer patients. Although Coley’s toxins established the plausibility of stimulating the immune response to fight cancer, it would take decades to begin unraveling the complex host-tumor interactions.

**Host-tumor interactions: immunoediting theory**

Although cancer cells arise from host-derived cells, malignant transformation is associated with mutations that can generate neoantigens. Neoantigens are epitopes derived from native proteins which contain a potentially immunogenic mutation. Thus, transformed cells can be distinguished from normal cells, and can potentially be eliminated by the immune system\(^{38}\). Nonetheless, it is clear that tumors arise in immunocompetent hosts, so determining the signals and mechanisms involved in a tumor’s escape from the host immune response has been the subject of significant research efforts\(^{39}\). The compiled results of these experiments has given rise to the immunoediting theory\(^{38}\).

According to immunoediting theory, the interaction between the host immune system and a tumor involves three stages: (1) elimination, (2) equilibrium, (3), escape. During the elimination stage, danger signals released from tissues containing transformed cells (HMGB1) and inflammatory cytokines such as Type I IFNs recruit innate immune cells. The neoantigens expressed by the transformed cells stimulate an adaptive immune response driven by CD4 and CD8 T cells\(^{38}\). Although it is difficult to confirm this stage in humans or even in preclinical mouse models, it is hypothesized
that many nascent transformed cells are eliminated at this stage\textsuperscript{38}. If malignant cells are able to persist despite the initial immune response, they enter the equilibrium phase, where tumor cell eradication is roughly matched by tumor cell growth, which results in an overall stabilization in the volume of the tumor. Further selection and “editing” of resistant clones may take place at this stage\textsuperscript{38}.

Finally, for a clinically detectable tumor to develop, it must escape the immune response, and it is reversal of this escape that is the goal of tumor immunotherapy. There are many mechanisms behind escape. Tumor cells become resistant to cell killing mechanisms used by the immune system by overexpressing anti-apoptotic molecules STAT3 or BCL-2\textsuperscript{38}. A widely used method of escape, and one that is likely selected for by the elimination and equilibrium stages of the process is loss of antigen expression. This can proceed through several mechanisms including the emergence of tumor cells which lack rejection antigens, the loss of MHCI expression, and loss of antigen processing machinery\textsuperscript{38}. Importantly, this project will explore the role of MHCI expression on tumor cells in the ability of the immune system to mount an effective response.

Another mechanism of escape is for malignant cells to establish an immunosuppressive microenvironment\textsuperscript{40}. In this case, the immune system may recognize tumor antigens, an inflammatory response may be mounted, but a wide variety of tumor-derived signals circumvent an effective immune response\textsuperscript{41}. There are many stages, signals, and cells involved in an effective immune response\textsuperscript{42}. Antigen
presenting cells must traffic to the tumor, take up antigen, and become activated to process and display antigen to T cells. T cells must receive proper signals to become activated. If the presentation takes place in lymph nodes, the T cells must subsequently traffic to and infiltrate the tumor. T cells must remain activated, recognize the appropriate antigen displayed via MHCI, and eliminate the malignant cell. Tumors have an ability to interfere with each of these steps, which is crucial to the tumor’s escape from the immune response.

Paracrine factors such as adenosine, prostaglandin E2, and TGF-β interfere with the proper infiltration and function of dendritic cells and polarize macrophages towards a protumoral M2 phenotype. Metabolic factors also alter the antitumor immune response. Lactate reduces dendritic cell maturation factors and T-cell costimulatory ligands CD80 and CD86. Arginine consumption and subsequent depletion by M2 macrophages inhibits the function of T cells. Cytokines such as IL-4 and IL-10 downregulate antigen presentation by dendritic cells. Checkpoint proteins, such as CTLA-4 and the PD-1/PD-L1 axis, along with many other signals, inhibit the activity of effector T cells. Myeloid derived suppressor cells (MDSC), which inhibit the activation of effector T cells, are induced by granulocyte-colony stimulating factor (G-CSF), and recruited to tumors by CCL2 produced in the tumor microenvironment. Regulatory T cells (Tregs) use a variety of mechanisms to maintain tolerance to tumor associated antigens and reduce the activity of antigen presenting cells and effector T
cells, and are recruited to the tumor via chemokines such as CCL4, CCL22, and CCL28. Thus tumors have an incredibly diverse array of mechanisms for preventing their destruction by the immune system.

**Stimulating an effective antitumor immune response**

The major goal of cancer immunotherapy is to generate or restore effective antitumor immunity. Thus, efficacious treatments must interfere with or repolarize the signals that the tumor established. Antigen release and reduction in immunosuppressive signaling from malignant cells can be achieved through treatment with chemotherapy or radiation. Antigen presentation can be promoted with vaccine or cytokine (IFN-γ or GM-CSF) administration. Activation of T cells can be enhanced with checkpoint blockade or cytokines (IL-2 or IL-12, for example). It is likely that successful immunotherapy will involve combinations of these approaches in order to reduce the likelihood of compensatory mechanisms and resistance from the tumor. Exploring new ways of delivering the components of these treatments will be explored in this project.

Checkpoint blockade in particular has revolutionized the field of cancer immunotherapy, demonstrating its clinical applicability and potential to greatly improve cancer treatment. Inhibition of CTLA-4 and PD-1 has demonstrated efficacy against melanoma and renal carcinoma, and is being tested in a variety of other tumors. Combination therapies are already being developed, and it will be critical to
understand how checkpoint blockade interacts with more traditional treatment modalities as it gains clinical adoption\textsuperscript{52}.

\textbf{Chemotherapy and the immune system}

Cytotoxic drugs are so-named for their ability to directly kill cells, so this class of drugs is generally thought to exert its effect through direct cell killing, independent of the immune system\textsuperscript{53, 54}. Furthermore, their side effects often involve bone marrow suppression, which leads to a decrease in leukocyte count. This led to concerns that cytotoxic chemotherapy would interfere with treatments involving the immune system\textsuperscript{54}. However, in clinical trials, cytotoxic chemotherapy was found to be additive or even synergistic with immunotherapies\textsuperscript{55}. Many of the basic mechanisms underlying this phenomenon have been elucidated. First, by killing tumor cells, tumor-derived immunosuppressive cytokines (ie TGF beta) are reduced\textsuperscript{50}. Second, cytotoxic drugs can exert immunomodulatory effects directly on leukocytic subsets\textsuperscript{50, 54, 56}. For example, doxorubicin can polarize immunosuppressive myeloid cells towards a more inflammatory phenotype\textsuperscript{57}. Third, and perhaps most importantly, some cytotoxic drugs induce immunogenic cell death, whereby inflammatory signals are released or displayed by dying tumor cells\textsuperscript{58}. Important mediators of immunogenic cell death include calreticulin display\textsuperscript{59}, HMGB1 secretion\textsuperscript{60}, ATP release\textsuperscript{61}, and formyl peptide receptor 1 expression on dendritic cells\textsuperscript{62}. This induces the production of immunostimulatory cytokines and chemokines, which stimulate the processing of tumor antigens and recruit
more leukocytes to the tumor\textsuperscript{53,63}. Indeed, it has been shown that specific effector cells (ie CD8) and cytokines (ie IFN-\(\gamma\)) are essential to the \textit{in vivo} efficacy of anthracyclines like doxorubicin\textsuperscript{64,65}. In certain tumor models, immunogenic cell death can lead to a cell-mediated immune response and long lasting immune memory which protects against re-challenge\textsuperscript{59}. However, most of the mechanistic work has been performed in immunogenic models using freely dissolved doxorubicin (Free Dox) and intratumoral administration of drug. This project will explore how delivering doxorubicin as a nanoparticle affects its immunomodulatory properties. As immunotherapy and nanoparticle delivery of chemotherapy achieve clinical adoption, it is critical to understand how they interact\textsuperscript{66,67}.

**Cytokine immunotherapy for cancer**

Cytokines are a diverse class of immunomodulatory agents with effects ranging from carcinogenesis and promotion of tumor growth to induction of immunosurveillance and tumor immunity\textsuperscript{68}. Clinically detectable solid tumors are often recognized by the immune system but have evolved immunosuppressive mechanisms that protect them from the antitumor immune response\textsuperscript{67,69}. Therefore, the overall strategy of cytokine immunotherapy is to circumvent the tumor-derived signaling and reinstate immunosurveillance\textsuperscript{70}. Systemic cytokine treatment leads to significant side effects, and the half-lives are short leading to rapid clearance and necessitating many injections. This has led to interest in intratumoral delivery, but the drug can still rapidly
diffuse out of the tumor due to the low molecular weight\textsuperscript{71}. Therefore, this project will aim to develop immunomodulatory reagents delivered by an ELP that will transition after intratumoral injection, leading to a long-lasting depot that will persist in the tumor and improve cancer immunotherapy treatments.

\textit{In situ vaccine}

The goal of cancer immunotherapy is to generate a systemic antigen specific antitumoral immune response that can generate tumor regression and potentially even a long-term cure\textsuperscript{72}. Antitumor immunization is a specific form of cancer immunotherapy typically involving the administration of either tumor antigen (Approach 1), tumor lysate (Approach 2), or genetically modified irradiated cancer cells (Approach 3), along with an immune adjuvant to generate a protective immune response. Despite promise in preclinical trials, these approaches must overcome several hurdles in order gain clinical adoption due to the challenge of pre-selecting an immunogenic and effective antigen that will be protective in heterogeneous human cancers and (Approach 1), or due to the need for potentially complex infrastructure to manipulate cancer cells \textit{ex vivo} (Approach 2 or 3). Furthermore, while these approaches may generate an immune response at the vaccination site or draining lymph node, the cells must enter the tumor and act in the immunosuppressive microenvironment\textsuperscript{73}.

A promising alternative that addresses many of these challenges is to inject immunomodulatory materials directly into the tumor, often termed \textit{in situ} vaccination.
With this approach, the tumor itself becomes the source of antigen\textsuperscript{74}. The treatment components used in this approach must (1) result in the generation of exposed tumor antigen and convert the tumor microenvironment into a site conducive to (2) antigen presentation and (3) effector T cell activity\textsuperscript{71, 74}. One potential approach for altering the tumor microenvironment to turn the tumor into a site of antigen generation and presentation is doxorubicin treatment\textsuperscript{53, 63}. Combined with the immunomodulatory regents delivered via ELP, we have the ingredients to develop an in situ vaccine with the potential to generate an immune response locally which could go on to clear disseminated disease.

\textbf{Elastin like polypeptides}

ELPs are a versatile platform technology for genetically encoded drug delivery systems\textsuperscript{75}. Derived from the peptide sequence found in the hydrophobic domain of human tropoelastin (the soluble precursor to the protein elastin found in connective tissue), they consist of repeats of the pentapeptide motif Valine-Proline-Glycine-X-Glycine, where X is any amino acid other than proline\textsuperscript{75}. ELPs exhibit lower critical solution temperature (LCST) phase behavior in aqueous solvents. For a given molecular weight (MW), composition (identity of X), concentration, and co-solutes, each ELP has a distinct cloud point temperature ($T_c$), below which the ELP is soluble, and above which it reversibly aggregates into a polymer rich coacervate phase\textsuperscript{76}. The phase transition can also be triggered isothermally by increasing the concentration of salts\textsuperscript{77}. This
environmental sensitivity allows them to be purified in large quantities from *E. coli* lysates. Because their physical properties—MW and composition—can be precisely controlled at the genetic level, ELPs can be tuned to have a specific $T_t$. Increasing the hydrophobicity of the X residue, and increasing the MW (number of pentamer repeats) decreases the $T_t^{78}$. Previous work from our lab has shown that when ELPs with a transition temperature below body temperature are injected intratumorally, they form an aggregate—or depot—that retains the therapeutic within the tumor, decreasing the systemic side effects and increasing the efficacy of the treatment$^{79-81}$.

In the current project, ELPs will be implemented in two ways (1) As genetically encoded fusion partners with therapeutic proteins administered as injectable depots, and (2) as a covalently-linked carrier for the chemotherapy drug doxorubicin.
Chapter 2: Doxorubicin-conjugated polypeptide nanoparticles inhibit metastasis in two murine models of carcinoma

*Introduction*

Engineered drug delivery vehicles for cancer treatment seek to improve the clinical efficacy of chemotherapeutics by increasing the amount of drug deposited in the tumor while decreasing its accumulation in healthy tissues. This is necessary because chemotherapeutics are commonly comprised of small hydrophobic molecules that have short plasma half-lives leading to poor bioavailability after systemic administration.

Sequestration of drugs into the hydrophobic core of a soluble nanocarrier has been shown to enhance the solubility and bioavailability of the drug, improve its biodistribution by preventing rapid renal clearance due to low molecular weight, and stabilize the active form of the drug within the plasma environment. Furthermore, nanocarriers ranging between 20 and 100 nm are ideally suited to both extravasate through the leaky vasculature characteristic of rapid and uncontrolled tumor growth and accumulate within the extracellular matrix due to the enhanced permeability and retention (EPR) effect.

These attractive features of drug-loaded nanoparticles led us to develop a class of recombinant chimeric polypeptide (CP) nanoparticles for the delivery of chemotherapeutics to solid tumors. CPs are comprised of two components: (1) a hydrophilic elastin-like polypeptide (ELP) domain consisting of repeats of the
pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is any amino acid except Pro, and (2) a C-terminal C(GGC)$_7$ peptide segment that provides eight unique cysteine residues that can be used as sites for drug attachment. Conjugation of 4-6 copies of the chemotherapeutic doxorubicin (Dox) to the C-terminal drug attachment domain through an acid-labile linker results in the spontaneous self-assembly of ~40 nm diameter spherical micelles within which the drug is sequestered. We have previously demonstrated the efficacy of these CP-Dox nanoparticles in the C26 murine colon carcinoma model where ~90% of a tumor-bearing cohort was cured following a single injection$^{62}$. While the efficacy of CP-Dox against primary tumors is encouraging, the greatest clinical need is for drugs that interfere with the metastatic cascade, as metastasis accounts for the vast majority of cancer deaths$^{93}$.

Therefore, we examine herein the overall and metastasis-free survival rate for mice bearing two syngeneic metastatic tumors, mammary 4T1 carcinoma (4T1) and Lewis Lung Carcinoma (LLC) engineered to express firefly luciferase to enable in vivo tracking of metastasis. Furthermore, we used a clinically relevant treatment model in which the mice were treated with a combination of chemotherapy and surgical removal of the primary tumor, enabling us to directly correlate mortality with metastatic disease.
**Methods**

**Cell culture**

4T1-luciferase murine mammary carcinoma cells were provided by Prof. Mark Dewhirst at Duke University Medical Center (cells certified pathogen free on 6/26/13 by IMPACT Profile III). Lewis Lung carcinoma LL/2-Luc-M38 (LLC) cells were purchased from Caliper Life Sciences (certified pathogen free on 1/21/2011), after which the cells were passaged for less than 5 generations before use in animal experiments. Both cell lines were grown in DMEM supplemented with 10% FBS and cultured at 37°C in a humidified 5% CO₂ environment.

**Cytotoxicity Assays**

4T1 and LLC cells were seeded (10⁴ cells per well) in a 96-well plate and grown for 24 h, after which they were exposed to CP-Dox or free Dox (0-100 µM equivalents) for 24 h. Cell viability was determined based on their ability to reduce tetrazolium dye (MTT assay; Promega, Madison, WI). Viability was normalized to untreated controls, and the concentration required to achieve 50% inhibition of signal (IC₅₀) was calculated.

**CP-Dox Synthesis**

**Synthesis and expression of chimeric polypeptides.**

The gene encoding the CP was synthesized from custom oligomers purchased from IDT Inc. by recursive directional ligation, as described previously. The gene was cloned into a pET25b+ expression vector (Novagen, Madison, WI) and transformed into
BL21 (DE3) *E. coli* cells (EdgeBio, Gaithersburg, MD). Transformed cells were used to inoculate 50 mL flasks supplemented with 100 µg/mL ampicillin and grown overnight at 37°C and 190 rpm. Each 50 mL flask was used to inoculate six 1 L cultures of Terrific Broth (MOBIO, Carlsbad, CA) supplemented with ampicillin (100 µg/mL), which were grown overnight in a shaker incubator at 37°C and 190 rpm. Protein expression was induced 5 h following inoculation by the addition of IPTG to a final concentration of 0.5 mM. Purification of the CP was carried out by inverse transition cycling (ITC), as described previously. 

**Conjugation of Dox to the CP.**

Dox was conjugated to the CP as described previously. Briefly, Dox was activated by conjugation to n-ß-maleimidopropionic acid hydrazide (BMPH, Pierce Biotechnology, Rockford, IL) via an acid-labile hydrazone bond by stirring for 16 h in methanol supplemented with 0.1% (v/v) TFA. Separately, the purified CP was dialyzed overnight in deionized water and then reduced for 30 min in 20 mM tris carboxyethyl phosphine hydrochloride, pH 7.4 (TCEP, Pierce Biotechnology, Rockford, IL). The CP phase transition was triggered by the addition of 2.8 M NaCl, and the CP was concentrated by centrifugation (14,000 rpm for 10 min at 30°C), after which the CP pellet was re-solubilized in 100 mM phosphate buffer (pH 7, without saline). The activated Dox-BMPH conjugate dissolved in methanol was then added dropwise to the phosphate buffer and CP solution. The final ratio of methanol to PB was 2:1. After 3 h, TCEP was
added to a final concentration of 30 mM to ensure the availability of free cysteine residues for maleimide bond formation. The reaction was then left to stir overnight. The reaction solution was centrifuged using 10K MWCO Amicon centrifugal ultrafilters (Millipore, Billerica, MA) and washed with a 30% acetonitrile and 70% PBS solution for multiple cycles at 2,000 rpm for 45 min to solubilize and remove unconjugated Dox-BMPH until the sample was >98% pure by gel-filtration HPLC. Finally the buffer was exchanged with PBS with additional centrifugal ultrafiltration, and endotoxin was removed by passing the CP-Dox solution through a bed of Detoxi-gel™ resin (Pierce Biotechnology, Rockford, IL). The solution was sterilized by filtration (0.2 µm pore size, VWR, Radnor, PA) and concentrated by another centrifugal ultrafiltration step (Amicon 10K MWCO, 2000 rpm, 60 minutes). The topographical atomic force microscopic (AFM) images were collected in tapping mode using silicon nitride cantilevers (DNP-S, Bruker, 0.35 N/m nominal spring constant; 65 kHz nominal resonant frequency) with Multimode (Bruker, Santa Barbara, USA) in liquid. AFM images were obtained at a resolution of 512×512 pixels using 1 Hz scan rates.

**Animal Studies**

All animal experiments were performed in accordance with protocols approved by the Duke Institutional Animal Care and Use Committee (IACUC). BALB/c mice (Charles River, 6-10 weeks old) were inoculated with 8x10⁵ 4T1-luciferase cells in the 4th mammary fat pad. Albino BL6 mice (Charles River, 6-10 weeks old) were shaved and
inoculated subcutaneously on the flank with 1x10^6 LLC-luc cells. Mice were treated on day 8 (post-inoculation) with free Dox or CP-Dox at the maximum tolerated dose (5 mg/kg and 20 mg/kg, respectively). Mice were sacrificed if they appeared moribund or lost more than 15% of their baseline body weight, or if the tumor volumes exceeded 2000 mm^3.

**Pharmacokinetics and Biodistribution**

Mice were inoculated with 4T1 and treated with FreeDox (5mg/kg) or CPDox (20mg/kg) on Day 8 as described above. At 2, 24, 28, and 72 hours after treatment, mice were sacrificed and blood and tissue samples (tumor, liver, lung, heart, spleen, kidney and paw) were obtained, processed, and analyzed for doxorubicin content by fluorescence. Samples were homogenized and treated with acidified isopropanol to extract the doxorubicin, and the fluorescence of the doxorubicin in the supernatant was quantified using 485nm for excitation and 590nm for emission. Background fluorescence was subtracted according to calibration curves made for each organ. Drug concentration in tissues was calculated as percent of the total injected dose per gram of tissue, using calibration curves made from serial dilutions of known standards.

**Primary Tumor Regression Study (4T1)**

Mice were treated on day 8 and day 15 post-inoculation with free Dox or CP-Dox at their maximum tolerated dose (MTD, 5 mg/kg and 20 mg/kg, respectively), or an equivalent volume of PBS. Tumor dimensions (length and width) were measured every
other day, and tumor volumes were calculated using the formula Volume (mm$^3$) = (length * width$^2$/2. Groups contained 5-8 mice.

**Metastasis Inhibition Studies**

Mice were inoculated with either 4T1-luc or LLC-luc, as described above (10-12 mice per treatment group). On day 8 post-inoculation, mice were treated with free Dox (5 mg/kg) or CP-Dox (20 mg/kg). On day 15, mice were anesthetized and tumors were surgically resected. On day 22, mice were again treated with free Dox or CP-Dox at the MTD. Mice were monitored for metastasis and primary tumor recurrence 2x/week using the IVIS Xenogen bioluminescent imaging system (Caliper LS, Hopkinton, MA). Mice were sacrificed after observing a detectable metastatic signal in two consecutive imaging sessions, or if the mice became moribund (>15% weight loss or the presence of hyperventilation). The presence of metastases that were detected via bioluminescence was later confirmed by post-mortem dissection.

**Statistical Analysis**

Statistical analyses were performed using JMP Statistical Software and GraphPad Prism. Multiple groups were compared using ANOVA followed by Tukey-Kramer (Tukey’s HSD) test where applicable. Event-time plots were made using Kaplan-Meier technique and analyzed using the log-rank test. Unless otherwise noted, error bars are +/- standard error of the mean.
**Results and Discussion**

CP-Dox micelles demonstrate improved pharmacokinetics and biodistribution compared to free drug

Upon conjugation to doxorubicin, individual CP chains self-assemble into approximately 40 nm diameter micelles, as visualized by AFM, which we term CP-Dox (Figure 1a). The AFM results are consistent with previously reported dynamic light scattering (DLS) data where CP-Dox was found to have a hydrodynamic radius (Rh) of 21.5 nm\(^2\). CP-Dox maintains the ability to achieve maximal cytotoxicity when applied to 4T1 mammary carcinoma cells *in vitro*, exhibiting a slight increase in the IC\(_{50}\) relative to freely dissolved doxorubicin (0.46 µM and 2.0 µM for Free Dox and CP-Dox, respectively, Figure 1b). This effect, which is commonly observed for polymer-drug conjugates, is attributable to an impaired ability for the drug to diffuse across the cell membrane when attached to a hydrophilic carrier\(^8\).

Despite this slight decrease in potency *in vitro*, we anticipated that this nano-carrier would vastly improve the *in vivo* efficacy of the drug by increasing the maximum tolerated dose, lengthening the plasma half-life, and improving the biodistribution compared to that of the free drug. The maximum tolerated dose (MTD), as determined by the largest intravenous (IV) bolus dose that did not cause greater than 15% body weight loss, increased from 5 mg/kg for Free Dox to 20 mg/kg for CP-Dox in both BALB/c (Figure 3d) and Albino BL/6 mice (data not shown).
Figure 1: CP-Dox micelles demonstrate improved pharmacokinetics and biodistribution compared to free drug.

(a) Tapping mode atomic force microscopy (AFM) image of CP-Dox nanoparticles on mica in phosphate buffered saline (PBS). CP-Dox micelles have a diameter of approximately 40 nm. (b) In vitro cytotoxicity assay of Free Dox and CP-Dox against 4T1 mammary carcinoma. IC₅₀ values were 2.0 µM and 0.46 µM for CP-Dox and Free Dox, respectively. (c) After intravenous bolus administration of Free Dox (5mg/kg) and CP-Dox (20mg/kg) to BALB/c mice, CP-Dox extends the half-life of doxorubicin to 11.0 hours, with a corresponding clearance of 0.271 ml/hr (n=3 mice/group). Theoretical free doxorubicin concentration at time 0 is approximately 120 uM if the drug were confined to the intravascular space (see body text for details on calculation). (d) CP-dox improves the tumor accumulation compared to free doxorubicin in an orthotopic 4T1 mammary carcinoma model (n=3 mice/group).

We next examined the pharmacokinetics and biodistribution of doxorubicin, where free Dox and CP-Dox were injected at their respective MTDs. As shown in Figure
1c, the plasma level of free doxorubicin was essentially undetectable as early as 2 hours post-injection. The theoretical max concentration at time zero, if the drug were confined to the intravascular space and assuming a dose of 5 mg/kg, mouse body weight of 20 g, and a blood volume of 1.5 ml, is approximately 120 uM. The fact that it was undetectable at 2 hours is consistent with the short half-life of doxorubicin (~5 min) in mice and its large volume of distribution. In contrast, conjugating doxorubicin to the CP greatly extended the plasma residence time of Dox as well as increased the concentration within the plasma compartment, with low but detectable levels remaining even 72 hours following treatment. The elimination half-life of doxorubicin, when delivered as CP-Dox, is increased to 11.0 hours, and the plasma clearance is 0.271 ml/hr.

The intratumoral accumulation of Free Dox was poor, with less than 1% of the injected dose per gram of tumor tissue (%ID/g) detectable in the tumor at 2 hours, and undetectable drug levels at 24 hours (Figure 1d). On the other hand, CP-Dox displayed 5% ID/g of tumor tissue 24 hours post-injection. Remarkably, CP-Dox treated tumors retained significantly more doxorubicin after 72 hours than the free Dox treated tumors retained only 2 hours following administration (p<0.05, t-test).

On the basis of the %ID/g, the liver, kidney and paw were targeted to a greater extent by CP-Dox than Free Dox (Figure 2a, e, f). We examined the paw because of the propensity for doxorubicin nanoparticles to cause hand-foot syndrome, wherein the drugs leaks into the capillaries of the hands and feet, causing pain and redness. The
low level of the drug (<1% ID/gram) in this tissue is therefore reassuring. Concentrations of Dox in the lungs, heart, and spleen were similar for CP-Dox and free doxorubicin (Figure 2b-d).

![Figure 2: Biodistribution of CP-Dox and Free Dox in BALB/c mice.](image)

Mice were injected with CP-Dox (20mg/kg) or Free Dox (5mg/kg), tissues were harvested at times indicated by data points, and doxorubicin was quantified based on its fluorescence. Data is normalized to % of injected dose per gram (n=3 mice/group). (a) Liver, (b) Lung, (c) Heart, (d) Spleen, (e) Kidney, (f) Paw.

**CP-Dox prolongs survival and delays metastasis in an orthotopic murine mammary carcinoma model**

To investigate the effect of the improved pharmacokinetics and biodistribution on therapeutic efficacy, BALB/c mice were inoculated with 8x10⁵ tumor cells in the 4th mammary fat pad, and were then treated with CP-Dox or free Dox at their respective maximum tolerated doses on days 8 and 15 post-inoculation. 4T1-luc is a highly metastatic murine mammary tumor that metastasizes to the lungs, liver, and brain⁹⁷, and
responds poorly to free Dox treatment in vivo\textsuperscript{98, 99}. Mammary fat pad inoculation was used because orthotopic tumors are known to more aggressively metastasize and be more refractory to chemotherapy than s.c. tumors\textsuperscript{100, 101}. For these reasons, the 4T1 model presents many of the challenges faced in the clinic by chemotherapeutics.

Figure 3a illustrates that CP-Dox significantly inhibited primary tumor growth (p<0.05, ANOVA). Furthermore, treatment delayed the development of metastasis (Figure 3b), which resulted in improved overall survival of the mice when compared to a no-treatment control (PBS) or free Dox (Figure 3c, p<0.05, log-rank). Mouse body weights were followed as a surrogate marker for the toxicity of the drug treatments. This treatment schedule (2 treatments separated by 1 week) was well tolerated, with no mice losing more than 15% of their original body weight, as shown in Figure 3d.
Figure 3: CP-Dox prolongs survival and delays metastasis in an orthotopic murine mammary carcinoma model.

4T1 cells were implanted on day 0 and mice were treated with CP-Dox, Free Dox, or a vehicle control on day 8 and 15. (a) Primary tumor volume was significantly lower in CP-Dox compared to Free Dox and PBS groups (p<0.05, ANOVA). (b) Metastasis-free survival, and (c) Overall survival were significantly improved by CP-Dox treatment (p<0.05, log-rank). (d) Percent of initial body weight as a function of days post tumor inoculation. Note: Error bars in (a) and (d) are 95% confidence interval, n = 5-8/group.

The black arrows (labeled Drug Tx in the legend) show the days on which the animals were treated with either PBS, Free Dox or CP-Dox nanoparticles.
Two treatments demonstrated markedly reduced metastasis when compared to a single dose (8 days post-inoculation; Figure 4a-b). No further improvement in primary tumor or metastasis inhibition was observed with three doses (Figure 4c-d).

Figure 4: The effect of dosing schedules on the in vivo efficacy of CP-Dox against 4T1 mammary carcinomas. (a) Primary tumor growth with single dosing on day 8. (b) Metastasis-free survival for mice that were treated on day 8 post-inoculation. (c) Primary tumor growth and (d) metastasis-free survival for mice that were treated with PBS x3, Free Dox x3, CP-Dox x1 or x3. Black arrows denote days of drug treatment. Error Bars are 95% confidence intervals. (n=10 mice/group)
**CP-Dox enhances survival in two metastatic murine tumor models when combined with primary tumor resection**

We next instituted a treatment regimen that consisted of chemotherapy a week before surgery (neoadjuvant; day 8), followed by surgical resection of the primary tumor (day 15) and chemotherapy a week after surgery (adjuvant; day 22). We included surgical resection because it allowed us to isolate the effect of the primary tumor burden from metastatic disease in causing mortality. This treatment regimen also allowed us to test CP-Dox in a more clinically relevant scenario, which is significant to its translation to the clinic.

We found that surgical removal of the 4T1 mammary carcinoma was successful in achieving local control of disease, as no mice developed local recurrence in either treatment group. Furthermore, as seen in Figure 5a, the combination of CP-Dox and surgery greatly improved the therapeutic outcome over free Dox with surgery (p<0.05, log-rank) and stand-alone CP-Dox treatment without surgery (Figure 3b). In fact, over half of the CP-Dox treated mice survived to the end of the 80 day study, which in our experience is a sufficient length of time to ensure a long-term cure for this tumor model. For the free Dox cohort, only 20% of the mice achieved a long-term cure. Figure 5c shows a bioluminescent image from day 35, at which point the orthotopic 4T1 primary tumor has been surgically removed. The mouse treated with free Dox (middle) has developed a detectable lung metastasis, while the mice treated with CP-Dox (left and right), remain metastasis free.
Figure 5: Neoadjuvant and adjuvant treatment with CP-Dox significantly prolongs metastasis-free and overall survival in two metastatic murine of carcinoma in conjunction with surgery.

(a) 4T1 and (b) LLC survival is increased in CP-Dox treated mice over Free Dox (p<0.05, log-rank). (c) In vivo luciferase imaging of BALB/c mice bearing orthotopic 4T1-luc tumors. A free Dox treated mouse (middle) shows lung metastases, while the CP-Dox treated mice (left and right) are metastasis free at day 35. Note: n = 10-12/group. Black arrows = Drug Treatment (Free Dox or CP-Dox). Grey arrows = primary tumor resection.

To further demonstrate the ability of CP-Dox to inhibit metastasis, we used the same treatment regimen for subcutaneously implanted Lewis Lung carcinoma
engineered to express luciferase. Similar to 4T1, it is a syngeneic cell line that is widely used to study metastasis in immune-competent mice, and it responds poorly to free Dox \textit{in vivo}, despite exhibiting sensitivity to the drug \textit{in vitro} (Figure 6)\textsuperscript{102,103}. For this model, 1x10\textsuperscript{6} cells were inoculated into the flank of BL6 Albino mice on day 0, followed by CP-Dox or free Dox neoadjuvant treatment on day 8, primary tumor resection on day 15, and adjuvant chemotherapy on day 22. The LLC cells were more locally invasive than the 4T1 cells, leading to a significant number of mice exhibiting local primary tumor recurrence. The incidence of this primary tumor recurrence was, however, highly asymmetric, with only 7\% of the CP-Dox cohort experiencing tumor recurrence compared to 46\% of the free Dox cohort (p<0.05, Fisher’s Exact), despite identical surgical techniques for both groups. Metastasis-free survival and overall survival were significantly prolonged in the CP-Dox group (Figure 5b; p<0.05, log-rank).

**Figure 6: Cytotoxicity of CP-Dox nanoparticles against Lewis lung carcinoma.** The IC-50 values for CP-Dox and Free Dox are 0.082 uM and 1.7 uM, respectively.
**CP-Dox inhibits metastasis by delaying dissemination of viable cells from the primary tumor**

Our results clearly demonstrate the ability of CP-Dox to inhibit metastasis in two cell lines, so we next explored the question of which step of the metastatic process was interrupted by our treatment. The metastatic cascade involves multiple steps, including invasion from the primary tumor into the blood stream, travel through the circulatory system, arrest in a distant organ, and finally growth into a metastasis. To elucidate the timing of these steps in the 4T1 model, we removed primary tumors on Days 8, 10, 12, or 15 and then observed the metastasis-free survival of mice. As shown in Figure 7A, only about 20% of the mice develop metastases if the primary tumor is removed on Day 8 or earlier, whereas all mice that received surgery on Day 12 or Day 15 succumbed to metastasis. This means that for the majority of mice, cells with the potential to form metastases spread from the primary tumor between Day 8 and Day 12.

This experimental approach was then applied to LLC, with resection limited to Days 8 and 15. In LLC, all mice survive resection on Day 8, whereas approximately two-thirds of mice eventually succumb to metastasis if tumors resection is delayed to Day 15 (Figure 7B).
Figure 7: Surgery-only controls to determine time of metastatic cell dissemination. (A) Effect of surgical resection day on survival after inoculation with 4T1, demonstrating that in untreated mice, metastasis forming cells spread from the primary tumor between Day 8 and Day 12 for the majority of mice. (B) Effect of surgical resection day on survival after inoculation with LLC. In this model, approximately 60% of mice develop metastases if tumors are resected on Day 15, whereas all mice survive resection on Day 8.

For both tumor models, these results mean that on Day 8, the day of drug treatment in the experiments described earlier, the majority of mice have not yet developed viable micrometastases. However, most mice have developed disseminated disease by Day 15, the day of surgical removal, if no other treatments are applied. The fact that CP-Dox improves survival in this setting suggests that it more effectively delays the dissemination of cells from the primary tumor for at least another week so that surgery on Day 15 may be curative.

**Efficacy of CP-Dox in surgical models is fully attributable to the neoadjuvant treatment**

As most mice do not contain viable micrometastases on the day of Dox treatment (Day 8), I hypothesized that the survival benefit of CP-Dox stems largely from the
neoadjuvant treatment preventing the dissemination of viable cells from the primary
tumor rather than by exerting a direct cytotoxic effect on micrometastases. To test this
hypothesis, I repeated the 4T1 chemotherapy and resection experiments described
above, but included a cohort that only received the neoadjuvant treatment (CP-Dox
Neo). As shown in Figure 8A, withholding adjuvant treatment had no discernible effect
on survival with these sample sizes (p>0.05, log-rank, n=7 in each group). Furthermore,
in a small pilot study, adjuvant CP-Dox therapy on Day 19 after surgery on Day 12
(when all mice have developed micrometastases) conferred no survival advantage
compared to Free Dox or PBS (data not shown).

Finally, I tested multiple neoadjuvant treatments to determine if CP-Dox could
continue to delay metastasis if it is administered weekly. In this experiment,
neoadjuvant treatments were administered weekly starting on Day 8 once (CP-Dox Neo
x1), twice (CP-Dox Neo x2) or three times (CP-Dox Neo x3), with surgery one week after
the final treatment for each group. At this sample size (5-6 mice per group), there were
no statistically significant differences among the groups (Figure 8B, p>0.05, log-rank).
The highest rate of survival was achieved in the group with two neoadjuvant
treatments, but only 40% of mice survived in the CP-Dox Neo x3 group. While teasing
out the true trends in survival would require larger numbers of mice, some conclusions
relevant to the mechanism of metastasis inhibition of CP-Dox can still be drawn from
this experiment. If CP-Dox were capable of treating disseminated cells directly, one may
hypothesize that if mice were to succumb to metastasis, they would die at later time points if they received multiple neoadjuvant treatments, or at least later than mice that only received PBS before surgical removal. However, as shown in Figure 8B, in comparison to the surgery-only controls in black (reproduced from Figure 5A), if a mouse is going to die of metastasis, it generally happens within a relatively small window from Days 36-50, regardless of the treatment group. This suggests that the survival of the mice is mainly a function of the number of disseminated cells released from the primary tumor before surgical removal. This result is perhaps not surprising since CP-Dox was designed to take advantage of leaky vasculature and improve accumulation in the primary tumor. Micrometastases, the putative target of adjuvant treatment, may be relatively avascular and therefore difficult to target with macromolecular carriers\(^{105, 106}\).

Figure 8: Role of Neoadjuvant and Adjuvant CP-Dox treatments in 4T1 surgical model.

(A) Effect of adjuvant treatment with CP-Dox on metastasis free survival, demonstrating that the adjuvant CP-Dox treatment confers no survival benefit beyond that seen for neo-adjuvant CP-Dox (p>0.05, log-rank). The black arrow represents the Neoadjuvant treatment received by both cohorts of mice. The grey arrow denotes the day of surgery, while the blue arrow represents the Adjuvant treatment, only received by the CP-Dox Neo+Adj group. (B) Shown in blue is the
survival of mice after 1, 2, or 3 neoadjuvant treatments of CP-Dox, administered weekly starting on Day 8, with surgery one week after final treatment (Surgery day noted in parentheses of legend for each group). No significant differences were observed among groups with this sample size (5-6 mice/group). Shown in black is PBS treated mice with the day of surgical resection included in parentheses, demonstrating that if a mouse succumbs to metastasis, this takes place between days 35 and 50 for most mice, regardless of treatment group.

**Conclusion**

We have shown that CP-Dox inhibits metastasis and improves the survival of mice in two syngeneic metastatic cell lines. Our study is particularly relevant to clinical translation because 90% of cancer deaths in patients are caused by metastasis rather than the growth of the primary tumor. Our treatment approach of chemotherapy and surgical tumor resection expands the scope of a typical drug delivery study that only follows primary tumor growth. This focus on primary tumor growth by the field of drug delivery may be a contributing factor to the relatively slow adoption of new delivery systems in the clinic. Indeed, our drug delivery vehicle succeeded in enhancing doxorubicin’s accumulation in the primary tumor, and while our results demonstrate a strong inhibition of metastasis, our data suggest that the effect arises from improved control of the dissemination of invasive cells from the primary tumor (Figure 9). Nonetheless, the ability to prevent metastasis by controlling the primary tumor is clinically important in the setting of neoadjuvant treatments administered to downstage a tumor and enable surgery. During the treatment, it is critical that the cells are not
able to spread from the primary tumor. Our results suggest that our drug formulation would be a more effective neoadjuvant agent compared to freely dissolved drug.

Figure 9: Doxorubicin-conjugated polypeptide nanoparticles (CP-Dox) inhibit metastasis by preventing the escape of viable cancer cells from the primary tumor to a greater extent than freely dissolved doxorubicin (Free Dox).

This suggests that another approach must be taken in order to use CP-Dox to treat existing metastases. If the improved treatment of the primary tumor can be exploited to generate a systemic immune response, CP-Dox may still hold the potential to treat metastatic disease\textsuperscript{74}. The following chapter will explore how CP-Dox affects the host antitumoral immune response.
Chapter 3: Nanoparticle formulation improves doxorubicin efficacy by enhancing host antitumor immunity

Introduction

Cytotoxic chemotherapy is a mainstay of cancer treatment. This class of drugs was historically thought to be antagonistic to the immune response due to bone marrow suppression and subsequent reduction in leukocyte count, but a mounting body of evidence has shown that some cytotoxic chemotherapeutics can help stimulate an antitumor immune response involving both the innate and adaptive arms of the immune system. Many of the mechanisms underlying this phenomenon have been elucidated in recent years. By simply reducing the number of cancerous cells, cytotoxic chemotherapy can interfere with tumor-derived immunosuppressive signaling and create an environment conducive to more effective immunological responses. Cytotoxic drugs can also exert immunomodulatory effects directly on leukocyte subsets. For example, sub-lethal doses of doxorubicin can polarize myeloid cells towards a more inflammatory phenotype and stimulate antigen presentation. Some cytotoxic drugs, including doxorubicin, induce immunogenic cell death, whereby inflammatory signals are released or displayed by dying tumor cells. Immunogenic cell death can lead to a cell-mediated immune response and long lasting immune memory which protects against re-challenge. This work has changed the way that chemotherapy is
viewed and generated excitement for combinations of chemotherapy and immunotherapy\textsuperscript{111,112}.

In parallel to these advances in understanding the immunogenic properties of chemotherapy, there have been substantial improvements in the delivery of chemotherapy with the development and application of nanoparticle technologies\textsuperscript{28}. Tumors have leaky vasculature and a relative lack of lymphatics, so nanoparticles of the appropriate size (generally 10-100nm) passively accumulate in the tumor, a phenomenon known as the enhanced permeability and retention (EPR) effect\textsuperscript{90}. Nanoparticle delivery also reduces side effects by limiting the drug’s extravasation into off-target organs, thereby permitting the administration of larger doses\textsuperscript{27,113}. Despite advances in delivery approaches, it remains unclear if repackaging alters the aforementioned immunomodulatory effects of cytotoxic chemotherapy. Nanoparticle delivery changes many attributes of a drug, including its pharmacokinetics, biodistribution, intratumoral distribution, and mechanism of cell uptake\textsuperscript{114}. Furthermore, it is becoming increasingly appreciated that nanoparticles are logical delivery vehicles for immunomodulatory agents due to their preferential uptake by immune cell subsets\textsuperscript{66}. As nanoparticle delivery approaches gain widespread clinical adoption and are inevitably used in combination with immunotherapeutic strategies, it
is critical to understand how the immunomodulatory effects of chemotherapy are changed or potentially enhanced by a nanoparticle formulation.

Our lab has developed a nanoparticle delivery system for doxorubicin, named chimeric polypeptide-doxorubicin (CP-Dox)\textsuperscript{115}. The CP consists of a hydrophilic elastin-like polypeptide and a conjugation domain containing eight cysteines separated by a diglycine spacer. Doxorubicin is conjugated to n-ß-maleimidopropionic acid (BMPH) via an acid labile hydrazone bond, and then attached to the cysteine-rich domain of the CP via a maleimide bond. The relative hydrophobicity of doxorubicin triggers self-assembly into \textasciitilde40nm micelles\textsuperscript{115}.

CP-Dox has significantly more treatment efficacy than free doxorubicin. We have previously shown that CP-Dox can cure 90% of mice bearing C26 colon carcinoma as a monotherapy\textsuperscript{115}, and 60% of mice bearing 4T1 mammary carcinoma or Lewis Lung carcinoma in combination with primary tumor resection\textsuperscript{116}. The large improvement in efficacy of the nanoparticle formulation compared to free drug in these studies, in association with reports of immunogenic cell death by doxorubicin, led us to speculate that improved direct tumor cytotoxicity was not the sole reason for the increased efficacy of CP-Dox nanoparticles, and that an anti-tumor immune response may play a role. In the present study, we examine the role of the host anti-tumor immune response
in the efficacy of CP-Dox against the poorly immunogenic 4T1 mammary carcinoma. By comparing to freely dissolved doxorubicin (Free Dox), we determined whether repackaging a drug can enhance its immunomodulatory effects.

**Materials and Methods**

**CP-Dox Synthesis**

CP-Dox was synthesized as described in the previous chapter, with minor modifications. Briefly, the gene encoding the CP was inserted into a pET25b+ expression vector (Novagen, Madison, WI) and transformed into BL21(DE3) E. coli cells (EdgeBio, Gaithersburg, MD). Transformed cells were grown in Terrific Broth (MOBIO, Carlsbad, CA) supplemented with ampicillin (100 μg/mL), and protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Purification of the CP was carried out by inverse transition cycling (ITC), as described previously. Dox was activated by conjugation to n-ß-maleimidopropionic acid hydrazide (BMPH, Pierce Biotechnology, Rockford, IL), then combined with the purified and reduced CP. After overnight incubation, the reaction solution was centrifuged for multiple cycles at 3,000 x g for 45 min using 10K MWCO Amicon centrifugal ultrafilters (Millipore, Billerica, MA) and washed with a 30% acetonitrile and 70% PBS solution until unconjugated Dox-BMPH was removed and the sample was >98% pure by size exclusion HPLC. The buffer was exchanged with PBS with additional rounds of centrifugal ultrafiltration. The solution was sterilized and endotoxin was removed by passing the CP-Dox solution
through an Acrodisc Filter with a Mustang E Membrane (Pall, Port Washington, NY), then concentrated by another centrifugal ultrafiltration step (Amicon 10K MWCO, 3,000xg, 60 minutes). Endotoxin content in CP-Dox was found to be less than 0.1 EU/ml by the Limulus Amebocyte Lysate Assay (Lonza, Walkersville, MD).

**Cell Culture**

4T1-luciferase murine mammary carcinoma cells (4T1-luc) were provided by Prof. Mark Dewhirst at Duke University Medical Center. Lewis Lung carcinoma LL/2-Luc-M38 (LLC) cells were purchased from Caliper Life Sciences. CT26 cell line was provided by Duke Cancer Center Facility from ATCC (certified mycoplasma free). None of these cell lines are listed in the Database of Cross-Contaminated or Misidentified Cell Lines. Cells were passaged for less than 5 generations before use in animal experiments. All cell lines were tested and found to be free of mycoplasma. 4T1-luc and LLC-luc were grown in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS, 1mM sodium pyruvate, and 4mM glutamine. CT26 was grown in RPMI (Sigma-Aldrich) supplemented with 10% FBS, 1mM sodium pyruvate, 10mM HEPES, and 4.5g/L glucose. All cell lines were cultured at 37°C in a humidified 5% CO₂ environment.

**Animal Studies**

All animal experiments were performed in accordance with protocols approved by the Duke Institutional Animal Care and Use Committee (IACUC). BALB/c mice (Charles River, Female, 6-10 weeks old) were inoculated with 8x10⁵ 4T1-luciferase cells
in the 4th mammary fat pad, or shaved and inoculated subcutaneously with $2.5 \times 10^5$ CT26 cells on the flank. Albino BL6 mice (Charles River, Female, 6-10 weeks old) were shaved and inoculated subcutaneously on the flank with $1 \times 10^6$ LLC-luc cells. For all inoculations, cells were suspended in serum-free DMEM at a concentration appropriate for a 50 µl injection. Unless otherwise noted, mice were treated on day 8 (post-inoculation) with free Dox or CP-Dox at the maximum tolerated dose (5 mg/kg and 20 mg/kg, respectively). In metastasis prevention studies, tumors were surgically resected on day 15. Mice were sacrificed if they appeared moribund or lost more than 15% of their baseline body weight, or if the tumor volumes exceeded 2000 mm$^3$. Tumor volumes were calculated using the formula \[ \text{Volume (mm}^3) = \text{length} \times \text{width}^2 / 2. \] Samples sizes of 5-7 were used for tumor growth curves, which was sufficient to detect tumor volume differences of ~50%, and for survival curves. Sample sizes of 3-5 were sufficient for flow cytometry to detect differences among treatment groups. Mice were randomized to treatment groups using the list randomizer from random.org. Experimenters were blinded during tumor measurement but not during analysis. For CBC analysis, 100 µl of blood was drawn from mice into an EDTA coated tube (Sarstedt, Newton, NC). Samples were run on an Idexx Procyte (Idexx Operation, Inc., Memphis, TN).

**Depletion studies**

Mice were administered depleting antibodies or appropriate isotype control antibodies intraperitoneally (IP) for each experiment as follows: CD8 Depletion: 250 µg
of anti-CD8a clone 2.43 (BioXCell, Lebanon, NH), starting day 6 and weekly thereafter. CD4 Depletion: 250 µg of anti-CD4 clone GK1.5 (BioXCell), starting day 6 then weekly thereafter. BALB/c NK cell depletion: 20 µl of anti-asialo GM1 rabbit serum (Wako Chemicals, Richmond, VA) or control rabbit serum, starting day 6, repeated every 4 days for a total of 4 injections. BL/6 NK cell depletion: 200 µg of anti-NK1.1 clone PK-136 (BioXCell) starting day 6, repeated every 4 days for a total of 4 injections. IFN-γ depletion: 100 µg of anti-IFN-γ clone R4-6A2 (BioXCell), on days 7, 9, 15 and 21. After repeated antibody injections, some mice developed a fatal anaphylactic reaction, which correlated with tumor burden. These mice were censored from survival curves since they did not meet the experimental endpoint, and antibody treatments were discontinued for the remaining mice. When more than half of the mice in a treatment group died or were sacrificed, the group was censored from the tumor regression curves to avoid skewing the mean.

**Flow Cytometry**

Tumors were mechanically dissociated and then enzymatically degraded for 60 min at 37°C in HBSS buffer containing 5 mg/ml Collagenase Type I Gibco, Grand Island, NY) and 0.2 mg/ml DNAase I (Roche, Indianapolis, IN) supplemented with 5% FBS. The solution was diluted in PBS and passed through 70 µm strainers. Cells were then pelleted by centrifugation and resuspended in ACK red cell lysis buffer (Quality Biological, Gaithersburg, MD) for 2 min, after which the solution was diluted with PBS.
Cells were pelleted and counted by Trypan blue exclusion. One million cells were used for antibody staining. LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Grand Island, NY) or Zombie Live/Dead Aqua stain (Biolegend, San Diego, CA) was applied for 30 minutes. Cells were then blocked (5% rat serum, 5% mouse serum, 1% CD16/32 (clone 93, eBioscience, San Diego, CA)) in FACS buffer (PBS with 3% FBS and 30 uM EDTA) for 30 min. Cells were then stained antibodies for 30 min, washed 2x with PBS, and then fixed with 0.4% paraformaldehyde in PBS. The following antibodies were used: PE/Cy7 anti-CD3e (clone 145-2C11; BD Biosciences, San Jose, CA); FITC anti-CD8a (clone 53-6.7; BD Biosciences); APC anti-CD4 (clone RM4-5; BD Biosciences); PE anti-CD80 (clone 16-10A1; eBioscience; San Diego, CA) or PE Armenian hamster IgG isotype (eBio299Arm; eBioscience); PE-Cy5.5 anti-CD11c (clone 35-0114; eBioscience); PE-Cy7 anti-F4/80 (clone BM8; eBioscience); APC anti-CD206 (C068C2; Biolegend) or APC rat IgG2a isotype (clone RTK2758; Biolegend); AF700 anti-Ly6G (clone RB6-8C5; eBioscience); APC-Cy7 anti-CD11b (clone M1/70; eBioscience); V450 anti-Ly6C (clone HK1.4; eBioscience); Qdot605 anti-CD45 (clone 30-F11; eBioscience); Qdot655 anti-IA/IE (clone M5/114.15.2; eBioscience); PE anti-H-2Kd (clone SF1-1.1; Biolegend); PE anti-H-2kb (clone AF6-88.5; Biolegend). Cells were then run on a BD LSRII flow cytometer in the Duke DHVI Research Flow Cytometry Facility or a BC FACSCanto in the Duke Cancer Center Flow Cytometry Facility. Compensation and voltage settings were
determined by samples stained with only one antibody. Data was analyzed in FlowJo Flow Cytometry Analysis software (Tree Star, Ashland, OR).

**Cytokine and Chemokine Analysis**

Tumors were homogenized in lysis buffer (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20, 20-201 protease inhibitor cocktail (Roche, Indianapolis, IN)) and analyzed for protein content with a BCA assay (ThermoFisher, Waltham, MA). Samples were diluted to 1 mg/ml. 20 µl of blood was drawn into EDTA tubes for plasma analysis. Cytokine and chemokine analysis was performed on tumor and plasma samples using a Milliplex Kit (EMD Millipore, Billerica, MA) according to the manufacturer’s instructions. One outlier was removed for CP-Dox tumor samples for IL-6 level and Free Dox for IL-4 level (p<0.05, Grubbs). One outlier mouse was removed from CP-Dox plasma chemokine analysis due to hemolysis. Overall data trends and conclusions drawn were unaffected.

**Statistical Analysis**

Statistical analyses were performed using Prism 6 (GraphPad Software). Tumor growth curves and grouped bar graphs were analyzed by two-way ANOVA or one-way ANOVA where applicable, followed by Tukey-Kramer (Tukey’s) when global tests achieved significance. Event-time plots were made using Kaplan-Meier technique and analyzed using the log-rank test or for fraction of long-term survival achieved by
Fischer’s Exact Test. Error bars are +/- standard error of the mean. * indicates p<0.05, which was used as the cutoff for statistical significance.

**Results**

**The immune system contributes to the efficacy of CP-Dox**

Although cytotoxic chemotherapies are classically thought to function via direct cell killing, the host anti-tumor immune response has repeatedly proven to be a crucial factor in the full efficacy of certain chemotherapeutics, including anthracyclines. We hypothesized that the improved delivery of doxorubicin by CP-Dox would enhance the stimulation of a host anti-tumor immune response. To test this hypothesis, we inoculated BALB/c mice with CT26 subcutaneously. Eight days later, mice were treated with CP-Dox or freely dissolved doxorubicin (Free Dox) at their maximum tolerated dose (20 mg/kg and 5 mg/kg, respectively) or vehicle control (PBS). As shown in Figure 10A-B, Free Dox and CP-Dox were both effective in delaying primary tumor growth and prolonging survival compared to PBS treated mice. Unlike Free Dox, however, CP-Dox achieved long-term cure in 2 out of 5 mice. Three weeks after tumors were last palpable, these mice were re-challenged with CT26 on the opposite flank. As seen in Figure 10C, these mice were resistant to re-challenge, suggesting that the mice had developed a protective memory immune response.

CT26, similar to many other cell lines often used for studying the immune response’s role in chemotherapy, was derived from a chemically-induced tumor.
Chemically induced cell lines harbor a greater number of neoantigens on average and therefore are potentially more immunogenic than spontaneous tumors. We hypothesized that nanoparticle delivery may reveal the immunomodulatory properties of doxorubicin in more challenging models. We chose 4T1 mammary carcinoma, a poorly immunogenic cell line widely used for studying metastasis. To test our hypothesis, we compared the drug’s efficacy in BALB/c nu/nu mice, which lack functional T cells, to its efficacy in immunocompetent BALB/c mice. On day 0, mice were injected orthotopically with 4T1-luc, and then treated intravenously (IV) on day 8 with CP-Dox or Free Dox (at the maximum tolerated doses, 20 mg/kg and 5 mg/kg, respectively) or vehicle control (PBS). In both strains of mice, CP-Dox was more effective at reducing primary tumor growth than Free Dox or PBS treatment (Figure 10D, p<0.05 ANOVA, Tukey’s), whereas Free Dox had no significant effect compared to PBS. Consistent with our hypothesis, the efficacy of CP-Dox was greater in magnitude and the duration of the response to treatment was longer lasting in immunocompetent BALB/c mice. A global 2-way ANOVA test for interaction between mouse strain and treatment on Day 24 onward was significant (p<0.05), permitting comparisons between strains and within treatment groups. This analysis demonstrated a significant difference between strains only for the CP-Dox treated mice (p<0.05, Tukey’s). Overall survival is shown in Figure 10E, where CP-Dox significantly improves survival in both strains compared to Free Dox and CP-Dox, but only in the CP-Dox group is survival improved.
between the two strains (p<0.05, log-rank). These results suggest that CP-Dox efficacy is associated with the presence of T cells, as indicated by the increased survival benefit in immunocompetent mice. Based on this data we decided to further investigate changes in immune cells after treatment with CP-Dox.

Figure 10: The immune system contributes to the efficacy of CP-Dox. Immunocompetent BALB/c mice were inoculated with CT26 subcutaneously, then treated with CP-Dox (20 mg/kg), Free Dox (5 mg/kg) or PBS 8 days later. (A) Primary tumor volume, with complete responders (CR) noted and (B) Survival (n=5 CP-Dox, 5 Free Dox, 3 PBS). (C) Primary tumor volume of mice previously cured after CP-Dox treatment and naïve controls after inoculation with CT26. The number of mice resistant to tumor growth are indicated in parentheses in the figure legend (n=2 CP-Dox, 4 Naïve). (D-E) Immunocompetent BALB/c mice (dashed lines) or Nude (BALB/c (nu/nu), solid lines) mice lacking functional T cells were inoculated with 4T1 mammary carcinoma in the mammary fat pad. (D) Primary tumor volume and (E) Survival (n=7 CP-Dox, 7 Free Dox, 6 PBS for Nude and BALB/c groups in D,E). Tumor growth curves analyzed by two-way ANOVA and Tukey’s post hoc, survival by log-rank. *p<0.05
CP-Dox normalizes the white blood cell count of 4T1 mammary carcinoma-bearing mice

Tumor-bearing mice experience widespread immune dysfunction, due in part to tumor-derived signaling, which subverts the host anti-tumor immune response 42,120. In many tumor models, including 4T1, this includes the mobilization of poorly functional or even immunosuppressive myeloid cells from the bone marrow 121, 122. Consistent with these findings, we observed splenomegaly in mice bearing 4T1. To test the effect of CP-Dox treatment and 4T1 tumor burden on peripheral blood cell counts, immunocompetent BALB/c mice were inoculated orthotopically with 4T1-luc mammary carcinoma and then treated on day 8 IV with Free Dox, CP-Dox or vehicle control (PBS). Tumor-free mice were also treated with the same doses of Free Dox or CP-Dox to serve as controls to determine the effects of drug treatment alone. On day 7 post-treatment (day 15 post-inoculation), blood was collected and complete blood count (CBC) was performed. Mean red blood cell count (RBC), hemoglobin level (HGB), and platelet counts (PLT) were within normal limits for all groups regardless of tumor presence or drug treatment, although tumor-bearing mice treated with CP-Dox were near the lower limit for platelet count. (Figure 11A-C). Monocyte count was increased by 4T1 tumor, but unaffected by drug treatment (Figure 11D), while eosinophil and basophil count were within normal range for all mice regardless of tumor presence or drug treatment (Figure 11E-F).
Figure 11: CP-Dox does not induce widespread hematopoietic dysfunction. Effect of tumor presence and drug treatment on (A) Red blood cell count, (B) Hemoglobin, and (C) Platelet count. Mean values were within normal limits for all groups, although tumor-bearing mice treated with CP-Dox were near the lower limit for platelet count. (D) Monocyte count, which tumor presence increased, but was unaffected by drug treatment. (E) Eosinophil count, which was increased by tumor presence, and moderately decreased by CP-Dox treatment. (F) Basophil count, which was unaffected by tumor presence and drug treatment. Note: Lines indicate mean (solid) and 95% confidence interval limits (dashed) reported by Charles River for female BALB/c mice.

As seen in Figure 12A, CP-Dox treatment decreases the white blood cell count (WBC) of mice, consistent with the known myelosuppressive effects of doxorubicin\textsuperscript{123}. Nonetheless, the mean was within the reported reference range for BALB/c mice. 4T1 tumor burden causes a dramatic increase in the white blood cell count (WBC) of mice compared to non-tumor bearing controls regardless of treatment (p<0.05, Two-way ANOVA). Within tumor bearing mice, CP-Dox (and to a lesser extent, Free Dox) caused a dramatic decrease in the WBC, moving it into the normal range. Interestingly, WBC
was correlated with tumor burden (Figure 12A, bottom, R²=0.61). Neutrophil count (NEUT) was unaffected by drug treatment in tumor-free mice (Figure 12B, top). The presence of 4T1 tumor induced a large increase in neutrophil count (p<0.05, Two-way ANOVA), consistent with our observation of an expansion in CD11b+/Ly6G+ cells in the spleen and blood (data not shown), a subset of cells consistently associated with immunosuppression in the context of cancer. Importantly, CP-Dox treatment significantly decreased the neutrophil count to near the normal range seen in tumor-free mice (p<0.05, Tukey’s). Neutrophil count was similarly correlated with tumor burden (Figure 12B, bottom, R²=0.69). Lymphocyte count was slightly decreased by CP-Dox treatment, but remained within normal limits, and the presence of 4T1 tumor had no overall effect (p>0.05, two-way ANOVA, Figure 12C, top). In contrast to WBC and NEUT, lymphocyte count was only slightly correlated with tumor burden (Figure 12C, bottom, R R²=0.31).
Figure 12: CP-Dox normalizes the white blood cell count of 4T1-bearing mice. Mice were inoculated orthotopically with 4T1 mammary carcinoma and treated 8 days post-inoculation IV with the maximum tolerated dose of Free Dox or CP-Dox (5 mg/kg and 20 mg/kg, respectively) or vehicle control (PBS). On day 15 post-inoculation, blood was collected and CBC or cytokine analysis was performed on tumor-bearing and non-tumor bearing mice (which had also undergone the same drug treatment 7 days prior) to quantify and compare (A) white blood cell count (WBC), (B) neutrophil count (NEUT), (C) lymphocyte count (LYMPH) and (D) granulocyte-colony
stimulating factor (G-CSF). Note: Lines indicate mean (solid) and 95% confidence interval limits (dashed) reported by Charles River for female BALB/c mice.

These results clearly demonstrate that 4T1 tumors affect hematopoiesis, especially in stimulating neutrophil production; this led us to hypothesize that the hematopoietic cytokine granulocyte-colony stimulating factor (G-CSF) may be increased in 4T1-bearing mice. Indeed, as shown in Figure 12D, G-CSF plasma levels increased more than 10-fold in both Free Dox and PBS treated mice bearing 4T1 carcinoma (Figure 12D, top). This increase in G-CSF levels was significantly mitigated by CP-Dox treatment, whereas CP-Dox treatment did not significantly lower G-CSF in non-tumor bearing mice. Similar to WBC and NEUT, G-CSF levels were highly correlated with tumor burden (Figure 12D, bottom, R²=0.66). Interestingly, 4T1 tumors increased the plasma levels of IL-5 and IL-6 (Th2 cytokines, Figure 13A), while decreasing the levels of IL-12 p40 (Th1 cytokine, Figure 13B). Taken together, these results suggest that CP-Dox normalizes hematopoiesis and cytokine levels in mice inoculated with 4T1 carcinoma and that this effect is achieved through a significant reduction in tumor burden.
Figure 13: 4T1-bearing mice exhibit Th2-polarized plasma cytokine levels. (A) Th2 cytokines. IL-5 and 6 are significantly increased in 4T1 carcinoma-bearing mice. Drug treatment had no overall effect. (B) Th1 cytokines. IL-12 p40 is significantly decreased in 4T1 carcinoma-bearing mice. *p<0.05, data analyzed by two-way ANOVA

**CP-Dox increases intratumoral cell death and leukocyte infiltration**

Having demonstrated that CP-Dox modulates the systemic immune response via a process as fundamental as hematopoiesis in tumor bearing mice, we next wanted to determine if CP-Dox could alter the intratumoral leukocytic infiltrate. We hypothesized that because CP-Dox is more efficient at delivering doxorubicin to the tumor than Free
Dox, CP-Dox would induce greater cell death, thereby generating inflammation within and increasing leukocyte recruitment to the tumor. To test this hypothesis, 4T1-bearing mice were treated with PBS, Free Dox (5 mg/kg) or CP-Dox (20 mg/kg). One week later, tumors were harvested and either (1) processed to a single cell suspension for analysis by flow cytometry, or (2) homogenized to quantify cytokine and chemokine levels via a multiplexed bead assay.

As seen in Figure 14A, there was a significantly larger fraction of dead cells in tumors treated with CP-Dox compared to Free Dox (8.0% vs. 2.7%, p<0.05, Tukey’s), which is unsurprising given our previous study demonstrating the difference in primary tumor control between the two treatments. We next investigated the intratumoral levels of 7 chemokines (CCL2-5, CXCL1, 2, 10) involved in the recruitment of leukocytes to the tumor, and noted a general trend towards higher levels in CP-Dox treated tumors (Appendix A, Figure 47). To confirm this impression quantitatively, the levels for each cytokine were normalized to the mean level for each cytokine in PBS treated mice, then the normalized values were averaged by drug treatment. Consistent with our hypothesis, this PBS-normalized grand average of chemokine levels was significantly increased in CP-Dox treated mice (p<0.05, ANOVA, Figure 14B). The increased cell death and chemokine expression achieved by CP-Dox treatment as compared to free drug and PBS controls led to a significant increase in leukocyte invasion and
accumulation within the tumor. This was effect was measured by the percentage of live cells expressing the pan-leukocyte marker CD45 (p<0.05, Tukey’s, Figure 14C).

Remarkably, one week after treatment, more than 2 out of 3 live cells in a CP-Dox treated tumor was a CD45+ leukocyte.

Figure 14: CP-Dox treatment increases tumor cell death and intratumoral leukocyte infiltration.

One week after treatment of 4T1 mammary carcinoma with CP-Dox, Free Dox, or PBS, tumors were processed to a single-cell suspension and examined by flow cytometry or homogenized and analyzed for chemokine levels. (A) Percentage of dead cells (n=5 CP-Dox, 6 Free Dox, 3 PBS). (B) Normalized and averaged values for 7 chemokines (raw data found in Supplementary Figure 1) (n=3 CP-Dox, 4 Free Dox, 4 PBS). (C) Leukocyte (CD45+) as a percentage of Live cells (n=5 CP-Dox, 6 Free Dox, 3 PBS). (D-F) CD45+ cells were then gated for quantification of (D) T cells (CD3+), then (E) CD8+ and (F) CD4+ cells as a percentage of live cells (n=3 CP-Dox, 3 Free Dox, 3 PBS for D-F). Data analyzed by ANOVA and Tukey’s post-hoc. *p<0.05.

To dissect the identity of tumor infiltrating leukocytes, CD45+ cells were then gated by flow cytometry on the pan-T cell marker CD3 and quantified as a percentage of live cells in the tumor (Figure 14D). CP-Dox increased the percentage of T cells in the
tumor compared to PBS (p<0.05, ANOVA, Tukey’s). CD8+ cells made up a larger percentage of the live cells within the tumor after CP-Dox treatment in comparison to Free Dox (p<0.05, ANOVA, Tukey’s, Figure 14E). There was also a trend towards increased helper T cells — defined as CD4+ T cells — in CP-Dox treated mice compared to PBS (p=0.056, ANOVA, Tukey’s, Figure 14F). Overall these results demonstrate that CP-Dox mobilizes the recruitment of lymphocytes involved in the adaptive immune system.

**CD8 cells, but not CD4 cells or NK cells, are required for full efficacy of CP-Dox in 4T1**

Having demonstrated the importance of functional T cells in the efficacy of CP-Dox, as well as an increase in their intratumoral recruitment, we next asked which subsets in particular were contributing to the efficacy of CP-Dox. To answer this question, we depleted CD8 or CD4 T cells with antibodies. On Day 6 post-treatment and weekly thereafter, mice were treated intraperitoneally (IP) with 250 µg of anti-CD8 antibody (Clone 2.43), anti-CD4 antibody (Clone GK1.5), or appropriate isotype control. As shown in Figure 15A, CD8 depletion drastically reduced the efficacy of CP-Dox (p<0.05, Tukey’s, log-rank), but conversely had no effect on tumor growth or survival in Free Dox or PBS treated mice. In contrast, CD4 depletion had no significant effect on the tumor growth or survival in any of the drug treatment groups. These results suggest that the activity of CD8+ T cells accounts for the majority of the difference in efficacy of CP-Dox in nude vs. immunocompetent mice. In fact, the growth of tumors was
remarkably similar in nude and CD8-depleted mice after treatment with CP-Dox; each
group had a tumor volume of approximately 600mm$^3$ on Day 30 (Figure 10D and Figure
15A).

We next investigated the role of NK cells, another subset of immune cells with
the potential for anti-cancer activity, in the efficacy of CP-Dox. Mice were inoculated
with 4T1 and treated with drug as described previously, but with the addition of 20 µl of
anti-asialo GM1 rabbit serum or control rabbit serum delivered IP on Day 6, then
repeated every 4 days for a total of 4 injections. As shown in Figure 15C, the depletion of
NK cells had no significant effect on primary tumor growth or survival in any of the
drug treatment groups.

Figure 15: CD8+ T cells, but not CD4+ T cells or NK cells, are required for full efficacy
of CP-Dox.
Mice were inoculated with 4T1 and treated with drug and administered depleting
antibodies as described in the methods section. Primary tumor growth (top) and
survival (bottom) in the setting of (A), CD8 depletion (n=5:5 CP-Dox, 4:4 Free Dox, 4:3 PBS for α-CD8:Iso Ctrl) (B) CD4 depletion (n=5 CP-Dox, 5 Free Dox, 4 PBS for α-CD8 and Iso Ctrl), and (C) NK cell depletion(n=6:5 CP-Dox, 4:4 Free Dox, 3:3 PBS for α-asialo GM1:Serum Ctrl). Tumor growth curves analyzed by two-way ANOVA and Tukey’s post hoc. Survival data analyzed by log-rank. *p<0.05

Due to evidence for CD8, but not NK cell involvement, we decided to investigate levels of MHCI expression on 4T1 cells. Consistent with the role of CD8 cells in slowing the growth of 4T1 tumors treated with CP-Dox, we found that 4T1 cells express substantial levels of MHCI in vitro (Figure 16, left). To further explore the connection between MHCI expression and the relative importance of CD8 vs. NK cells, we examined the effect of CD8 and NK cell depletion in the Lewis Lung Carcinoma (LLC or LL/2) model, a cell line syngeneic to C57Bl/6 mice that expresses undetectable levels of MHCI in vitro (Figure 16, middle).

**Figure 16: Contribution of CD8 vs NK cells correlates with MHCI expression on 4T1 and LLC.**

Cell lines were cultured in vitro and stained with anti-H2Kd (4T1 and CT26), H2Kb (LLC) or isotype control. 4T1 and CT26 express MHCI, while MHCI is undetectable on LLC.

CD8 depletion had no effect on primary LLC tumor volume (p>0.05, two-way ANOVA, Figure 17A). Combined with primary tumor resection on Day 15, there was a
non-statistically significant trend towards increased survival in isotype control mice in the Free Dox and PBS groups, but CD8 depletion had no effect on the survival of CP-Dox treated mice (p>0.05, log-rank, Figure 17A). In contrast, NK depletion with anti-NK1.1 antibody (compatible with C57Bl/6) had a global effect on primary tumor volume (p<0.05, two-way ANOVA, Figure 17B), and decreased the survival of CP-Dox treated mice (p<0.05, log-rank, Figure 17B. Note: primary tumors were not resected in this experiment).

Figure 17: Efficacy of CP-Dox in the setting of CD8 and NK cell depletion in the LLC model.
(A, top) Day 15 tumor volumes. (A, bottom) Survival of mice inoculated with LLC and treated with CD8 depleting antibody or isotype control along with drug treatment on Day 8 and primary tumor resection on Day 15. In this model, there was a trend towards decreased survival in CD8 depleted groups for Free Dox and PBS treated mice, but there is no evidence for the involvement of CD8 T cells in the efficacy of CP-Dox, as survival was not decreased in CP-Dox treated mice setting of CD8 depletion (p>0.05, log rank) (n= 7:6 CP-Dox, 4:4 Free Dox, 3:3 PBS for α-CD8:Iso Ctrl).
Mice were treated with anti-NK1.1 antibody for the depletion of NK cells. (B, top) Day 17 tumor volumes, demonstrating an overall increase in tumor volume in mice lacking NK cells (p<0.05, Two-way ANOVA). (B, bottom) Survival of mice was slightly decreased by NK cell depletion in the CP-Dox treated mice (p<0.05, log-rank) (n=6:5 CP-Dox, 4:4 Free Dox, 3:3 PBS for α-NK1.1:Iso Ctrl). Note that primary tumors were not resected for the NK cell depletion experiment.

CP-Dox treatment increases the intratumoral ratio of Th1 to Th2 cytokines and is less efficacious in the setting of IFN-γ depletion

CD8 T cells are critical in a Th1 polarized, cell-mediated adaptive immune response, and stimulating this type of immune response is the ultimate goal of most cancer immunotherapy strategies. We hypothesized that the CD8 T cell response stimulated by CP-Dox would correlate with an increase in Th1 signaling. To test this hypothesis, mice were inoculated and treated with drug as previously described. On Day 15 post-inoculation (one week after drug treatment), tumors were homogenized and assessed for cytokine levels by multiplex bead assay. The unprocessed results of the 14 cytokines that had detectable levels within the tumor are included in Appendix A, Figure 48. In particular, we were interested in two crucial Th1 cytokines (IFN-γ, IL-12 p70) and three Th2 cytokines (IL-4, IL-5, and IL-6). Although often classified as a pro-inflammatory cytokine, IL-6 has been implicated in pro-tumoral myeloid polarization and in the enhancement of Th2 polarization, so IL-6 was included in the Th2 cytokine analysis. CP-Dox treatment significantly increased intratumoral IFN-γ levels compared to Free Dox and PBS (p<0.05, two-way ANOVA, Tukey’s), and there was a
trend towards increased IL-12 p70 for both CP-Dox and Free Dox compared to PBS (Figure 18A). While IL-4 and IL-5 levels were similar across treatment groups, CP-Dox significantly decreased IL-6 levels compared to Free Dox (p<0.05, two-way ANOVA, Tukey’s, Figure 18B). To assess the overall effect of drug treatment on Th1 and Th2 cytokines, the level for each cytokine was normalized to the mean of PBS-treated mice and averaged. As seen in Figure 18C (left), Th1 cytokines were approximately 2-fold higher, while Th2 cytokines were present at about 80% of the level of PBS treated mice. This resulted in a significant increase in the ratio of Th1 to Th2 cytokines compared to Free Dox and PBS treated mice (p<0.05, two-way ANOVA, Tukey’s, Figure 18C right). These results confirmed our hypothesis regarding cytokine polarization, but did not directly prove their necessity for achieving full efficacy of CP-Dox. To examine the mechanistic role of IFN-γ in the efficacy of CP-Dox, mice were treated with IFN-γ depleting antibodies on Day 7, 9, 15 and 21. As shown in Figure 18D, antibody depletion significantly reduced the efficacy of CP-Dox (p<0.05, Student’s t-test), while having no effect on PBS or Free Dox treated mice. Taken together, these results demonstrate that CP-Dox increases intratumoral IFN-γ levels, and this increase is critical for the efficacy of CP-Dox.
Figure 18: CP-Dox treatment increases the intratumoral ratio of Th1 to Th2 cytokines and is less efficacious in the setting of IFN-γ depletion.

Mice were inoculated with 4T1 and treated with drug as described earlier. Tumors were homogenized and analyzed for cytokine levels. (A) Th1 cytokine levels: IFN-γ, left and IL-12 p70, right (n=4/group) (B) Th2 cytokine levels: IL-4, left (n=4/group), IL-5, middle (n=4/group), and IL-6, right (n=3 CP-Dox, 4 Free Dox, 4 PBS). (C) Normalized and averaged Th1 vs. Th2 cytokine levels by drug treatment (left) and Th1/Th2 ratio (right) (n=4/group). (D) Primary tumor growth in the setting of IFN-γ depleting antibody on days 7, 9, 15 and 21 (n=5:5 CP-Dox, 4:4 Free Dox, 4:3 PBS for α-IFN-γ:Iso Ctrl). Data for bar graphs analyzed by ANOVA and Tukey’s post hoc, tumor growth curves analyzed by two-way ANOVA and Tukey’s post hoc. *p<0.05
**CP-Dox alters the phenotype of infiltrating myeloid cells**

Myeloid cells are a highly plastic subset of immune cells, which include a variety of phenotypes ranging from pro-tumor myeloid derived suppressor cells to anti-tumor antigen-presenting cells. This cellular phenotype can be influenced by the environment. Thus, based on our findings that the cytokine milieu is significantly changed by CP-Dox treatment, we hypothesized that the phenotype of intratumoral myeloid cells would be altered as well.

To examine the myeloid cell infiltrate in detail, tumors were processed to a single cell suspension and stained with a variety of myeloid cell markers, then subjected to the gating analysis outlined in Appendix B, Figure 49. CD11b+ (myeloid) cells were displayed on a plot of F4/80 (macrophage marker) and Ly6G to highlight granulocytic cells (CD11b+, Ly6G+, F4/80-, Appendix B, Figure 49, bottom left), a subset consistently associated with suppressing the anti-tumor immune response. CP-Dox showed a trend towards fewer Ly6G+ cells (p=0.10, ANOVA, Figure 19A). The remaining cells (CD45+/CD11b+/Ly6G-) can be described as mononuclear phagocytes, which were displayed on a plot of IA/IE (MHCII) vs. Ly6C in order to highlight Ly6C\textsuperscript{hi}/MHCII- monocytes (Appendix B, Figure 49, bottom right), a subset that contains immature myeloid cells such as inflammatory monocytes that are capable of differentiating into other myeloid subsets. CP-Dox treated tumors contained a significantly larger
percentage of these Ly6C<sup>hi</sup>/MHCII-cells compared to PBS or free Dox (p<0.05, Tukey’s HSD, Figure 19B) demonstrating CP-Dox treatment induces a larger infiltrate of inflammatory monocytes. These cells were removed from subsequent phenotypic analysis because the goal was to observe what these plastic cells differentiated into after entering the tumor and, based on their marker expression, they are still immature cells.

Figure 19: CP-Dox skews the myeloid infiltrate toward a monocytic phenotype. 4T1 tumors were treated on post-inoculation Day 8, then on Day 15 processed to a single cell suspension and examined by flow cytometry, then gated for CD45 expression. The percentage of cells that expressed Ly6G (A) or Ly6C (B) was quantified. There was a trend in decreased Ly6G expressing cells that did not reach statistical significance, and a significant increase in the fraction of Ly6C positive cells in CP-Dox treated mice (p<0.05, Tukey) (n=5 CP-Dox, 5 Free Dox, 3 PBS).

After this gating process, CD11b+/MHCII+ cells remained, a group of cells often described as tumor-associated macrophages (TAMs), a diverse group of mononuclear phagocytes potentially including macrophages and dendritic cells. These cells have widely come to be described as a spectrum that ranges from classically activated anti-tumor phenotypes (type M1), commonly defined by high expression of the cell surface marker CD80 (B7, T-cell co-stimulatory ligand) to the alternatively activated, pro-tumor
phenotypes (type M2) defined by high expression of CD206 (mannose receptor)\textsuperscript{130, 132, 133}. Interestingly, when these cells were plotted against CD11c (Integrin alpha x) and IA/IE (MHCII) expression, three distinct sub-populations emerged on the basis of differential CD11c expression, (Figure 20A, named CD11c\textsuperscript{low, int, and high}) a receptor which has been described as a marker of M1 polarization\textsuperscript{130}. As a percentage of total MHCII\textsuperscript{+} cells, there was a lower number of CD11c\textsuperscript{low} cells in CP-Dox treated mice compared to free Dox or PBS (9.6\% vs. 16.0\% and 12.6\% respectively, p<0.05, Tukey, Figure 20B). The other two subsets (CD11c\textsuperscript{high, int}) had similar percentages regardless of treatment.

The effect of treatment on the polarization of these cells was explored by examining the expression of CD206 and CD80 by flow cytometry. Histograms revealed that the CD11c\textsuperscript{low} subset expressed relatively low levels of both CD206 and CD80 in PBS and Free Dox treated mice (Figure 20C). Interestingly, in the CD11c\textsuperscript{low} subset, CP-Dox induced down-regulation of CD206 (p<0.05 CP-Dox vs. Free Dox and PBS, Tukey) concurrent with up-regulation of CD80 (p<0.001, Tukey) (Figure 20F). The CD11c\textsuperscript{int} subset of cells expressed high levels of CD206 in PBS and Free Dox treated tumors in a bimodal pattern, while in CP-Dox treated tumors, this cell population only retained the population with lower expression of CD206 (Figure 20D). In contrast, this CD11c\textsuperscript{int} subset of cells displayed a clear up-regulation of CD80 in CP-Dox treated tumors compared to Free Dox or PBS treated tumors (Figure 20D). Upon quantifying these shifts
for the CD11c<sup>int</sup> subset, we found that CP-Dox treated mice have significantly decreased expression of CD206 (p<0.05, Tukey) and increased expression of CD80 (p<0.05, Tukey) compared to PBS and Free Dox treated tumors (Figure 20G). For the CD11c<sup>high</sup> subset, PBS-treated mice expressed relatively low levels of CD206 but very high levels of CD80 (Figure 20E). Neither treatment with Free Dox or CP-Dox significantly altered the expression of CD206 or CD80 in the CD11c<sup>high</sup> subset (Figure 20H). Overall, these results suggest that CP-Dox treatment skews the mononuclear phagocyte infiltrate towards a more anti-tumor, M1 phenotype, which is consistent with the higher levels of Th1 signaling described earlier (Figure 18).
Figure 20: Treatment with CP-Dox alters the phenotype of mononuclear phagocytes in 4T1 mammary carcinoma.

Mice were inoculated with 4T1 mammary carcinoma and treated with drug as described earlier. One week after drug treatment, cells were processed to a single cell suspension and analyzed by flow cytometry. (A) Flow cytometry plot showing CD45+/CD11b+/Ly6G-/IA/IE+ myeloid cells (TAMs) for a PBS-treated mouse, displayed as CD11c vs. IA/IE (MHCII), revealing three subsets of cells on the basis of CD206/CD80 expression levels.
their CD11c expression. (B) Breakdown of each subset as a percentage of IA/IE+ cells for each treatment group (n=5 CP-Dox, 5 Free Dox, 3 PBS). (C-E) Flow cytometry histograms for the CD11c<sub>low</sub>, CD11c<sub>int</sub> and CD11c<sub>high</sub> subsets, respectively for CD206 and CD80 expression for treatment with PBS (black), free Dox (red) or CP-Dox (blue). (F-H) Quantification of CD206 and CD80 expression for the (F) CD11c<sub>low</sub>, (G) CD11c<sub>int</sub>, and (H) CD11c<sub>high</sub> subset for different treatments (n=5 CP-Dox, 5 Free Dox, 3 PBS). (*p<0.05). Note: Geometric MFI = Geometric mean fluorescence intensity, defined as the nth root of the product of n numbers.

**Blockade of CCL2 or CD11b does not alter the efficacy of CP-Dox**

Having demonstrated that treatment with CP-Dox causes the myeloid infiltrate to display markers associated with an antitumoral phenotype, we next sought to determine how manipulations of the myeloid cell infiltrate affect treatment with CP-Dox. Work from Ma et al has shown that myeloid subsets required for full efficacy of doxorubicin express CCR2, a chemokine receptor for CCL2, involved in the recruitment of inflammatory monocytes<sup>134</sup>. Our analysis of CCL2 after CP-Dox treatment showed a trend towards increased levels (327 pg/ml vs 208 pg/ml and 238 pg/ml for Free Dox and PBS respectively (Appendix A, Figure 47). We hypothesized that treatment with a CCL2-neutralizing antibody would prevent the infiltration of inflammatory monocytes (shown to be recruited to the tumor in large numbers by CP-Dox in Figure 19B) and alter the efficacy of CP-Dox. Mice were inoculated with 4T1 and treated with CP-Dox, Free Dox, or PBS on Day 8 as previously described, with the addition of 250 ug anti-CCL2 antibody (Clone MCP-1) or isotype control IP starting on Day 6 and repeated weekly thereafter. Mice were followed for primary tumor growth and overall survival.
Depletion of CCL2 did not significantly alter primary tumor growth in drug any
treatment group (p>0.05, Two-way ANOVA, Figure 21A). Interestingly, there was a
trend towards increased survival in the setting of CCL2 depletion in Free Dox and PBS
groups (Figure 21B). This is consistent with prior studies demonstrating that CCL2
depletion plays a larger role in metastasis than in primary tumor growth. However,
the survival of mice treated with CP-Dox was not affected by the depletion of CCL2.

Figure 21: CCL2 depletion does not alter the efficacy of CP-Dox.
Mice were inoculated with 4T1 and treated with CP-Dox, Free Dox, or PBS on Day 8
as previously described. On Day 6 and weekly thereafter, mice were treated with 250
ug of CCL2 depleting antibody or isotype control IP. (A) CP-Dox significantly
inhibited primary tumor growth (p<0.05, Two-way ANOVA), however tumor growth
was not significantly altered by the depletion of CCL2 (p>0.05, Two-way ANOVA).
(B) Overall survival of mice, demonstrating a trend towards increased survival for
CCL2 depleted mice within the PBS and Free Dox treated groups, but no difference
between the CP-Dox groups.

Since the lack of effect from CCL2 depletion could have been due to preventing
the infiltration of an irrelevant cell type, we hypothesized that interfering with myeloid
cell trafficking at the most basic level (by blocking CD11b, a part of the integrin receptor
complex) could have a larger effect. To test this hypothesis, mice were inoculated with
4T1 and treated with drug as described previously, with the addition of 100ug of CD11b blocking antibody (Clone M1/70) injected IP every two days to maintain active blockade of the receptor. As shown in Figure 22A, CD11b blockade had no significant overall effect on primary tumor volume (p>0.05, Two-way ANOVA), although there was a trend towards smaller tumor volume in anti-CD11b CP-Dox treated mice. While CD11b+ cells can contain important antigen-presenting cell subsets, it can also include cells with immunosuppressive activity (commonly called myeloid derived suppressor cells, or MDSCs), which could account for this small effect. CD11b blockade had no effect on the survival of mice (Figure 22B).

Overall, these results suggest that myeloid cell phenotype alterations made by CP-Dox may reduce their suppression of T cell responses, so further depleting or preventing their infiltration with antibodies does not affect CP-Dox treatment.

Figure 22: CD11b blockade has no significant effect on primary tumor growth or survival.
Mice were inoculated with 4T1 and treated with CP-Dox, Free Dox, or PBS on Day 8 as previously described. Additionally, mice were treated with CD11b blocking antibody or isotype control every 2 days. (A) Primary tumor volume and (B) Overall
survival were not significantly affected by CD11b depletion, although there was a slight trend towards smaller tumors in CP-Dox anti-CD11b treated mice.

**CP-Dox requires the presence of CD8+ cells in order to prevent metastasis in a surgical model of 4T1 mammary carcinoma**

Drug delivery studies often focus solely on efficacy against primary tumors, despite the fact that metastasis causes the majority of cancer deaths. In a prior publication we demonstrated that CP-Dox in combination with surgery delays the dissemination of 4T1 cells from the primary tumor, leading to cure in approximately 60% of mice. To determine the role CD8 cells play in CP-Dox’s ability to interfere with metastasis, mice were inoculated with 4T1-luc mammary carcinoma in the 4th mammary fat pad on Day 0. On Day 6, mice were treated with either CD8 depleting antibody or an isotype control antibody; this treatment was repeated weekly thereafter for the duration of the experiment. On Day 8 mice were treated with CP-Dox (20 mg/kg), Free Dox (5 mg/kg), or PBS. On Day 15, primary tumors were surgically resected. Mice were then monitored for the development of metastasis with luciferase imaging. Metastasis-free survival of the mice is shown in Figure 23. CP-Dox treatment prolonged the survival of mice in both the CD8 depleted (p<0.05, log-rank) and the isotype control groups (p<0.05, log-rank); however, CD8 depletion had no effect on the survival of mice in the PBS and Free Dox treatment groups, as all mice in those groups developed metastatic disease between days 20 and 40. In the CP-Dox treatment group, isotype control mice achieved long-term survival at a rate of 60%, consistent with our previous results [116]. However, in the CD8 depleted mice treated with CP-Dox, all mice succumbed to metastatic disease
by day 60 as confirmed by luciferase imaging and post-mortem examination, resulting in a significantly lower overall survival rate (p<0.05, CD8 Depleted vs. Isotype Control, Fisher’s Exact). 4T1 cells express significant levels of MHCI in vitro, increasing the susceptibility of 4T1 cells to a CD8 T cell response (Figure 16). These results show that the ability of CP-Dox to prevent metastasis and achieve a long-term cure in combination with surgery requires the presence of CD8+ cells.

Discussion

We have shown in this study that a single intravenous injection of a nanoparticle formulation of doxorubicin dramatically alters the host antitumor immune response, stimulating CD8+ T cells to limit tumor growth and prevent metastasis in

Figure 23: CP-Dox prevention of metastasis is CD8+ T cell dependent in a surgical model of 4T1 mammary carcinoma. Metastasis free survival of mice inoculated with 4T1 and treated with CD8 depleting antibody or isotype control along with drug treatment and primary tumor resection (n=7:5 CP-Dox, 6:5 Free Dox, 4:3 PBS for α-CD8:Iso Ctrl). CD8 depletion had no effect on the survival of Free Dox or PBS mice, but in CP-Dox treated mice, no mice survived to the end of the experiment in the setting of CD8 depletion (p<0.05, Fisher’s Exact).
immunocompetent mice with 4T1 mammary carcinoma (Figure 24). We also shown that CP-Dox increases the ratio of Th1 to Th2 cytokines in the tumor and that IFN-\(\gamma\) depletion reduces the efficacy of CP-Dox. Our data extends the work of many other groups who have elucidated the intricate signals mediating the host antitumor immune response and its involvement in the efficacy of chemotherapy. The critical role of IFN-\(\gamma\) and CD8+ T cells in the efficacy of intratumorally injected doxorubicin was recently demonstrated in carcinogenically induced tumors\(^{109}\). Similarly, a recent study showed that Doxil and doxorubicin were more effective in immunocompetent mice and demonstrated synergy with checkpoint blockade in CT26 colon carcinoma\(^{138}\). This study is particularly relevant to our work because Doxil also employs a nanoparticle delivery system, a PEGylated liposome. However, our current work is distinct from prior studies by demonstrating the immunomodulatory effects of doxorubicin in mice treated with a nanoparticle delivery system in poorly immunogenic 4T1 mammary carcinoma. Here we define immunogenicity of a cell line based on whether pre-treatment with irradiated cells confers a survival advantage to mice after subsequent live cell challenge, which is effective in CT26,\(^{59, 139}\) but not 4T1\(^{119, 140}\).

Despite the poor immunogenicity of 4T1, there is evidence that 4T1 harbors mutations capable of recognition by the immune system\(^{141, 142}\), albeit fewer than CT26\(^{118}\). Furthermore, 4T1 expresses MHCI, which should render the neo-antigens detectable.
Nonetheless, we clearly demonstrate that CD8 depletion, or even injection into nude mice, does not affect 4T1 tumor growth rate in PBS treated mice. This suggests that immunosuppressive signals may neutralize the host antitumor immune response to 4T1. Based on our data, it appears likely that CP-Dox alters the tumor microenvironment, reducing tumor-derived signaling, and activating a latent host antitumor immune response (Figure 24).

Figure 24: Model of CP-Dox’s enhancement of the host antitumor immune response in 4T1 mammary carcinoma.

CP-Dox treatment generates inflammatory chemokines and IFN-γ, and reduces the production of IL-6 and G-CSF. Chemokine production leads to the recruitment of leukocytes including CD8+ T cells and inflammatory monocytes. CD8+ T cells, in the presence of increased IFN-γ, contribute to controlling the primary tumor and preventing metastasis. Inflammatory monocytes become biased to an antitumor “M1”
phenotype in the presence of increased IFN-γ and decreased IL-6. Reduced G-CSF production decreases the levels of neutrophils, which share phenotypic markers with poorly functional myeloid derived suppressor cells (MDSC).

Nonetheless, the CD8 response stimulated by CP-Dox did not lead to cure, but rather a sustained repression of growth for approximately two weeks following treatment. This suggests that the cells within the tumor eventually circumvent the immune response, a phase in the immunoediting process often referred to as “escape”138. This can involve multiple mechanisms including selection for cells that do not express rejection-stimulating antigens, down-regulation of MHCI, or re-establishment of an immunosuppressive environment138. Future studies will work to identify the antigens mediating the CD8 cell response and then focus on the period of tumor re-emergence to determine the mechanism of escape from the immune response.

Consistent with the expanding literature describing the release of inflammatory mediators after doxorubicin treatment, we show that CP-Dox increased cell death and the intratumoral production of chemokines, which ultimately led to a dramatic increase in the number of tumor-infiltrating leukocytes (Figure 2). Interestingly, the increase in chemokine levels in CP-Dox treated tumors was largely driven by CXCL10 and CCL5, which are produced by Th1-polarized dendritic cells143. These chemokines are ligands for the receptors CXCR3 and CCR5, which are markers of Th1 cells and suggest the preferential recruitment of Th1 cells after CP-Dox treatment144,145. These results are consistent with changes in intratumoral cytokine expression, providing further evidence
of a re-polarization towards a Th1 response that is mediated by CP-Dox and largely
driven by increased IFN-\(\gamma\) levels. IFN-\(\gamma\) induces a variety of anti-tumor responses,
including the promotion of antigen presentation and cytotoxic T lymphocyte activity\textsuperscript{146}.
Promoting Th1 immune responses and generating a CD8 T cell response are central
goals of tumor immunotherapy, making CP-Dox a promising complementary treatment
in immunotherapy regimens\textsuperscript{120, 147, 148}.

Our finding that 4T1 mammary carcinoma caused abnormal increases in
neutrophil count that correlated with tumor burden and G-CSF levels has many
parallels with the existing literature on myeloid derived suppressor cells (MDSC).
Myeloid derived suppressor cells (MDSC) are a well-studied group of
immunosuppressive cells that are induced by certain tumors, which can be divided into
monocytic (Ly6C+, monocyte marker) and granulocytic (Ly6G+, neutrophil marker)
subtypes\textsuperscript{47, 122}. Our work is consistent with that of Waight, et al., who found that 4T1
expresses large amounts of G-CSF that induces the development of immunosuppressive
granulocytic MDSC \textit{in vivo} and that blocking G-CSF and MDSC development slowed
tumor growth\textsuperscript{149}. Work from Alizadeh et al. showed that two doses of doxorubicin could
lower MDSC levels in 4T1 and improve immunotherapeutic approaches\textsuperscript{121}. We showed
that CP-Dox lowered plasma G-CSF (Figure 1D), peripheral neutrophil count (Figure
1B), and intratumoral granulocytic (CD11b/Ly6G+) cells (Supplemental Figure 8A).
Reducing the abundance of immunosuppressive cells may account for the observed improvement in adaptive immunity after CP-Dox treatment and may provide a niche for more effective immune cell subsets to proliferate and exert their anti-tumor effects.\(^5\)

Within the tumor we focused on characterizing the myeloid infiltrate and tumor associated macrophages (TAMs). TAMs can be classified into an anti-tumor (M1) subset and a pro-tumoral (M2) subset, and there is considerable effort being spent on strategies to repolarize these pro-tumoral M2 cells into the M1 subset that may help the immune system target and destroy tumor cells.\(^1\),\(^3\),\(^2\),\(^5\). We identified a group of cells highly skewed toward the pro-tumor M2 phenotype (herein referred to as CD11c\(^{int}\), a subset of the CD11b\(^+\), MHCII\(^+\), Ly6G\(^-\) cells) based on high levels of CD206 and low levels of CD80 expression in PBS and free Dox treated tumors.\(^1\),\(^5\). While the expression of mannose receptor (CD206) itself does not directly promote tumor growth, its expression is induced by the same genetic programs that promote tissue remodeling, angiogenesis, and immunosuppressive cytokines. Furthermore, the expression of CD206 is downregulated by IFN-\(\gamma\).\(^5\). The CD206\(^{hi}\) subset has been shown to promote the motility and metastasis of cancer cells.\(^1\),\(^5\). The phenotypic repolarization of these cells was consistent with increased Th1 signaling in the tumor, and may relieve immunosuppression and promote cell-mediated immunity. Given that CD8 cells are involved in the efficacy of CP-Dox, we hypothesized that antigen presentation by a
subset of myeloid cells could be involved. Recent work by Ma, et al. suggests that after doxorubicin treatment, Ly6C+ cells infiltrate and mature within the tumor into functional antigen presenting cells. Similarly, work by Movahedi et al. suggests that Ly6C\textsuperscript{hi} monocytes are the source of other intratumoral myeloid subsets. Interestingly, CP-Dox increased the recruitment of Ly6C+ inflammatory monocytes into the tumor (Supplemental Figure 8B). However, unlike in the Ma et al. study, CD11b and CCL2 blockade failed to modulate chemotherapeutic efficacy, a difference that may be attributed to the specific tumor models studied. As described recently by Broz et al., functional intratumoral antigen presenting cells may be quite rare or harbor a unique phenotype, necessitating more precise strategies to avoid depleting both pro- and anti-tumoral cells which leads to no overall benefit. Identification of the specific antigen-presenting cell subset after CP-Dox treatment will be the subject of future investigation. Overall, our studies add to the growing evidence that anticancer therapies manipulate infiltrating myeloid cells.

In this study, we present the most extensive exploration to date of the host anti-tumor immune response’s role in the efficacy of a nanoparticle formulation of a cytotoxic chemotherapy. Our data suggests that in order for doxorubicin to achieve immunomodulatory effects after systemic administration — particularly in challenging or poorly immunogenic cancers — the adoption of better drug delivery strategies may be
necessary. Furthermore, this work reveals mechanisms and expands our understanding of how drug delivery strategies improve the efficacy of free drug. By involving the host immune response, nanoparticle delivery strategies may achieve a level of efficacy beyond that which would be predicted by simply considering the fold-increase of drug delivered to the tumor. Our data adds to the growing body of evidence supporting the use of nanoparticle delivery systems in future combinations of chemotherapy and immunotherapy. 158.
Chapter 4: ELP-based immunomodulatory reagents

The immune system holds great potential for fighting cancer, but tumors circumvent immunological attack by a variety of mechanisms, including the generation of immunosuppressive signals\(^\text{40}\). Immunomodulatory agents such as cytokines or toll-like receptor (TLR) agonists may stimulate a more effective immune response, but their dosing may be limited by severe systemic side effects\(^\text{159}\). Furthermore, the action of these agents may be improved by localizing them to a specific location, where high concentration gradients recruit and activate the appropriate cell types. Thus, using ELP-based delivery strategies are ideal in this setting. By tuning the T\(_t\) below body temperature, soluble ELP-fusion can be injected intratumorally and a coacervate will form, localizing the fusion partner to within the tumor to reduce systemic side effects and recruit the proper effector cells directly into the tumor. This chapter will cover the development and characterization of ELP-based delivery strategies for IL-2, GM-CSF, IL-10, and immunostimulatory oligodeoxynucleotides (ODNs) such as Cytosine phosphate guanine (CpG).

**Synthesis and characterization of ELP/IL-2 fusion protein**

**Introduction**

The cytokine that has gained the most widespread clinical use is systemic IL-2 for treatment of melanoma and renal cell carcinoma. In metastatic melanoma, the response rate is relatively low (15-20%), but when patients respond, it often leads to long-term
Due to the high rate of side effects, the cytokine must be administered in an inpatient setting, and most patients cannot tolerate the whole treatment course. This has sparked interest in local IL-2 therapy, whereby the dose limiting toxicities of systemic administration may be avoided. Intratumoral injections of IL-2 have repeatedly outperformed systemic IL-2 with fewer side effects in both humans and preclinical models. This local therapy strategy has shown the ability to induce systemic immunity and clear metastatic disease in multiple preclinical models.

A major concern for the use of IL-2 in the setting of cancer is the risk of promoting the proliferation and activity of regulatory T cells (Tregs), which express IL-2 receptor, CD25. However, evidence suggests that the relative effects of IL-2 on regulatory T cells vs. cytotoxic T cells is dose dependent, where low concentrations promote the activity of regulatory T cells.

An ELP/IL-2 fusion protein holds the potential to improve cancer treatment by addressing two prior concerns about IL-2 immunotherapy: (1) induction of Treg activity and (2) systemic side effects. Local treatment with ELP/IL-2 will create an intratumoral depot for prolonged release of IL-2 at high concentrations into the tumor microenvironment (addressing concern 1) with limited systemic exposure (addressing concern 2).
Methods

Assembly of ELP/IL-2 Gene

To generate an ELP/IL-2 fusion protein for intratumoral delivery, the gene for E4-60 ([VPGVG]60) was cloned to the gene for murine IL-2 and inserted into a pET24-based vector using PRe-RDL. E4-60 was chosen as the fusion partner because its Tt is around 35°C at relevant concentrations (Appendix D, Figure 53). The gene encoding the secreted portion of murine IL-2 (Ala 21-Gln 169, full sequence in Appendix C, Figure 50) was purchased in a plasmid with the appropriate flanking restriction enzyme sites (BseR1 on 5’ and 3’ end) from Genart (ThermoFisher). The plasmid was amplified in Eb5alpha cells (EdgeBio, Gaithersburg, MD) then purified by mini-prep (Qiagen). The purified plasmid was digested with BseR1 (New England Biolabs, Ipswich, MA) to generate the appropriate GG/CC overhangs for vector insertion. In parallel, a modified pET24 vector, referred to as JMD5, was similarly digested with BseR1 and treated with Alkaline Phosphatase, Calf Intestinal Phosphatase (CIP) (New England Biolabs, Ipswich, MA) to prevent vector re-ligation. Both the amplified and digested IL-2 insert and the digested vector were run on a 1.5% agarose gel, and the appropriate bands were removed and purified with a gel extraction kit (Qiagen, Valencia, CA). The elutions for each purified band were combined in equal ratios in Quick Ligase Buffer and Quick Ligase Enzyme (New England Biolabs, Ipswich, MA). The product was then transformed into competent *E. coli* strain Eb5alpha (EdgeBio, Gaithersburg, MD). The transformed cells were plated on Kanamycin agar plates, resulting colonies were
screened for correct insert size by colony PCR, grown up in 4 ml Terrific Broth (MO BIO, Carlsbad, CA) cultures, mini-prepped (Qiagen), then plasmid DNA was sent for sequencing using T7 promoter and terminator primers at Eton (Research Triangle Park, NC). A culture from a colony with the correct insert was stored in 3.5% DMSO at -80°C.

With the murine IL-2 gene inserted into the JMD5 vector, it could then be combined with E4-60 using previously published PRe-RDL technique\textsuperscript{166}. Briefly, the E4-60 containing plasmid was “A cut” and the IL-2 containing plasmid was “B cut”, to yield a reconstructed plasmid with an insert of E4-60 upstream of IL-2. The ligation product was transformed into the BL-21 (DE3) strain of \textit{E. coli} (EdgeBio, Gaithersburg, MD). Clones were screened and sequenced as described earlier, and correct clones were frozen at -80°C.

**Expression**

A frozen stock of a BL-21 (DE3) clone containing the E4-60/IL-2 plasmid was used to inoculate 50ml of TB culture with 45 ug/ml kanamycin and incubated overnight at 37°C with orbital shaking at 250rpm. The culture was then spun down at 3,000 rpm for 10 minutes, resuspended in fresh media, and split evenly between two 4L flasks containing 1L of TB media supplemented with 45 ug/ml kanamycin. These flasks were placed in an incubator with orbital shaking at 200 rpm at 25°C. After 5-6 hours, 1ml of sterile 0.2M isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) was added to each flask for a final concentration of 0.2 mM, the temperature was adjusted to 16°C, and induction
continued overnight. The next morning, cultures were spun down at 3,000 rpm for 10 minutes, resuspended in 25 ml of PBS, yielding a total volume of ~40 ml, then frozen at -80°C.

**Purification**

Frozen cell pellets were thawed and then lysed by sonication with a Misonix Sonicator 3000 (Misonix, Inc., Farmingdale, NY) on ice with the following program: 10 seconds on, 40 seconds off, for a total on time of 3 minutes. Polyethylenimine 10% (v/v) was added to the cell lysate at a ratio of 2 ml for every 1L of growth media and then shaken vigorously. The resulting mixture was spun at 14,000 rpm at 4°C for 10 minutes. The pellet, containing precipitated nucleic acids and insoluble proteins, was discarded. The supernatant was confirmed to contain the full fusion protein by SDS PAGE (~42 kD, mIL-2 MW: 17 kD, E4-60 MW: 25 kD). The protein was then purified by inverse transition cycling (ITC)\(^8\). Briefly, ELP transition was triggered by the addition of 0.2-0.4 M ammonium sulfate, then pelleted by centrifugation at 14,000 rpm at 30°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in approximately 4x the pellet volume of PBS on ice. The resolubilized construct was then incubated on ice for 30 min to encourage proper disulfide bond formation. The solution was then centrifuged at 14,000 rpm at 4°C for 10 minutes to remove insoluble aggregates and contaminating proteins. This cycle was repeated 3x, then the final cold spin supernatant was analyzed by SDS PAGE (Figure 25). The purified protein was then
passed through a sterilizing and endotoxin binding filter (Mustang E membrane, Pall). Protein concentration was quantified using Beer-Lambert law with predicted extinction coefficients and molecular weights (detailed in Appendix C, Table 1), along with A280 determined a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

**E4-60/IL-2 Characterization**

Phase transitions for protein-ELP fusions were characterized using a Cary 300 Bio UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA) by changing the temperature from 10 to 40°C and back while monitoring absorbance at 350nm. Hydrodynamic radius was determined using dynamic light scattering with a DynaPro Plate reader from Wyatt (Santa Barbara, CA), at 10°C and a concentration of 25 uM to ensure solubility. Size exclusion chromatography was run with a Superdex 200 10/300 GL column in an AKTA protein purification system. Mobile phase was 50mM Tris, pH 8, 250mM NaCl and the flow rate was 0.5 ml/min.

**CTLL-2 culture:**

The cell line is derived from a cytotoxic T lymphocyte from C57BL/6 mouse, and is dependent on IL-2 for growth. We obtained the cell line from Duke cell culture facility, originally provided by ATCC. The cell line was maintained according to ATCC recommendations. Briefly, the media used was RPMI 1640 (Sigma R8758) supplemented with 10% non-heat inactivated FBS (Hyclone), as well as the following additives (per
500 ml of base media): 2.8 ml of 45% glucose (Sigma), 5 ml of 100x HEPES (Gibco), 5 ml of 100 mM Na Pyruvate (Gibco), and 5 ml of 200 mM glutamine (Gibco). For each passage of the cells, 1.5-2 ul of mouse recombinant IL-2 (eBioscience, #14-8021-64) was added per 15 ml of bulk culture. Cells were kept at 37°C under a 5% CO2 atmosphere. Cells were maintained at a concentration of 1x10^4 to 2x10^5 cells/ml, which resulted in 1:4 or 1:5 splits every 2-3 days.

**IL-2 Bioassay**

CTLL-2 cells were washed to remove residual IL-2, then counted and plated in a 96 well plate at a density of 2x10^5 cells/ml in a total volume of 100 ul per well. Free IL-2 (eBioscience #14-8021-64) and E4-60/IL-2 were tested in triplicate. For free IL-2, the top concentration was 0.1 uM which was 5-fold serially diluted across 11 wells. For E4-60/IL-2, the top concentration was 1 uM, which was 4-fold serially diluted across 11 wells. The final column of wells contained no IL-2. After 48 hours of incubation at 37°C and 5% CO2, 40ul of a 1:1 mixture of base media: Cell Titer 96 Aqueous One Solution (Promega, Madison, WI) was added to the cells to perform an MTT assay. The assay determines the ability of the cells to reduce tetrazolium dye which is a reflection of metabolic activity and cell number. After 2 hours of incubation at 37°C and 5% CO2, the plate was imaged for absorbance at 490nm and 650nm (background) on a VICTOR plate reader (Perkin Elmer, Waltham, MA).
**In vivo experiments (sq test, 4T1, and B16 experiment)**

*Subcutaneous cell recruitment test:* 10 week old female C57BL/6 mice from Charles river were injected subcutaneously on the flank with 200ul of 50uM E4-60/IL-2. Five days later, mice were euthanized and the skin surrounding the injection and skin from the contralateral flank were removed and processed to a single cell suspension by digestion with Collagenase I. Cells were stained with antibodies against CD45, CD11b, MHCII, Ly6C, CD4, and CD8 and analyzed by flow cytometry.

*B16F10 IL-2 experiment:* 8 week old female C57BL/6 mice from Charles river were injected intradermally on the right flank with 1x10⁶ B16F10 cells. When tumor volumes reached 150-200 mm³ (Day 8-10), they were injected intratumorally with 1mM E4-60/IL-2 or E4-60 (ELP only control) at dose normalized to 1/3 of the tumor volume (as calculated by V=½l*w²). Tumor volume and survival were subsequently monitored.

**Results and Discussion**

*Expression and purification of E4-60/IL-2*

E4-60/IL-2 was expressed in *E. coli* as a soluble fusion protein, and purified by inverse transition cycling (ITC).

As seen in Figure 25, after 3 rounds of ITC a pure protein with the expected molecular weight of 42 kD (mIL-2 MW: 17 kD, E4-60 MW: 25 kD) was attained. The yield was approximately 50 mg/L of culture, easily and cheaply providing ample material for downstream testing.
E4-60/IL-2 is active in vitro

Before proceeding with further characterization of the construct, we wanted to confirm that the expressed protein displayed the bioactivity of IL-2. The protein was tested in a proliferation assay with CTLL-2 cells, which are dependent on IL-2 for growth, and compared to Free IL-2. As shown in Figure 26, E4-60/IL-2 is bioactive, and shows the same maximum efficacy as Free IL-2. E4-60/IL-2 was less potent than Free IL-2 (ED50 of 143 ng/ml vs. 4.7 ng/ml), which is typical for proteins fused to high molecular weight carriers\textsuperscript{167}. However, it could also be attributable to a mixture of properly folded protein with improperly folded intermediates, multimers, and aggregates, so further characterization of the fusion was performed.
Figure 26: Bioactivity of E4-60/IL-2 fusion protein. E4-60/IL-2 is less potent than Free IL-2 \textit{in vitro} (ED50 of 143 ng/ml vs. 4.7 ng/ml), but is still capable of stimulating CTLL-2 proliferation.

**Characterization of E4-60/IL-2**

We next sought to characterize E4-60/IL-2 to investigate its potential for \textit{in vivo} applications and elucidate potential causes for its lower \textit{in vitro} potency. Turbidity profiles are shown in Figure 27A, which reveals a Tt of approximately 23°C, confirming that the protein will form an insoluble coacervate \textit{in vivo}, as desired. Interestingly, the Tt dropped from free E4-60 by several degrees (Appendix C, Figure 53), and became concentration independent. This concentration independence is typically associated with self-assembled structures such as micelles, suggesting that the majority of the construct is likely multimers, bringing the ELP chains close enough to each other in solution that they transition as a function of this local concentration, independent of the bulk
concentration in solution. On the other hand, IL-2 acts a homo-dimer, which could bring ELP chains close enough to one another to behave this way. Another interesting feature of the turbidity profiles is that the protein does not resolubilize in the same way upon cooling. While this could suggest irreversible aggregation and misfolding of the IL-2 fusion partner, the fact that the protein continues to re-solubilize over time even upon re-heating (while remaining below the Tt) suggests that this is largely a kinetic issue, and that the protein is capable of regaining solubility with sufficient time. Furthermore, the bioactivity assay was run at 37°C, well above the Tt, and the protein still displayed activity, which is reassuring for potential in vivo applications.

To further investigate the nature of the protein in solution, we performed dynamic light scattering (Figure 27B). The results suggest that the protein is a heterogeneous mixture of different states. The 5 nm peak is consistent with the expected size for a unimer (Free E4-60 has an Rh of approximately 3-4 nm), while the 42 nm and 158 nm peaks are larger scale multimers/aggregates. This notion was supported by size exclusion chromatography, which revealed a large aggregate peak and a smaller peak corresponding the the expected unimer elution volume (Appendix C, Figure 55). Taken together, these results suggest that the potency of E4-60/IL-2 could be improved by re-folding, optimizing the buffer conditions during purification, or isolating the unimer peak.
Figure 27: Characterization of E4-60/IL-2.
(A) Turbidity profile, revealing a concentration independent Tt of 23°C. (B) DLS data suggesting that the protein exists as a mixture of unimer and larger scale aggregates/multimers.

E4-60/IL-2 is bioactive in vivo

Although the potency could likely be improved, the cell proliferation assay results suggested that the protein is bioactive, so we proceeded to in vivo testing. We injected E4-60/IL-2 or E4-60 alone subcutaneously into the flank and analyzed the cells recruited to the injection site five days later by flow cytometry. The results are explained in more detail in the E4-60/GM-CSF chapter, but briefly, the protein induced the recruitment of leukocytes (Figure 32), with a notable increase in non-myeloid (CD11b-) cells compared to ELP injection alone (Figure 33).

To test the potential for E4-60/IL-2 as an anticancer agent, we treated B16F10 bearing mice with intratumoral injections and monitored subsequent tumor growth. As shown in Figure 28, E4-60 significantly delayed tumor growth compared ELP-only control (p<0.05, ANOVA). The treatment did not appear to be toxic to the mice, as they did not experience any weight loss (data not shown). Comparisons to free IL-2 are
necessary in the future in order to assess the role of the ELP in potentially enhancing the intratumoral exposure to IL-2. Nonetheless, this experiment serves as an important proof of principle that ELP-cytokine fusions can maintain their activity as an intratumoral depot.

Figure 28: E4-60/IL-2 inhibits tumor growth in the B16 melanoma model. BL/6 mice inoculated with B16F10 melanoma were treated intratumorally (I.T.) with E4-60/IL-2 fusion or with ELP control. E4-60/IL-2 significantly inhibited tumor growth (p<0.05, ANOVA).

**Synthesis and characterization of ELP/GM-CSF fusion protein**

**Introduction**

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), which stimulates the recruitment, proliferation and activation of dendritic cells, and therefore has been studied extensively as a vaccine adjuvant\textsuperscript{168-170}. In appropriate pre-clinical
models, GM-CSF along with tumor vaccination increases antigen presentation, leading to the generation of effector T cells and durable immune memory\textsuperscript{170, 171}. The most common approach taken in the literature is to transfec tumor cells with GMCSF and re-inject them after radiation\textsuperscript{171}. The approach has been effective, but clinical translation of this approach may be limited due to the technical and regulatory challenges of genetically manipulating tumor cells and re-administering them. ELP/GMCSF offers the potential for local release of the cytokine in the tumor microenvironment without any genetic manipulation. The goal of this section is to generate bioactive ELP/GM-CSF that will form a coacervate upon \textit{in vivo} injection for use in cancer vaccine protocols.

\textbf{Methods}

\textit{Assembly of E4-60/GM-CSF/8xHis gene}

To generate the gene for E4-60/GM-CSF, the gene encoding the secreted portion of murine GM-CSF (Ala 18 – Lys 141) was purchased as a gBlock from IDT (Coralville, IA) with appropriate flanking restriction enzyme sites (BseR1 on 5’ and 3’ end) and 50 base pairs of complementarity with modified pET24 vector “JMD5” (full sequence in Appendix C, Figure 51). The gBlock was cloned into JMD5 by Gibson Assembly using Gibson Assembly Master Mix (New England Biolabs) according to manufacturer instructions. Clones were screened and confirmed to have the correct insert. Next the gene for E4-60 ([VPGVG]\textsubscript{6}) was cloned upstream to the gene for murine GM-CSF using PRe-RDL as described previously, followed by cloning of the sequence encoding 8
histidine residues downstream of E4-60/GM-CSF with PRe-RDL. This plasmid was transformed into BL-21 (DE3) strain of *E. coli* (EdgeBio, Gaithersburg, MD), as well as SHuffle T7 Express strain of *E. coli* (New England Biolabs).

**Expression, purification, and re-folding of E4-60/GM-CSF/8xHis**

Cells were grown up and induced as described in the IL-2 methods section. The construct was expressed insolubly (regardless of *E. coli* strain and orientation of the ELP and GM-CSF), so the protein was purified under denaturing conditions (6M Guanidine HCl) from the inclusion body fraction (Figure 29, Lane 1) according to manufacturer’s instructions (Nickel NTA resin, Thermo Scientific). The protein was then re-folded by several stages of dialysis in decreasing concentrations of guanidine along with glutathione to encourage disulfide bond shuffling and arginine to prevent aggregation. Buffers are outlined in Appendix C, Table 2). The his tag column eluted protein was diluted 1:1 with “Resuspension Buffer”, then 1:1 again with “Dialysis Buffer #1”, then dialysed against buffer #1 with stirring, at 4°C for 24 hours in Spectra/Por 2 12-14 kD MWCO tubing (Spectrum Labs). The next day the tubing was transferred to Buffer #2, and dialysis continued for 24 hours. Buffer #2 was diluted 1:1 with DI water, then the protein was dialysed for another 24 hours. The process concluded with 24 hours of dialysis against Buffers #3 and #4 each. The protein concentration throughout re-folding was approximately 0.5 mg/ml of GM-CSF equivalents. The resulting protein was
sterilized, purified of endotoxin, and quantified by A280 as described previously, using values found in Appendix C, Table 1).

Characterization

The construct was subjected to analysis by DLS, Cary Scan, and size exclusion chromatography as described in the IL-2 section. MALDI-MS was performed with a PE Biosystems Voyager-DE instrument, equipped with a nitrogen laser (337 nm). Samples in Tris buffered saline at an approximate concentration of 25 uM were diluted 1:5 into a sinapinic acid matrix before analysis.

MC/9 Culture

MC/9 cells are a mast cell line derived from a liver of a fetal mouse. The cell line is dependent upon cytokines for growth and can be used to test the bioactivity of GM-CSF. The cells were grown in suspension with DMEM (D6429 Sigma) supplemented with 10% non-heat inactivated FBS and 2mM additional glutamine. During each passage, beta-mercapto ethanol was added to a final concentration of 0.05 mM and T-Cell culture supplement with Con-A (T-Stim, Corning) was added to 10% of total volume. Cells were maintained at a concentration between 2x10^5 and 2x10^6 cells per ml.

Bioactivity assay

MC/9 cells were washed to remove residual T-stim, then counted and plated in a 96 well plate at a density of 2x10^5 cells/ml in a total volume of 100ul per well. Free GM-CSF (GoldBio) and E4-60/GM-CSF/8xHis were tested in triplicate. Proteins were tested at
a concentration starting at 100 ng/ml, followed by serial 3 fold dilution across 11 wells, with the final column containing only media. After 48 hours of incubation at 37°C and 5% CO2, and MTT assay was performed as described in the IL-2 section.

**In vivo testing**

*Subcutaneous cell recruitment test:* 10 week old female C57BL/6 mice from Charles river were injected subcutaneously on the flank with 200ul of 50uM E4-60/GM-CSF. Five days later, mice were euthanized and the skin surrounding the injection and skin from the contralateral flank were removed and processed to a single cell suspension by digestion with Collagenase I. Cells were stained with antibodies against CD45, CD11b, MHCII, Ly6C, CD4, and CD8 and analyzed by flow cytometry.

**Results and Discussion**

**Expression, purification, and re-folding of E4-60/GM-CSF/8xHis**

Similar to many eukaryotic proteins expressed in prokaryotic organisms, GM-CSF was produced in inclusion bodies\textsuperscript{173}, which necessitated the use of his tag purification from the insoluble fraction of the cell lysate. As shown in Figure 29, Lane 2, this resulted in a pure protein at the expected MW of ~40 kD (mGM-CSF MW: ~14 kD, E4-60 MW: ~25 kD, 8xHis MW: ~1 kD). Yield at this stage was >100mg of protein per liter of BL21 culture.
Figure 29: Purification of E4-60/GM-CSF/8xHis fusion protein. Nickel affinity chromatography under denaturing conditions resulted in a pure protein with the expected MW (40 kD). Lane 1: resuspended inclusion body fraction (IBF). Lane 2: His tag column eluent containing purified E4-60/GM-CSF

Due to the denaturing conditions used for resolubilization and purification, the protein needed to be re-folded in order to be tested for bioactivity. A modified version of protocol previously published for human GM-CSF proved successful\textsuperscript{173}. Briefly, the protein was reduced with DTT and dialyzed against decreasing concentrations of guanidine in the presence of glutathione in oxidized and reduced form (detailed buffer recipes found in Appendix C, Table 2). Approximately 75% of the starting material was recovered as soluble protein after re-folding, an impressively high yield for re-folding. Analysis by MALDI mass spectrometry showed a clean peak at 39.9 kD, which is in agreement with the theoretical molecular weight (Appendix C, Table 1).
Re-folding results in bioactive E4-60/GM-CSF/8xHis

To confirm that protein re-folding was successful, the construct was assayed for bioactivity compared to free mGM-CSF based on the proliferation of MC/9 cells. As shown in Figure 30, E4-60/GM-CSF is bioactive, and shows the same maximum efficacy as Free GM-CSF. Similar to E4-60/IL-2, E4-60 GM-CSF was less potent than its freely dissolved counterpart, but in this case only by a factor of approximately 2.7 (ED50 of 27 pg/ml vs. 10 pg/ml). This suggests that the presence of an ELP on the N-terminus and a His tag on the C-terminus of GM-CSF does not interfere with receptor binding or protein folding to a large degree, and that the construct holds great potential for therapeutic applications.

**Figure 30:** E4-60/GM-CSF/8xHis is bioactive *in vitro.* MC/9 proliferation assay reveals ED50 of 10 pg/ml for Free GM-CSF and 27 pg/ml for E4-60/GM-CSF/8xHis.
Next we checked the turbidity profile of the fusion as a function of temperature, which revealed a transition temperature between 25 and 30°C (Figure 31A). Interestingly, the construct appeared to begin to transition at 25°C regardless of concentration, but the slope and maximum signal of the transition was highly dependent upon the concentration.

We next analyzed the construct with DLS at 25uM and 10°C. The construct is a homogeneous, single population with an average hydrodynamic radius of 4.7 nm. This is consistent with the expected size for this construct as a unimer, since E4-60 has an $R_h$ of approximately 3-4 nm, and the globular GM-CSF domain would not contribute as significantly to the hydrodynamic radius as the disordered ELP. Size exclusion chromatography further supported the notion that the fusion exists as a unimer, since only a small fraction of the protein eluted in the “Peak 1” aggregate fraction, while the majority eluted in “Peak 2” at 12.2 ml, only slight before when E4-60 alone elutes (12.3 ml) (Appendix C, Figure 56). MALDI mass spectrometry on the eluted peaks confirmed that both peaks had a molecular weight corresponding to E4-60/GM-CSF/8xHis (data not shown). Further support for the hypothesis that the two peaks correspond to aggregate or misfolded unimer (Peak 1) and unimer (Peak 2) was provided by an activity assay, where Peak 1 was essentially inactive, while Peak 2 displayed the potency of the construct before chromatography (Appendix C, Figure 61).
While the bioactivity assay confirms that the construct is active at 37°C, the concentrations used in the assay are relatively low (compared to in the turbidity profile experiment) which could increase the Tt to above 37°C. Thus, the bioactivity assay does not rule out the possibility that at concentrations used for potential downstream in vivo applications (>50uM), the aggregation of the ELP at 37C might induce irreversible misfolding of the GM-CSF and destroy its activity. To address this concern, the construct was heated to 37°C for 10 minutes at a concentration of 50uM, which resulted in a visibly turbid solution. Without cooling, the protein was tested in a bioactivity assay, and found to retain its original activity as compared to a control E4-60/GM-CSF sample (Appendix C, Figure 62). Thus, ELP coacervation does not affect the in vitro activity of E4-60/GM-CSF, which was reassuring before proceeding with in vivo testing.

![Figure 31: Characterization of E4-60/GM-CSF/8xHis.](image)

(A) Turbidity profile reveals a transition temperature between 25 and 30°C, suitable for in vivo coacervate formation. (B) DLS found a homogenous population with a hydrodynamic radius (Rh) of 4.7 nm.
E4-60/GM-CSF/8xHis is bioactive in vivo

To explore the in vivo activity of E4-60/GM-CSF the construct was injected subcutaneously in the flanks of C57BL/6 mice and studied by flow cytometry for immune cell recruitment. E4-60 alone was used as a negative control, and E4-60/IL-2 to study how cytokine identity affects the phenotype of the immune cell subsets that are recruited. Results for each mouse were normalized to untreated skin from the opposite flank. Mice injected with the GM-CSF or IL-2 fusions demonstrated a clear increase in the fraction of CD45+ cells present at the injection site (Figure 32), confirming that E4-60 GM-CSF induced a local inflammatory response.

![Graphs showing flow cytometry results](image)

**Figure 32: Subcutaneous injection of E4-60/GM-CSF and E4-60/IL-2 increases the fraction of CD45+ cells in skin harvested from the injection site.**

To further explore the phenotype of the leukocytes, we next examined the fraction of CD11b+ cells, which is a marker of myeloid cells. Consistent with the role of GM-CSF in recruiting and activating myeloid cells, there was an increase in CD11b+ cells...
overall, as well as in Ly6C+ (inflammatory monocyte marker) and MHCII+ (antigen presentation to CD4+ cells) cells in mice treated with GM-CSF (Figure 33). GM-CSF did not have any effect on CD11b- cells. IL-2 most strongly increased the number of CD11b- cells, slightly increased CD11b+/Ly6C+ cells, but did not increase CD11b+ cells overall, nor the number of MHCII+ cells. As expected, ELP did not significantly change the cell subsets compared to untreated skin.

![Graph showing changes in cell subsets after treatment with different factors.](image)

**Figure 33:** GM-CSF recruits myeloid cells, while IL-2 recruits non-myeloid cells (CD45+/CD11b-) to the skin of mice 5 days after subcutaneous injection. Data are expressed as fold change from untreated skin.

The results from this preliminary experiment clearly demonstrate that E4-60/GM-CSF is active *in vivo,* so the construct will be tested for the ability to inhibit B16 melanoma growth with and without CpG oligonucleotide (which has been shown to enhance the effect of GM-CSF vaccines\(^ {174}\)) and compared to freely dissolved mGM-CSF\(^ {175}\), and in prophylactic vaccine settings before being combined with CP-Dox to form an *in situ* vaccine\(^ {175}\).
Synthesis and characterization of E4-60/K12 for the delivery of CpG ODN

Introduction

CpG, shorthand for Cytosine-phosphate-Guanine, are unmethylated dinucleotides that are present at a much higher frequency in the bacterial genome than the mammalian genome. They are a pathogen associated molecular pattern (PAMP) that, upon binding to Toll-like receptor 9 (TLR9) on myeloid cells, stimulate an innate immune response and the production of Th1 cytokines. Therefore, they have attracted much attention from the cancer immunotherapy field as an immune adjuvant, where they have been shown to enhance the efficacy of many different immunotherapeutic approaches, including GM-CSF based cancer vaccines.

ELPs offer the opportunity to improve the delivery of CpG for several reasons. First, the efficacy of CpG is improved when maintained in close proximity to the tumor vaccine, and an ELP depot would maintain high local intratumoral concentrations. Second, ELP aggregates or nanoparticles may induce cellular uptake through endocytosis and deliver CpG to the target cells of the immune system where TLR9 is expressed in endolysosomes. Third, ELPs may protect CpG from degradation by DNAase, improving its in vivo efficacy. I hypothesized that including a cationic K12 trailer on an ELP would generate ELP-DNA complexes with the potential to improve the efficacy of CpG.
Methods

Assembly of gene, expression, and purification of E4-60/K12

A sequence encoding 12 lysines was cloned downstream of E4-60/K12 by PRe-RDL gene assembly. The construct was transformed into BL21 (DE3) cells and purified by ITC using a PEI addition (2m of 10% PEI for every liter of original bulk culture), bakeout cycles (65C for 10 min, 4C for 10 min with subsequent centrifugation), and relatively high salt (2M NaCl) to induce phase transition.

Characterization of E4-60/K12

The construct was analyzed by MALDI MS for molecular weight, Cary UV Vis spectrophotometer for phase transition temperature, DLS for hydrodynamic radius as a function of temperature as described in the IL-2 and GM-CSF chapters.

CpG in vitro bioactivity

RAW264.7 cell culture: RAW264.7 is an immortalized mouse macrophage cell line which is activated by pathogen associated molecular patters such as CpG and LPS. It was acquired from ATCC via Duke cell culture facility. The cell line was maintained according to ATCC guidelines. Briefly, the cells were cultured in DMEM (Sigma D6429) + 10% FBS at 37C in a 5% CO2 atmosphere. Cells were passaged by scraping and splitting at a 1:4 ratio every 2-3 days.

Griess Assay: Griess Assay is a colorimetric assay for detecting nitrite, which is formed by the spontaneous oxidation of nitric oxide (NO) under physiological
conditions. RAW264.7 cells produce NO after stimulation with CpG, so this assay was used to assess the bioactivity of E4-60/K12 in association with CpG. The assay was run according to manufacturer (ThermoFisher) instructions.

Results and Discussion

Purification of E4-60/K12

To design an ELP-based system for delivering CpG, I cloned a 12 lysine (K12) trailer to E4-60 and expressed the construct in BL-21 (DE3) cells. Lysine was chosen for its ability to complex DNA, due to its positive charge. As expected, the first round of ITC resulted in a protein that co-purified with a large amount of DNA from the E. coli lysate. Interestingly, this DNA was resistant to degradation by treatment with DNAase, which is encouraging for future in vivo applications (data not shown). The DNA was eventually removed by 3 rounds of ITC with high salt (2M NaCl) and heat (65°C), which disrupted the ionic interaction. DNA removal could be tracked by A260 without interference from the protein at 280nm, since tryptophan or tyrosine trailer (usually included for quantifying protein concentration by A280) was purposefully omitted. The protein was analyzed by SDS PAGE, which revealed a prominent band at the expected MW (~26 kD), but also a persistent fainter band with a slightly higher apparent MW. This band persisted through cation exchange on immobilized heparin (Figure 34), suggesting that the additional band is not a negatively charged E. coli protein bound to the construct. The protein was analyzed with mass spectrometry, which revealed a single peak at 26.2
kD (Appendix C Figure 59) further supporting the purity and identity of the final product. Most likely the second band is an artifact due to the protein running irregularly on SDS page due to its high positive charge density.

**Figure 34: Purification of E4-60/K12.**

Purification E4-60/K12 fusion protein with 3 rounds of ITC resulted in a prominent band at the expected MW of 26kD, as well as faint band with a slightly higher apparent higher MW. The second band is likely an artifact on SDS page due to the high positive charge density provided by the Lysine trailer.

**Characterization of E4-60/K12**

To determine the phase transition behavior of E4-60/K12, and how the behavior is affected by DNA complexation, the turbidity profile of the construct as a function of temperature was determined. As shown in Figure 35A, E4-60/K12 demonstrates several interesting behaviors. First, the construct without CpG (dotted lines) begins to aggregate, but then forms micelles between 37 and 40°C, then persists as micelles up to 60°C (confirmed by DLS, Figure 35B), likely as an aggregated ELP with the lysine block...
displayed on the corona. When mixed with CpG (solid lines) at an N to P ratio of seven (molar ratio of amino groups provided by lysine to phosphate groups on DNA backbone), E4-60 displays entirely different behavior. At 100, 50, and 25 uM, the construct transitions at 36, 36.5 and 38°C respectively. This relative insensitivity of the Tt to concentration change, as well as the full transition rather than formation of micelles is strong evidence for an interaction between E4-60/K12 with CpG which condenses the DNA and increases the local concentration of ELP chains.

![Figure 35: Turbidity profile of E4-60/K12 as a function of temperature with and without DNA.](image)

The presence of CpG caused the Tt to decrease and become relatively concentration independent. The CpG also caused aggregation rather than the formation of micelles upon ELP-block transition observed in the absence of CpG (behavior confirmed by DLS). The results confirm the successful interaction of E4-60/K12 and CpG.

To confirm that this interaction was not disrupted by heat (and therefore would persist after in vivo injection) E4-60/K12 was mixed with CpG as described above, transitioned by heating to 40°C and centrifuged. The supernatant was significantly
depleted of DNA as measured by A260, and the majority of the CpG was found in the resuspended pellet (Appendix C, Figure 63).

Finally, E4-60/K12 + CpG was tested for its ability to stimulate NO production from RAW 264.7 macrophages, based on a protocol adapted from previously published work. Briefly, CpG ODN or scrambled Ctrl ODN were incubated with or without E4-60/K12 for 24 hours at a concentration of 10ug/ml and an N:P ratio of seven. Positive (LPS) and negative (E4-60/K12 alone) controls were also included to aid the interpretation the results. Nitrite was detected by the Griess method. The results are shown in Figure 36, which clearly demonstrate that the only combination with appreciable immunostimulatory activity was E4-60/K12 (p<0.05, Two-way ANOVA). While one might expect the CpG ODN delivered without E4-60/K12 to maintain some activity, it is likely that the freely dissolved ODN is not taken up efficiently, as prior studies have shown that uptake is greatly improved by nanoparticle formation. Toll-like receptor 9 is expressed in endolysosomes, not on the cell surface, so CpG must be taken up by the cell in order to reach the receptor. Freely dissolved ODN is also more susceptible to nuclease activity, while E4-60/K12 protects the CpG from degradation. These results demonstrate that E4-60/K12 holds great promise for improving the delivery of CpG ODN.
Figure 36: CpG delivered by E4-60/K12 is more effective at stimulating NO production from macrophages. RAW264.7 cells were treated with immunostimulatory or scrambled control ODN, with or without complexation with E4-60/K12. NO production was determined by Griess method. E4-60/K12 + CpG was the only treatment to result in an appreciable amount (p<0.05, Two-way ANOVA), demonstrating the ability of E4-60/K12 to enhance the in vitro activity of CpG.

**Synthesis and characterization of E4-60/IL-10 fusion protein**

**Introduction**

IL-10 is a pleiotropic cytokine, although its immunosuppressive capabilities are usually highlighted in the context of cancer. However, some of the early-discovered functions of IL-10 were that it induced thymocyte proliferation via upregulation of CD3 and CD8 (for which it was deemed “B-Cell derived T-cell Growth Factor, B-TCGF), and increased the cytotoxic activity of CD8 T cells\textsuperscript{182}. Therefore, in the proper context, IL-10 can reduce tumor-associated inflammation, while simultaneously increasing the activity
of cytotoxic T-cells, leading to tumor regression in a variety of preclinical models\cite{183}. This effect is mediated by induction of IFN\(\gamma\) by IL-10 and increased expression of MHC molecules within the tumor, and can lead to durable immune memory\cite{184,185}. IL-10 has specifically been shown to have anti-tumor immunostimulatory activity in the 4T1 model\cite{185}. The current leading agent in the literature is pegylated IL-10 injected subcutaneously, but this approach is very expensive, causing the authors to resort to mini-circle plasmid injection in order to study IL-10’s effects\cite{185}. The ability to cheaply produce ELP/IL-10 from \textit{E. coli} cultures would facilitate studies with this cytokine, and provide a local treatment strategy to compare to systemic administration.

\textbf{Methods}

\textbf{Assembly of E4-60/IL-10/8xHis gene}

The gene encoding the secreted portion of murine IL-10 (Ser19-Ser178) was purchased as a gBlock from IDT (Coralville, IA) with appropriate flanking restriction enzyme sites (BseR1 on 5’ and 3’ end) along with appropriate forward and reverse primers (Sequence shown in Appendix C, Figure 52). The gBlock was amplified by PCR and the reaction product was confirmed to be of the correct size (~500 bp) by gel electrophoresis, then the reaction product was digested with BseR1 (New England Biolabs, Ipswich, MA) to generate the appropriate GG/CC overhangs for vector insertion. The gene for E4-60 was cloned upstream, and the gene for an 8xHis was
cloned downstream of mIL-10 by PRe-RDL as described earlier. The plasmid was transformed into SHuffle T7 Express cells (New England BioLabs, Ipswich, MA).

**Expression, purification and re-folding of E4-60/IL-10/8xHis**

Expression of E4-60/IL-10/8xHis was carried out using the same conditions as described in the E4-60/IL-2 section. Similar to E4-60/GM-CSF, the protein was expressed insolubly, so purification as carried out by Immobilized Metal Affinity Chromatography (IMAC) under denaturing conditions as described in the E4-60/GM-CSF section. Re-folding was attempted by two different techniques. First the denatured protein was reduced in “resuspension buffer” (6 M guanidine HCl, pH 8.5 and 5mM DTT) then rapidly diluted twenty-fold into 50 mM Tris pH 8.0, 2 mM glutathione (reduced form), 0.2 mM glutathione (oxidized form), 50 mM NaCl, 5 mM EDTA. After stirring for 4 days at 4°C, the volume was reduced by ultracentrifugation back to its original concentration and exchanged into Tris buffered saline (pH 8) containing 0.5M Arginine, which lowers the tendency for the protein to aggregate. Second, the same buffers and technique used for E4-60/GM-CSF were applied to E4-60/IL-10, with a modified Dialysis Buffer #4 (containing 0.5 M Arginine rather than 0.1 M Arginine, Buffers Described in Appendix C, Table 2).
Bioactivity Assay for E4-60/IL-10

MC/9 cells were cultured as described in the GM-CSF section. Bioactivity assay was based on the proliferation of MC/9 cells, as was carried out as described in the GM-CSF section, with the addition of 10 pg/ml of IL-4 (GoldBio) as a co-factor.

Characterization of E4-60/IL-10/8xHis

The construct was analyzed by MALDI MS for molecular weight, Cary UV Vis spectrophotometer for phase transition temperature, DLS for hydrodynamic radius as a function of temperature as described in the IL-2 and GM-CSF chapters.

Results and Discussion

Expression, re-folding, and bioactivity of E4-60/IL-10/8xHis

The synthesis of E4-60/IL-10 presented several challenges. Murine IL-10 contains 5 cysteines, 4 of which combine to form 2 disulfide bonds crucial to the activity of the protein. The 5th unreacted cysteine is reactive and prone to forming intermolecular disulfide bonds that reduce the activity of the protein. For this reason, a mutated form of mIL-10, containing a cysteine to tyrosine substitution (C149Y), was used. Previous studies have shown that this version, which substitutes the amino acid found in the analogous position in human IL-10, enhances the stability of the protein. This gene was then cloned to E4-60 using PRe-RDL. Despite expressing the construct in SHuffle T7 Express cells (designed for expressing di-sulfide containing proteins, New England BioLabs), the protein was expressed in inclusion bodies. To overcome this, an 8xHis tag
was added to the C-terminus of the protein and the inclusion body fraction (Figure 18A, Lane 1) was resuspended in 6M guanidine and purified by nickel chromatography under denaturing conditions, resulting in a pure protein at the predicted molecular weight (44 kD, Lane 2).

![Image](image.png)

**Figure 37**: Purification of E4-60/IL-10/8xHis. The protein was expressed insolubly in the inclusion body fraction (IBF). After IMAC purification the elution contained pure protein at the expected molecular weight (~45 kD).

Due to the denaturing conditions used to purify and extract the construct, the protein was then subjected to re-folding based on a protocol adapted from a previously published study\(^\text{187}\) involving rapid dilution as described in the methods section. This process proved less efficient than the GM-CSF re-folding, as only ~10% of the starting material was recovered as soluble protein. Nonetheless, the yield was on the order of 10 mg of IL-10 equivalents per L of protein, enough to proceed with testing bioactivity.

After sterilization and endotoxin removal, the construct was tested against Free IL-10 (GoldBio) for the ability to induce proliferation of MC/9 cells. As shown in Figure
38, the construct demonstrated bioactivity, but in this case had a much higher ED50 (127.5 ng/ml) than Free IL-10 (1.1 ng/ml).

![Graph showing MC9 Proliferation vs Log [IL-10 (ng/ml)] with two curves: Free IL-10 and E4-60/IL-10.]

**Figure 38:** E4-60/IL-10 is bioactive *in vitro* based on an MC/9 proliferation assay. The ED50 of Free IL-10 was 1.1 ng/ml and for E4-60/IL-10 was 127.5 ng/ml.

This is likely due to the inefficiency of the re-folding and the persistence of inactive protein in the final product throwing off the true concentration of active construct. To try to improve the activity of the construct, we applied the re-folding technique used for GM-CSF to E4-60/IL-10. The resulting protein remained soluble, and appeared to have improved activity in the MC/9 proliferation assay compared to the “old” technique (Figure 39).
Figure 39: Dialysis re-folding technique appears to improve the activity of E4-60/IL-10. EC50 is 27 ng/ml for E4-60/IL-10 vs. 0.71 ng/ml for Free IL-10.

Interestingly, the maximum efficacy of the re-folded construct was higher, suggesting that the protein has higher intrinsic activity than Free IL-10, but also raising concern that the protein may be signaling through a different receptor. To help elucidate the behavior of this protein, we proceeded to characterization.

Characterization of E4-60/IL-10/8xHis

The turbidity profile of E4-60/IL-10/8xHis is shown in Figure 40A. Similar to E4-60/IL-2, the transition temperature of E4-60/IL-10 is concentration independent. All concentrations transitioned at 25°C, raising concern that a significant fraction of the protein is aggregated or in misfolded multimers. DLS supported this notion, as there were two populations centered around 27 and 159 nm, with no evidence of protein in the expected range for unimer or dimer (<10 nm) (Figure 40B).
Figure 40: Characterization of E4-60/IL-10/8xHis.
(A) Turbidity profile reveals a Tt of 25°C independent of concentration. (B) DLS shows a bimodal population with Rh values of 27 and 159 nm for each sub-population.

To further characterize the protein and its aggregation level, size exclusion chromatography was performed. As expected based on DLS, the majority of the protein eluted at a volume corresponding to aggregate (7.6 ml, Figure 57A). Furthermore, in addition to the unimer peak (12.2 ml), a third peak appeared at an elution volume of 19.4 ml, raising the possibility of Free IL-10 contaminant. At first, the difference in elution volumes appeared to be too large for the differences in molecular weight between full construct (44 kD) and free IL-10 (~19 kD), especially considering that free E4-60 (~12.3 ml and ~25 kD) and full construct (13 ml and 44 kD) elute so closely. However, separation in size exclusion is based more on hydrodynamic radius rather than molecular weight, and ELPs have disproportionately large hydrodynamic radii for a given molecular weight due to their lack of order. In contrast, IL-10 is a globular protein and does not contribute as largely to the hydrodynamic radius. Furthermore, the molecular weight of Free IL-10 (~19 kD) is greater than the cutoff used for dialysis during the re-folding (12-
14 kD), so it is plausible that free IL-10 was retained during re-folding. Although the concentration of Peak 3 was too low to be detected by protein gel, the identity of Peak 1 and 2 was confirmed by SDS PAGE (Figure 57B). Further evidence for the hypothesis that Peak 3 is IL-10 came from a bioactivity assay on Peak 1 and 2 (Figure 57C). Peak 1 contains almost no activity, while Peak 2 contains severely limited potency compared to the pre-SEC sample. In fact, the dose response curve for Peak 2 appears quite similar to the results from E4-60/IL-10 re-folded by rapid dilution (Figure 38). While it is possible that the activity was lost due to the buffer exchange during chromatography, that would still hinder the in vivo application of the protein. Indeed, the fusion lacked any activity against 4T1 in vivo (Figure 41), despite prior evidence in the literature its activity against 4T1$^{185}$.

![Graph](image)

**Figure 41:** E4-60/IL-10/8xHis displays no in vivo activity against 4T1 mammary carcinoma after intratumoral (IT) or subcutaneous (SQ) administration.
Nonetheless, this does not explain why the maximum efficacy of E4-60/IL-10 (even if most of the activity comes from free IL-10) than commercial IL-10. Concerned that this was simply an issue of poorly active commercial IL-10 from GoldBio, we also tested eBioscience IL-10 but it had the same potency and maximum efficacy (data not shown). It is possible that the re-folding process more effectively generates IL-10 homodimers, the active form of IL-10. However, the bioassay is performed on MC/9 cells which express many receptors and can respond to different growth signals. The data presented herein cannot rule out that the protein is signaling through a different receptor with greater potential to stimulate cell growth. To confirm that the protein is signaling through IL-10 receptor, the bioassay would need to be performed in the presence of antagonistic IL-10 receptor antibodies.

The data presented herein cannot lead to a conclusive interpretation of the behavior of E4-60/IL-10. Although more experiments (pooling, concentrating, and characterizing “Peak 3” from size exclusion chromatography) could elucidate the feasibility of successfully making the construct, the more straightforward and predictable behavior of our other regents (IL-2, GM-CSF, and CpG) led us to focus our attention away from E4-60/IL-10.
Chapter 5: *In vivo* efficacy of CP-Dox and immunotherapy combination treatment strategies

*Introduction*

Cytotoxic chemotherapy can enhance the antitumoral immune response, as demonstrated in Chapter 3 of this project and by many prior studies\(^50,54\). Nonetheless, chemotherapy alone has not achieved satisfactory efficacy for cancer patients, suggesting that stimulating effective antitumor immunity will require combinations with immunotherapy. Cytokine immunotherapy in combination with chemotherapy has shown promise in preclinical models\(^159\). The literature contains multiple examples of doxorubicin enhancing the effects of IL-2\(^188,189\). Checkpoint blockade has been shown to interact positively with doxorubicin treatment\(^138\). Vaccination and adoptive T cell treatment strategies have been shown to be enhanced by doxorubicin treatment\(^121,190,191\). Therefore, this chapter will begin our exploration of combining CP-Dox with immunomodulatory reagents.

*Methods*

**CP-Dox + IL-2 experiment**

C57BL/6 mice were inoculated with 1x10\(^6\) B16-F10 cells intradermally on the right flank. On Day 9-11, after tumors had reached 200-300 mm\(^3\), mice were treated with drug IV at their MTD (20 mg/kg for CP-Dox, 5 mg/kg for Free Dox), and intratumorally with E4-60/IL-2 or E4-60 at a concentration of 1mM and a dose of 1/3 of the tumor volume.
**Checkpoint blockade administration**

BALB/c mice were inoculated with $8 \times 10^5$ 4T1-luc cells on the 4th mammary fat pad on Day 0. Mice were treated with either 200 ug of anti-PD-L1 (clone 10F.9G2, BioXcell), 200ug of anti-CTLA-4 (clone 9H10, BioXcell), or appropriate isotype controls starting on Day 8 and repeated every 3 days for a total of 4 treatments. CP-Dox or Free Dox was administered on Day 8 at their MTD (20 mg/kg for CP-Dox, 5 mg/kg for Free Dox).

**Results and Discussion**

**Efficacy of CP-Dox and IL-2 combination treatment**

To determine how CP-Dox and E4-60/IL-2 interact in the treatment of B16, a pilot experiment was undertaken in which B16 melanoma tumors were treated with either CP-Dox (20 mg/kg) or Free Dox (5 mg/kg) IV, with or without E4-60/IL-2 IT (1/3 of tumor volume @ concentration of 1mM) when tumor volumes reached 250-500mm³. Due to the heterogeneity of tumor volumes, tumor volumes were normalized to the size on the day of treatment administration. As shown in Figure 42, both CP-Dox and IL-2 the CP-Dox was more effective than Free Dox (Blue vs. Red), and IL-2 treatment was more effective than vehicle control (solid lines vs. dashed lines). Since E4-60/IL-2 improved both Free Dox and CP-Dox by similar proportions, the effects appear to be additive rather than synergistic. Nonetheless, these results show that CP-Dox can be effectively combined with E4-60/IL-2. Importantly, the combination treatments were well tolerated.
as no significant weight loss observed in the mice that received CP-Dox and E4-60/IL-2. This suggests that further dose escalation could enhance these effects. Future studies, such as the mechanistic studies applied in Chapter 3 to CP-Dox treatment alone, may elucidate the cell types and immunological signals involved in IL-2’s combination with CP-Dox.

![Figure 42: E4-60/IL-2 enhances the activity of CP-Dox.](image)

BL/6 mice were inoculated with B16 on the right flank and treated with either CP-Dox or Free Dox IV, with or without E4-60/IL-2 IT when tumor volumes reached 250-500mm³. Tumor volumes were subsequently measured and normalized to the volume on the day of treatment. E4-60/IL-2 appears to increase the efficacy of CP-Dox in an additive manner.

**Intratumoral injection of depot-forming CP-Dox is not effective**

Because E4-60/IL-2 is designed to be administered intratumorally, I also wanted to explore intratumoral CP-Dox administration. There is precedent for creating gel-like release systems of doxorubicin for intratumoral delivery. By synthesizing CP-Dox with ELPs that have lower transition temperatures such that in vivo administration would result in depot formation, high delivery and retention of doxorubicin would be
possible, and all treatment components (doxorubicin and adjuvants) could potentially be administered in one injection. However, when these treatments were administered in mice bearing B16 melanoma in comparison with Free Dox (IT and IV), they were largely ineffective (Figure 43). Free Dox IT showed better efficacy than the CP-Dox treatments, and there was an unexpected trend where the more hydrophobic constructs were least effective. This suggests that hydrophobic depots may form a necrotic core and then fail to diffuse and further limit tumor growth. Due to the lack of efficacy, this treatment approach was not pursued any further.

![Graph showing tumor volume over days for different treatments.](image)

**Figure 43: Intratumoral CP-Dox is not effective against B16 melanoma.** Mice were inoculated with B16 melanoma on the right flank. When tumor volumes reached approximately 250 mm³, mice received the treatments indicated on the legend. For the intratumoral injections, Free Dox was most effective. The ELP-based constructs showed little effect, and there was a general trend towards poorer performance with increasing hydrophobicity (lower Tt). Free Dox IV was included as a comparative reference.
Efficacy of CP-Dox and checkpoint blockade

Since CP-Dox appears to stimulate a CD8 T cell response, we hypothesized that combining it with checkpoint blockade would improve its efficacy. As shown in Figure 44A, 4T1 expresses relatively low amounts of PD-L1 compared to unstained control in vitro. The low amounts of PD-L1 could be due to studying the cells in vitro, as previous work has shown that PD-L1 expression is induced in vivo. Indeed, examining in vivo levels of PD-L1 on dissociated tumors and spleen after gating on singlets and live cells revealed a larger shift from unstained controls (Figure 44B). Published work has demonstrated that CTLA-4 is not very active as monotherapy against 4T1, but enhanced the effect of radiation therapy. If doxorubicin is similarly able to generate tumor antigen like radiation therapy, it may also combine effectively with CTLA-4 inhibition. Therefore, we proceeded with testing CP-Dox with checkpoint blockade.

![Figure 44: Induction of PD-L1 on 4T1 grown in vivo.](image)

(A) 4T1 cells cultured in vitro expressed low levels of PD-L1. (B) Tumors and spleens were harvested from 4T1 mammary carcinoma-bearing 15 days after inoculation and processed to a single cell suspension and analyzed by flow cytometry. After gating on live cells, PD-L1 expression was determined. PD-L1 expression was slightly higher in
tumor compared to the spleen, and higher than in cells grown *in vitro* compared to unstained samples.

Consistent with previous results, CP-Dox had a significant effect compared to Free Dox (p<0.05, 2 way ANOVA, Figure 45). While there was no significant overall effect of antibody treatment in this small pilot experiment (n=3 mice/group), there was a trend towards smaller tumors in the PD-L1 and CTLA-4 groups. However, that pattern was seen for both CP-Dox and Free Dox treatment, so it appears that any effect of checkpoint blockade in combination with CP-Dox is simply additive.

Figure 45: Primary tumor growth of 4T1 treated with PD-L1 or CTLA-4 in combination with CP-Dox or Free Dox.

CP-Dox significantly delayed tumor growth compared to Free Dox (p<0.05, two-way ANOVA), but there was no overall effect of checkpoint blockade compared to Isotype control at this sample size (n=3-4 mice/group, p<0.05, two-way ANOVA).
Thus, despite the evidence that CP-Dox stimulates a CD8 T cell response, checkpoint blockade does not significantly enhance the effect. Further studies will be required to understand the interaction between doxorubicin and checkpoint blockade in the 4T1 model. Prior studies showed synergy between doxorubicin and checkpoint blockade in CT26, so comparative studies of the markers expressed on T cells induced by CP-Dox treatment in the two models could be elucidating.

**Towards an in situ cancer vaccine**

Overall, this project has developed several regents that could be implemented for an in situ vaccine, and demonstrated that a nanoparticle formulation of doxorubicin will be an effective treatment for properly remodeling the tumor microenvironment to potentiate an immune response. As reviewed by Marabelle, et. al., an effective tumor vaccine should release tumor antigen, increase antigen presentation, and stimulate cytotoxic T cells\(^74\) (Figure 46). Indeed, Chapter 3 of this work provides strong evidence that CP-Dox as a monotherapy contributes in all three areas, despite not being a what is conventionally considered an immunotherapy. In Chapter 4, I described the synthesis and characterization of three immunomodulatory agents which will supplement the presentation of antigen (GM-CSF and CpG) and the activity of cytotoxic cells (IL-2). By generating antigen in situ, we will avoid needing to pre-select antigen or removing cells and manipulating them ex vivo, which would ease the clinical translation of our approach. By packaging the immunomodulatory reagents as ELP fusions for
intratumoral delivery, we can achieve high concentrations for long periods of time, which are necessary for generating immune responses. Furthermore, we can avoid the toxicities of systemic administration. Overall, we believe this treatment approach holds great promise for treating cancer, and turning local immunomodulation into a systemic immune response capable of clearing metastatic disease.

Figure 46: *In situ* cancer vaccine requirements. An effective *in situ* tumor vaccine must (1) generate tumor antigen, which can be achieved with CP-Dox, (2) activate antigen presentation, which will be stimulated with GM-CSF and CpG delivered by ELP, and (3) stimulate the activity of cytotoxic T cells, which is accomplished with CP-Dox and IL-2. Adapted from^74^.
Appendix A: Cytokine and chemokine levels

Figure 47: Intratumoral chemokine levels in 4T1-bearing mice. Although individual chemokine levels did not reach statistical significance (p>0.05, ANOVA), there was a clear overall trend towards higher chemokine levels in CP-Dox treated mice.
Figure 48: Intratumoral cytokine levels in 4T1-bearing mice.
Note: TNF-a, IL-12 p40, IL-13 and IL-17 levels were below LOD and are therefore not pictured. *Indicates cytokine used in Th1 cytokine level. **Indicates cytokine used in Th2 cytokine level.
Appendix B: Flow Cytometry Myeloid Cell Gating Strategy

Mice were inoculated with 4T1, treated with PBS, Free Dox or CP-Dox on Day 8. On Day 15, tumors were removed, processed to a single cell suspension and stained with a variety of leukocyte markers. (A) Schematic demonstration of gating strategy, where...
arrows denote the discriminatory marker used, and boxes represent the identity of the populations at each step. The grey path follows the cells of interest, the monocytic phagocytes (TAMs and dendritic cells). (B) Gating strategy shown with a representative data set. In summary, we selected singlets (not shown), cells (top left), live (top right), CD 45+ (middle left), CD 11b+ (middle right), Ly6G- (bottom left) and MHCII+ (bottom right) cells.
Appendix C: ELP fusion protein supplemental information

Gene sequences

Figure 50: Murine IL-2 sequence used for E4-60/IL-2 fusion protein.
This sequence was cloned downstream from E4-60 and expressed as a C terminal fusion protein.
Figure 51: Murine GM-CSF sequence used for E4-60/GM-CSF/8xHis fusion protein. The sequence was cloned downstream from E4-60, and upstream from an 8x His tag then expressed as a fusion protein.
Figure 52: Murine IL-10 sequence used for E4-60/IL-10/8xHis fusion protein. The sequence contains the secreted portion of the protein (Ser19-Ser178, which excludes the signal sequence) and a C149Y mutation which has been shown to improve solubility and activity after re-folding in recombinant expression systems.
Figure 53: E4-60 Turbidity profile as a function of concentration. E4-60 transitions between 32 and 37 °C at concentrations between 12.5uM and 100uM, making it an appropriate starting candidate for fusion proteins designed for coacervation after in vivo injection.
## Fusion protein information

### Table 1: Fusion protein parameters

<table>
<thead>
<tr>
<th>Construct</th>
<th>MW full Construct (kD)</th>
<th>MW free cytokine (kD)</th>
<th>Extinction Coefficient (L/mol/cm)</th>
<th>Tt (°C) at 50 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4-60 (Y)</td>
<td>24.8</td>
<td>-</td>
<td>1280</td>
<td>35</td>
</tr>
<tr>
<td>E4-60/IL-2</td>
<td>42.1</td>
<td>17.3</td>
<td>8610</td>
<td>23</td>
</tr>
<tr>
<td>E4-60/GM-CSF/8xHis</td>
<td>39.9</td>
<td>14.1</td>
<td>11290</td>
<td></td>
</tr>
<tr>
<td>E4-60/IL-10/8xHis</td>
<td>44.8</td>
<td>18.8</td>
<td>8160</td>
<td></td>
</tr>
<tr>
<td>E4-60/K12</td>
<td>26.2</td>
<td>-</td>
<td>-</td>
<td>36 (with DNA)</td>
</tr>
</tbody>
</table>

Assumptions for extinction coefficient calculations (from [http://biotools.nubic.northwestern.edu/proteincalc.html#helpexco](http://biotools.nubic.northwestern.edu/proteincalc.html#helpexco)):

<table>
<thead>
<tr>
<th>Residue</th>
<th>Moles cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>5690</td>
</tr>
<tr>
<td>Tyr</td>
<td>1280</td>
</tr>
<tr>
<td>Cys</td>
<td>120</td>
</tr>
</tbody>
</table>
## Refolding Buffers

Table 2: E4-60/GM-CSF/8xHis refolding buffers

<table>
<thead>
<tr>
<th>Component Name</th>
<th>Resuspension Buffer</th>
<th>Dialysis Buffer #1</th>
<th>Dialysis Buffer #2</th>
<th>Dialysis Buffer #3</th>
<th>Dialysis Buffer #4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GuHCl (M)</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DTT (mM)</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tris (mM)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Arginine HCl (M)</td>
<td>-</td>
<td>0.4</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>NaCl (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Reduced Glutathione (mM)</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Oxidized Glutathione (mM)</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>0.9</td>
<td>-</td>
</tr>
</tbody>
</table>
AKTA size exclusion chromatography runs

E4-60 Alone: 12.3 ml

Arginine: 20.7 ml

Figure 54: AKTA control runs, establishing elution volumes for E4-60 alone and buffer containing 0.5M Arginine
Figure 55: E4-60/IL-2 Size exclusion chromatography. Results suggest that the majority of the E4-60/IL-2 is aggregated.

Figure 56: E4-60/GM-CSF/8xHis Size exclusion chromatography. The results suggest that the construct is mostly a soluble unimer, consistent with DLS results and its relatively high potency in vitro. Both peaks had a molecular weight of 39.9 kD as measured by MALDI mass spectrometry.
Figure 57: E4-60/IL-10/8xHis Size exclusion chromatography, with subsequent SDS PAGE and bioassay of eluted peaks.

The protein largely elutes in the aggregate range, along with a small peak corresponding to unimer, which is consistent with DLS (both peaks confirmed to be full length construct by MALDI). A third peak elutes in a range that could correspond to IL-10 alone (B) SDS PAGE confirms that Peak 1 and 2 are full length construct, but concentration of Peak 3 was too low to detect protein. (C) Peak 1 (triangles) lacks activity. Peak 2 (circle with dotted line) has some activity, but much lower than than the pre-SEC sample (circle with solid line).
Figure 58: MALDI mass spectrometry for E4-60/GM-CSF/8xHis. Results show clean peak at expected molecular weight (39.9 kD).
Figure 59: MALDI mass spectrometry for E4-60/K12, demonstrating a clear peak at the expected molecular weight (26.2kD).

Figure 60: MALDI mass spectrometry for E4-60/IL-10/8xHis. The spectrum contains two main peaks which may include adducts, but they are in close agreement with the theoretical molecular weight of the full construct.
E4-60/GM-CSF Activity Assays

Figure 61: Activity of E4-60/GM-CSF size exclusion chromatography elution peaks. Peak 1 is largely inactive aggregate/multimer, while peak 2 is active unimer.

Figure 62: E4-60/GM-CSF retains activity after coacervate induction at 50uM.
**E4-60/K12 DNA capture**

Figure 63: UV absorbance analysis of CpG capture assay on E4-60/K12. E4-60/K12 (black curve) was mixed with CpG (red curve) at an N:P ratio of 7. The mixture (green curve) was heated to 37°C and centrifuged. The supernatant (light green + brown curves) were significantly depleted of DNA according to the decrease in A60, while the resuspended pellet (teal curve) contains the majority of the A260 signal, demonstrating the successful capture of CpG into the hot spin pellet.
References


8. T. Mitin, Radiation therapy techniques in cancer treatment. UpToDate; published online Jan 14, 2016


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Biography

Eric Mastria was born in Jacksonville, IL on July 27th, 1987. He graduated summa cum laude with a degree in Chemical Engineering from the University of Michigan in 2009. He then joined the MSTP at Duke University. He is a member of АΩΑ, a medical honor society. He was named Outstanding Teaching Assistant in 2013 for his work teaching Quantitative Physiology to undergraduate students. He is an author on the following articles:


