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Gold Nanovaccine Strategies for Cancer Immunotherapy

by

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ABSTRACT

Gold nanoparticles have excellent properties for cancer therapeutics because their tunable size and surface chemistry make them customizable for many applications. For immunotherapy applications in particular, we can leverage their natural biodistribution to the spleen and immune cells for delivering peptide antigen vaccines or tune their optical properties for photothermal therapy to ablate tumors, which results in tumor antigen circulation and an *in situ* vaccination effect. Our group has demonstrated the potential of gold nanoparticles to elicit systemic, anti-tumor immunity through several iterations of particle design, characterization, and *in vivo* testing. However, most of the animal testing was done using a B16-OVA model, which is less clinically relevant due to the transgene antigen inserted for vaccination and tumor detection. My work builds upon the strong foundation of proof-of-concept vaccination strategies and examines the use of these gold nanoparticle platforms for cancer immunotherapy applications in a more clinically relevant tumor model. Though many hurdles remain for the first gold nanoparticles to reach FDA approval, this work demonstrates the progression of gold nanoparticle-enabled cancer immunotherapy toward that end and illustrates novel immunotherapeutic outcomes and combinations that may inform future progress toward identifying a clinically viable gold nanoparticle cancer immunotherapy strategy.

Contents

Chapter 1

Introduction and Background[1]

1.1. Metallic Nanoimmunotherapy for Cancer

Cancer immunotherapy, or the utilization of the body's immune system to attack tumor cells, has gained prominence over the past few decades as a viable cancer treatment strategy. Recently approved immunotherapeutics have conferred remission upon patients with previously bleak outcomes and have expanded the number of tools available to treat cancer. Nanoparticles –including polymeric, liposomal, and metallic formulations – naturally traffic to the spleen and lymph organs and the relevant immune cells therein, making them good candidates for delivery of immunotherapeutic agents. Metallic nanoparticle formulations in particular are advantageous because of their potential for

[1] : ER Evans, P Bugga, V Asthana, R Drezek. Metallic nanoparticles for cancer immunotherapy. *Materials Today*. 14 Dec 2017.

dense surface functionalization and their capability for optical or heat based therapeutic methods. Many research groups have investigated the potential of nanoparticle-mediated delivery platforms to improve the efficacy of immunotherapies. Despite the significant preclinical successes demonstrated by many of these platforms over the last twenty years, few metallic nanoparticles have successfully entered clinical trials with none achieving FDA approval for cancer therapy. In this introduction, we will discuss preclinical research and clinical trials involving metallic nanoparticles (MNPs) for cancer immunotherapy applications and discuss the potential for clinical translation of MNPs.

Figure 1. Overview of metallic nanoimmunotherapeutic strategies

1.2. Initiating an Immune Response

Immune evasion is found in all types of cancer and contributes to tumor growth[1]. Under non-cancerous conditions, the body's immune system recognizes abnormal cells and facilitates their destruction[2]. Tumor cells evade such destruction by down-regulating the immune recognition and/or attack function of the T cells[3]. The field of cancer immunotherapy focuses on re-engaging the body's ability to recognize and destroy cancerous cells in order to restore the inherent immune system functions that have been compromised[4]. Reinvigoration of

this response can be achieved through a variety of strategies and materials, depending on the type of cancer and target cell or tissue[5].

Cytotoxic (CD8+) T cells are the primary cytotoxic components of the body's immune system and are responsible for killing abnormal, damaged, or infected cells. These T cells are typically activated in response to specific signals produced by antigen presenting cells (APCs)[6]. APCs, such as dendritic cells (DCs), recognize and internalize antigens and subsequently present these molecules on their surface via major histocompatibility (MHC) receptors[7]. MHC receptors presenting antigens interact with T cell receptors (TCR) on CD8+ T cells to initiate a cytotoxic immune response in which the CD8+ T cells become activated, differentiate, and expand to form a robust army of T cells specific to the antigen presented[8]. The T cells survey the body and release cytotoxic material into cells expressing that antigen, inducing cell death[6]. **Figure 2** illustrates how activation of specific T cells can be initiated *in vivo*.

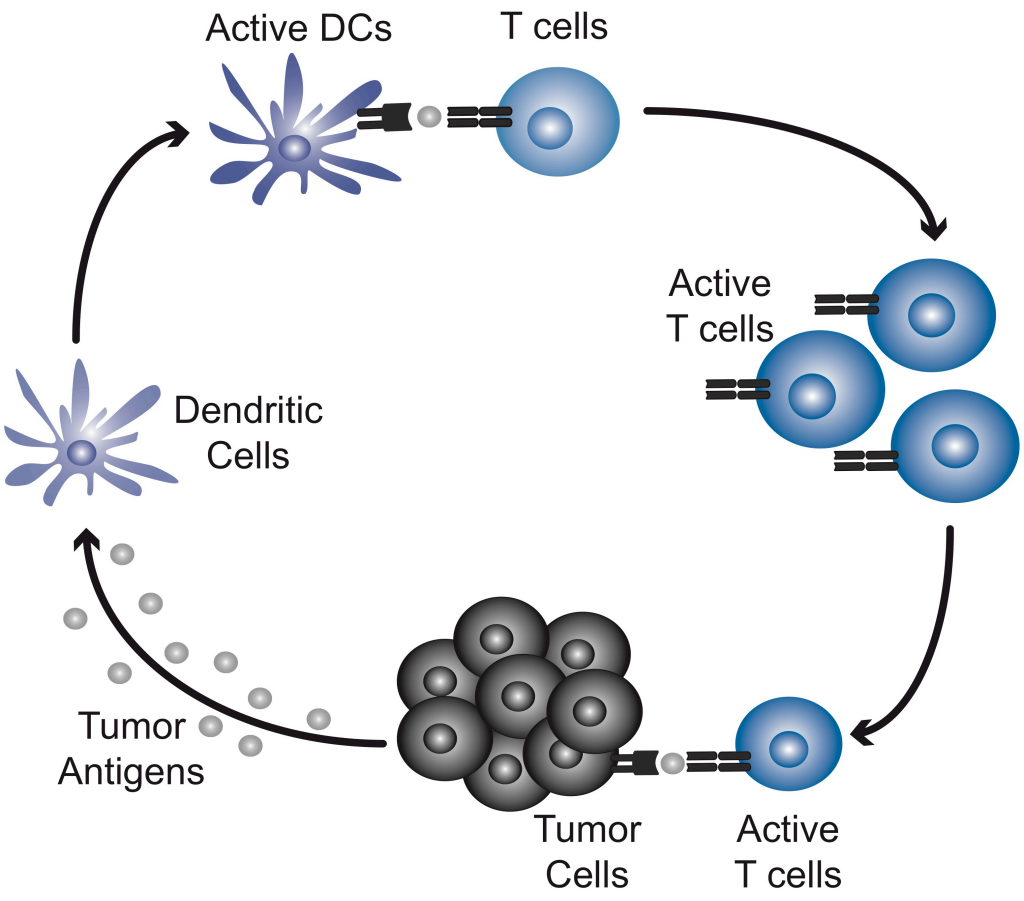

Figure 2. Overview of anti-tumor immunity cycle. Dendritic cells (DCs) uptake, process, and present tumor-associated antigens to T cells in lymphoid tissues. T cells are activated, differentiate, and expand before entering systemic circulation. When T cells identify tumor cells with the corresponding antigen, they release cytotoxic material into the cell, inducing apoptosis.

Cancer vaccines can initiate the production of antigen-specific T cells by delivering tumor antigens to APCs, which often reside in the spleen, skin, or lymph tissues[9]. The APCs then interact with CD8+ T cells in the spleen or lymph tissues, initiating maturation, expansion, and migration processes. These processes often require a boost in the form of adjuvant administration[10]. However, traditional adjuvants used to boost B cell vaccines are often insufficient to support CD8+ T cell activation; therefore, novel adjuvants such as toll-like

receptor (TLR) agonists are under clinical investigation to support cancer vaccines[11-13]. Effective adjuvants support anti-tumor immunity by inducing release of Th1 cytokines and type 1 interferons and promoting the activation of DCs, CD4+ and CD8+ cells. A selection of some of the pathways induced by CpG, a TLR9 agonist, are illustrated in **Figure 3**[14].

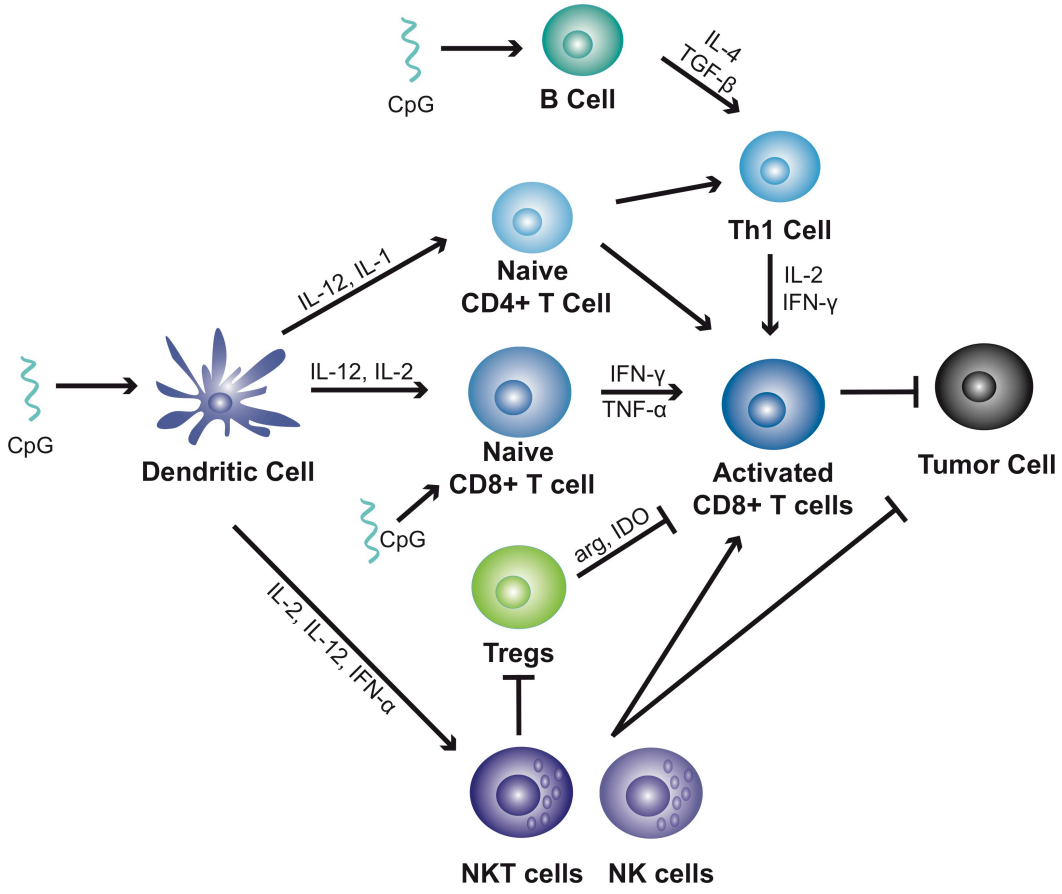

Figure 3. Immunostimulatory materials such as CpG can support T cell activation through several pathways. CpG-induced activation of antigen presenting cells (such as dendritic cells) leads to activation of CD8+ cytotoxic T lymphocytes, natural killer cells and natural killer T cells, which can kill tumor cells. CpG also induces CD4+ helper T-cell activation (particularly Th1 responses), which further supports CD8+ T cell activation. CpG may also directly promote cytotoxic T cell function. Overall, adjuvants such as CpG boost activation of antigen presenting cells, helper T cells, and cytotoxic cells. IL: interleukin. Arg: arginase. IDO: indoleamine 2,3-dioxygenase. IFN: interferon. TNF: tumor necrosis factor. TGF: transforming growth factor. Treg: regulatory T cell. Th, helper T cell.

Even with a robust army of primed and functional T cells, the tumor microenvironment can suppress T cell viability and function[15]. Tumor cells can interact with T cells via programmed cell death protein 1 (PD-1) and other pathways, causing T cells to lose cytotoxic activity[16]. Furthermore, the tumor microenvironment can inhibit T cell activity through other mechanisms including low pH, immune suppressive cytokines and immune cells, or physical barriers such as incomplete vasculature or excess extracellular matrix[3, 17]. Therapeutic modalities that mitigate T cell inactivity in the tumor microenvironment allow existing activated T cells to better perform their surveillance and cytotoxic functions and kill tumor cells[18].

1.3. Cancer Immunotherapy

Cancer immunotherapy harnesses the body's immune system to attack tumors. Numerous cancer immunotherapeutic approaches are being investigated including monoclonal antibodies, immune checkpoint inhibitors, adoptive cell therapies, and non-specific cancer immunotherapies[4, 19-23]. Some immunotherapies act at the site of the tumor microenvironment to directly facilitate immune cell killing of tumor cells[24, 25]. Other immunotherapies seek to enhance immunity against tumors by increasing the amount of tumor-specific cytotoxic T cells at the site of the cancer via approaches such as adoptive cell therapy or cancer vaccines[26, 27]. Adjuvant immunotherapies generally support the activation or efficacy of T cell responses through supporting pathways[14]. Nanoparticles have been and are currently being investigated to improve the delivery and/or efficacy of each of these approaches [5, 28-30].

Monoclonal antibodies are proteins that are engineered to target specific antigens. Upon binding to their respective substrates, monoclonal antibodies can perform a number of critical functions, including recruitment of immune cells, modulation of receptor or antigen functions, or local delivery of anti-cancer drugs[31]. Given the vast network of immune interactions and cancer cell antigens associated with tumors, monoclonal antibody treatments currently comprise an immense library of therapeutic agents[23]. To date, these treatments are considered one of the most successful forms of cancer immunotherapy for solid tumors and are frequently administered by clinicians for the treatment of a number of malignancies[32]

Some tumor cells overexpress immune checkpoint molecules on their surface in order to deactivate T cells and evade immunogenic cell death[33]. As illustrated in Figure 4, immune checkpoint inhibitor therapies prevent cancer cell evasion by interfering with T cell suppression signals[34]. Checkpoint inhibitors enable existing anti-tumor immune responses that have been exhausted or deactivated by the tumor. Currently, there are seven approved checkpoint inhibitors targeting PD-1, PD-L1, or CTLA-4 and several other checkpoint inhibitors are undergoing clinical evaluation[16, 18, 35]. Notably, Keytruda (pembrolizumab) is the first cancer therapy to be indicated based on a patient's biomarker status rather than the tissue origin of their tumor[36].

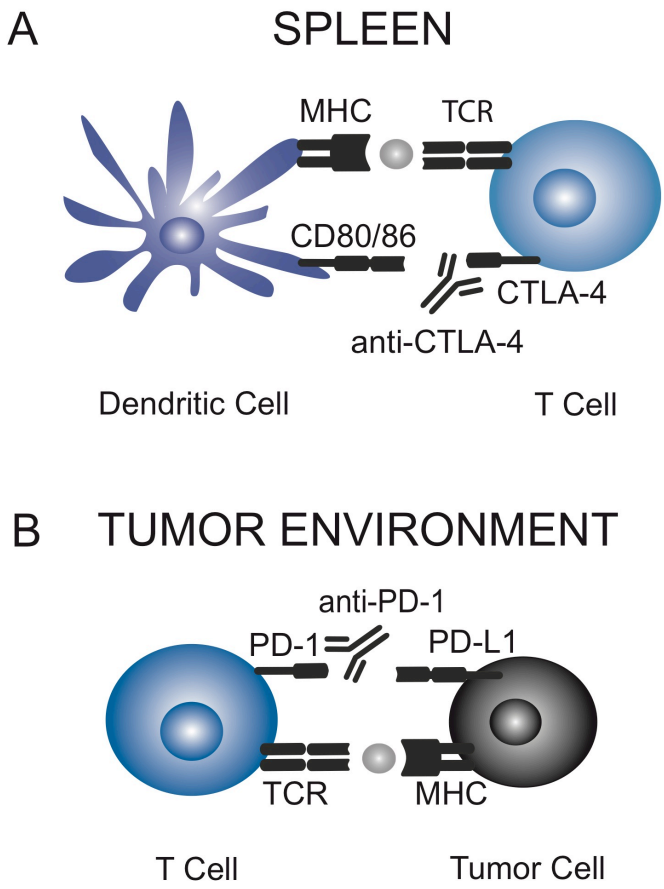

Figure 4. Clinically approved checkpoint inhibitors enable T cells to perform their cytotoxic activity by A) enabling T-cell activation by antigen-presenting cells or B) preventing tumors from deactivating T-cells via pathways including PD-1 and PD-L1.

Adoptive cell transfer therapies, also known as adoptive T cell therapies (ACT), are cancer treatment strategies in which isolated anti-tumor lymphocytes are expanded ex vivo then subsequently re-delivered into the patient, as shown in Figure 4[37]. The advantage of ACT is that it can augment the patient's existing immune response to the cancer cells through the provision of a large number of cytotoxic, anti-tumor T cells[38]. Isolated T cells can also be genetically modified to further enhance this immune response. Current studies

utilizing ACT can be classified into three treatment strategies: (1) isolation, expansion, and reinfusion of tumor-infiltrating lymphocytes (TILs) to produce a monoclonal population of tumor specific T cells; (2) antigen-specific expansion of peripheral blood lymphocytes (PBLs) to generate a polyclonal population of tumor specific T cells; and (3) gene modification of PBLs to confer tumor-specific antigen recognition in a population of T cells[37]. Data from clinical studies investigating ACT have shown this form of immunotherapy to be especially efficacious in the treatment of metastatic melanoma, with approximately 50% of patients exhibiting tumor regression[21]. The FDA recently approved Novartis's adoptive T cell therapy with Chimeric Antigen Receptors (CAR-T cells), making it the first of several anticipated approvals of CAR-T cell therapy in the United States[39].

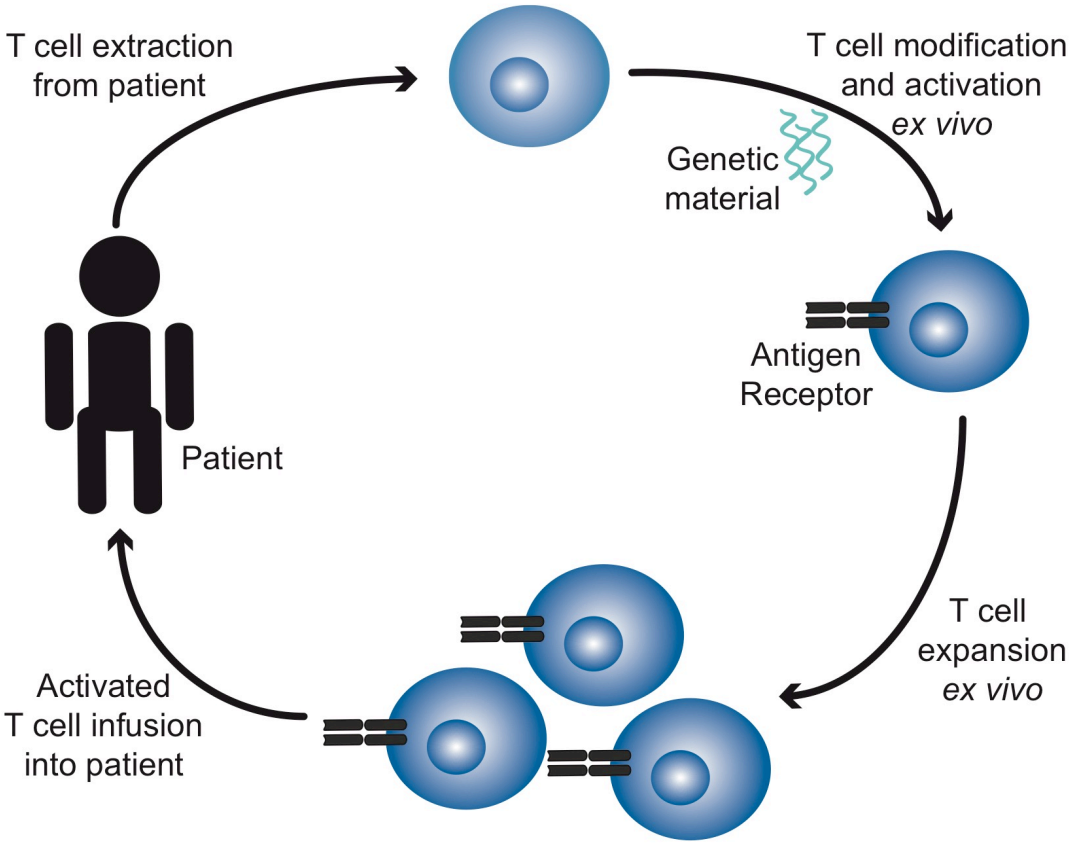

Figure 5. In adoptive T cell therapies, a patient's T cells are isolated then modified and expanded *ex vivo* before being reinfused into the patient.

Other cell transfer therapy approaches begin further upstream by activating dendritic cells. Dendritic cell vaccines involve extracting and reprogramming DCs ex vivo and administering the modified DCs to induce the activation and expansion of T cells in vivo[40, 41]. A clinically approved DC vaccine, Sipuleucel-T, is indicated for the treatment of prostate cancers. Dendritic cells are extracted from the patient and then modified with a unique antigen (prostatic acid phosphatase) found in approximately 95% of prostate cancers as well as with a granulocyte macrophage colony-stimulating factor (GM-CSF). Upon infusion into the patient, the modified DCs activate T cells specifically in response to

the prostatic acid phosphatase antigen, allowing for targeted attack of the prostate tumor[42].

Cancer vaccination strategies aim to elicit an immune response in vivo by delivering synthetic peptides mimicking tumor antigens to the lymph tissues where APCs reside to initiate immunity[9, 41, 43]. However, these therapies have failed to reach their therapeutic potential due to insufficient delivery of antigens to the lymph tissues caused by rapid degradation of peptides in circulation[44]. In addition, endogenous antigens are often not sufficient to elicit a response strong enough to overcome immune tolerance to self-antigens[10]. Neoantigens, or antigens specifically mutated by the tumor cells, have emerged as potential alternatives to tumor-associated antigens because they are not hindered by tolerance mechanisms and can be patient and tumor-specific[45].

Non-specific cancer immunotherapies include treatments that stimulate or enhance the anti-tumor immune response, without directly targeting tumor cells themselves[46]. These therapies commonly involve the delivery of cytokines or immunostimulatory molecules such as CpG[47]. Though non-specific immunotherapies can be administered independently, many function in concert with other forms of cancer therapy, serving to augment the overall therapeutic efficacy of these systems[48].

1.4. Leveraging the Properties of Metallic Nanoparticles for Immunotherapy

Nanoparticles have unique physical and chemical characteristics that can be engineered for use in many therapeutic applications including cancer immunotherapy[5,

28-30, 49, 50]. With sizes ranging from 1-100 nm, nanoparticles have high surface area to volume ratios and advantageous delivery kinetics[29, 51]. Nanoparticle designs can be customized to an intended application via modulation of particle properties including size, shape, and charge[52-54]. Early studies focused on nanoparticle delivery to tumors via the enhanced permeability and retention (EPR) effect which could be further enhanced by conjugating tumor-targeting antibodies to the nanoparticles[55-59]. While these delivery strategies are still commonly used in the field, many groups also leverage the natural biodistribution of nanoparticles to the lymphoid tissues – including the spleen, draining lymph nodes, and skin-resident dendritic cells – for cancer immunotherapy[60-62].

Metallic nanoparticles (MNPs) are particularly advantageous in cancer immunotherapy applications due to the precision with which their size, shape, charge, and surface modification can be controlled[53, 54, 63]. Compared to non-metallic nanoformulations of similar sizes, the higher density MNPs are more readily uptaken by cells, providing a benefit for cancer vaccination strategies[60, 64]. MNPs also have distinctive optical properties that can be leveraged for metallic nanoparticle-mediated tumor ablation combined with immunotherapy[65-67]. The following section will describe the variety of strategies, applications, and preclinical successes demonstrated using metallic nanoparticle immunotherapies, some of which are outlined in **Table 1**.

Table 1. Overview of the variety of metallic nanoparticles and examples of their cancer immunotherapy applications

MNP	Approach	Mechanism	Outcome	Citation
Aluminum oxide	Adjuvant	Enhances anti-cancer effects of tumor cell vaccines	Observed smaller tumor sizes and more CTLs when co-administered with a tumor cell vaccine	[68]
Cobalt oxide	Antigen delivery	Induce macrophage activation	Increased antigen-specific CTLs *in vivo*	[69]
Cuprous oxide	Alter tumor microenvironment	Alter expression of drosophila transcription factor	Induced myeloid infiltration and systemic immunity	[70]
Gold	Antigen/adjuvant delivery; Photothermal therapy	Increased CTL responses; tumor ablation released tumor antigens	Reduced tumor growth *in vivo*; prevented tumor growth *in vivo*	[28, 71]
Iron oxide	M1 macrophage polarization; Protein delivery; Photothermal therapy	Increased pro-inflammatory macrophage proliferation; IONP-HSP chaperoned antigens to APCs; thermal tumor ablation	Inhibited tumor growth; IONP-HSP70 led to tumor-specific CTL responses; ablation led to protective immunity	[72-74]
Silver	Reduce tumor-promoting cytokines	Decreased IL-1β signaling in tumor microenvironment	Inhibited fibrosarcoma tumor growth *in vivo*	[75]
Titanium dioxide	Immune stimulation induced by ultrasound	ROS generation increased pro-inflammatory cytokines and interleukins in the tumor	Suppressed tumor growth *in vivo*	[76]
Zinc oxide	Antigen delivery (pulsed DCs)	Improved antigen-specific CTL responses	Delayed tumor growth *in vivo*	[77]

CTL: cytotoxic T lymphocyte. IONP: iron oxide nanoparticle. HSP: heat shock protein. IL-1 β: interleukin 1 beta. ROS: reactive oxygen species. DC: dendritic cell. APC: antigen presenting cell.

Strategy: improving antigen and adjuvant delivery

Many cancer cells can be identified based on the expression of tumor-specific (mutated protein) or tumor-associated (up-regulated protein) antigens on their surfaces[45, 78]. Thus, there exists a potential to vaccinate patients against these tumor signatures to treat tumors and prevent recurrence of tumors with those same signatures[7, 79]. Delivery of peptide antigens alone to antigen presenting cells is insufficient to induce immunity due to the rapid degradation of peptides upon systemic administration[44]. Nanoparticles can overcome these delivery hurdles by preventing peptide degradation and improving the concentration of therapeutic molecules delivered to the target tissue[29].

Metallic nanoparticles enhance vaccine delivery by improving uptake of antigens by dendritic cells (and other APCs) and thus improving the resulting anti-tumor cytotoxic T cell response[28, 30]. In one of the earliest examples of this phenomenon, Chen et al. delivered antigens using gold nanoparticles (AuNPs) of varying sizes and observed significant sera antibody responses against the delivered antigen[80]. Others have since applied AuNP platforms to deliver tumor-associated antigens, often demonstrating proof-of-concept successes using ovalbumin (OVA) as a model antigen. For example, Ahn et al. demonstrated that gold nanoparticles deliver OVA to dendritic cells and facilitate cross-presentation, slowing tumor growth[81]. Peptide-coated AuNPs were shown to elicit a humoral response in vivo as measured by an increase in IgG secretion mediated by the blimp/pax5 pathway[82]. Almeida and colleagues demonstrated AuNP-mediated delivery of OVA antigens improved tumor burden and survival following both prophylactic and therapeutic administrations, while OVA administration alone did not induce immunity or improve survival[83].

The weak immune responses induced by peptide antigens can also be further boosted by co-administration of adjuvant molecules. Such adjuvants can also benefit from improved delivery to immune cells via incorporation on a nanoparticle carrier. Indeed, metallic nanoparticles have been used to improve adjuvant delivery, with particular focus on TLR-9 adjuvants such as CpG, a synthetic oligodeoxynucleotide that mimics bacterial DNA[84]. Several groups have shown that delivery of CpG using AuNPs improves CD4+ helper T cell and cytokine activation, leading to improved CD8+ responses downstream[85-87]. While most groups focus on initiating Th1 immunity, Brinas et al. showed that AuNPs

carrying tumor associated glycopeptides and a B-cell adjuvant induced production of IgG and IgM immunoglobulins[88].

Most successful nanoparticle vaccination strategies combine antigen and adjuvant delivery on the same particle to compensate for the generally weak immune responses induced by peptide antigens alone. Jewell and colleagues used a layer-by-layer approach to co-deliver a model antigen and the poly-IC adjuvant to DCs, leading to activation of the DCs and subsequent generation of an antigen-specific T cell response[89]. Lee et al. demonstrated that AuNPs and ferritin nanoparticles induced a CTL response against the model RFP antigen when co-administered with CpG[90, 91]. This effect was abscopal in that the local treatment provided systemic immune protection and prevented RFP-expressing melanoma growth in vivo[91]. Mirkin et al. demonstrated that 15 nm AuNP-CpG formulated with OVA antigens resulted in a substantial increase in IgG2a antibody titers as well as improved T cell activation leading to reduced tumor growth and improved survival in a lymphoma model system[92].

Recently, several groups have observed that metallic nanoparticles have the potential to act as an adjuvant themselves, prompting curiosity about the potential inherent immune-stimulating properties of these delivery vehicles[65]. Gold, traditionally considered bioinert, has demonstrated inherent immune activation properties that may be adapted for stimulating anti-tumor immunity[65]. Lee and colleagues observed that peptide coated gold nanoparticles elicited humoral immunity in vitro, in vivo, and ex vivo[82]. Almeida et al. observed that antigen-coated gold nanoparticles produced a sufficiently strong immune response without an adjuvant in a cancer vaccination model,

leading to T cell expansion in the spleen and tumor prevention in vivo [83]. Bare, non-functionalized metallic nanoparticles can also impact immunity. Mukherjee and colleagues have demonstrated a strong body of work in identifying and utilizing the inherent anti-tumor properties of bare AuNPs and relevant combinations to further improve cancer immunotherapies[93]. They observed that bare gold nanoparticles inhibited MAPK signaling and tumor growth and metastasis in two in vivo tumor models, altered signaling molecules in the tumor microenvironment leading to inhibition of tumor growth in vivo, and reduced tumor promoting angiogenic factors including human growth factors and VEGF[94-96]. Bare gold nanorods elicited innate immune signaling pathways including toll like receptors, NOD-like receptors, and MAP kinases in vivo [97]. Bare silver nanoparticles have demonstrated anti-tumor activity in vivo in a lymphoma model by inducing apoptosis and slowing angiogenesis[98-100]. Other particles comprised of a silver core and gold shell have also shown preliminary anti-tumor activity[101]. Despite these interesting results, further studies are required to elucidate the mechanisms driving the immune activation properties of these metallic nanoparticles. If metallic nanoparticles continue to demonstrate such inherent adjuvant properties and initiate anti-tumor immunity in vivo, these findings could provide motivation for using MNPs over biodegradable nanoformulations in cancer immunotherapy applications.

Strategy: leveraging optical properties to improve immunotherapy

A particularly interesting strategy that utilizes the unique properties of metallic nanoparticles for cancer immunotherapy is ablative therapy in which applied energy is converted to heat by certain compatible MNPs including hollow gold nanoshells, cuprous oxide

nanoparticles, and others. Ablative hyperthermia can be induced using techniques such as radiofrequency ablation, focused ultrasound, and NIR-mediated photothermal therapy (PTT). These treatments increase blood flow in tumors, induce cytotoxicity, and disrupt tumor vasculature[102-104]. As a result, tumor-specific antigens and danger signals are released from the tumor environment, alerting the immune system as illustrated in **Figure 6**[105, 106]. Dendritic cells uptake these antigens and interface with T cells in draining lymph nodes, leading to an activation of CTL immune responses[43]. Thus, the locally applied ablative therapy can elicit systemic immunity, demonstrating an abscopal effect. This is a particularly interesting phenomenon because the CTLs generated in response to the release of antigens and cytokines from the primary tumor site are able to migrate systemically to distal tumor sites, indicating a potential opportunity to treat metastatic tumors that express similar markers as the primary tumor. The abscopal effect is also observed with other methods of tumor ablation, including ablation with non-metallic nanoparticles in photodynamic therapy and clinically with the combination of radiotherapy with immunotherapy [107-117]. There is also some evidence to suggest that metallic nanoparticles combined with radiotherapy have the potential to initiate systemic anti-tumor immunity; however, further studies are needed to elucidate the mechanisms that cause immune activation[118-121].

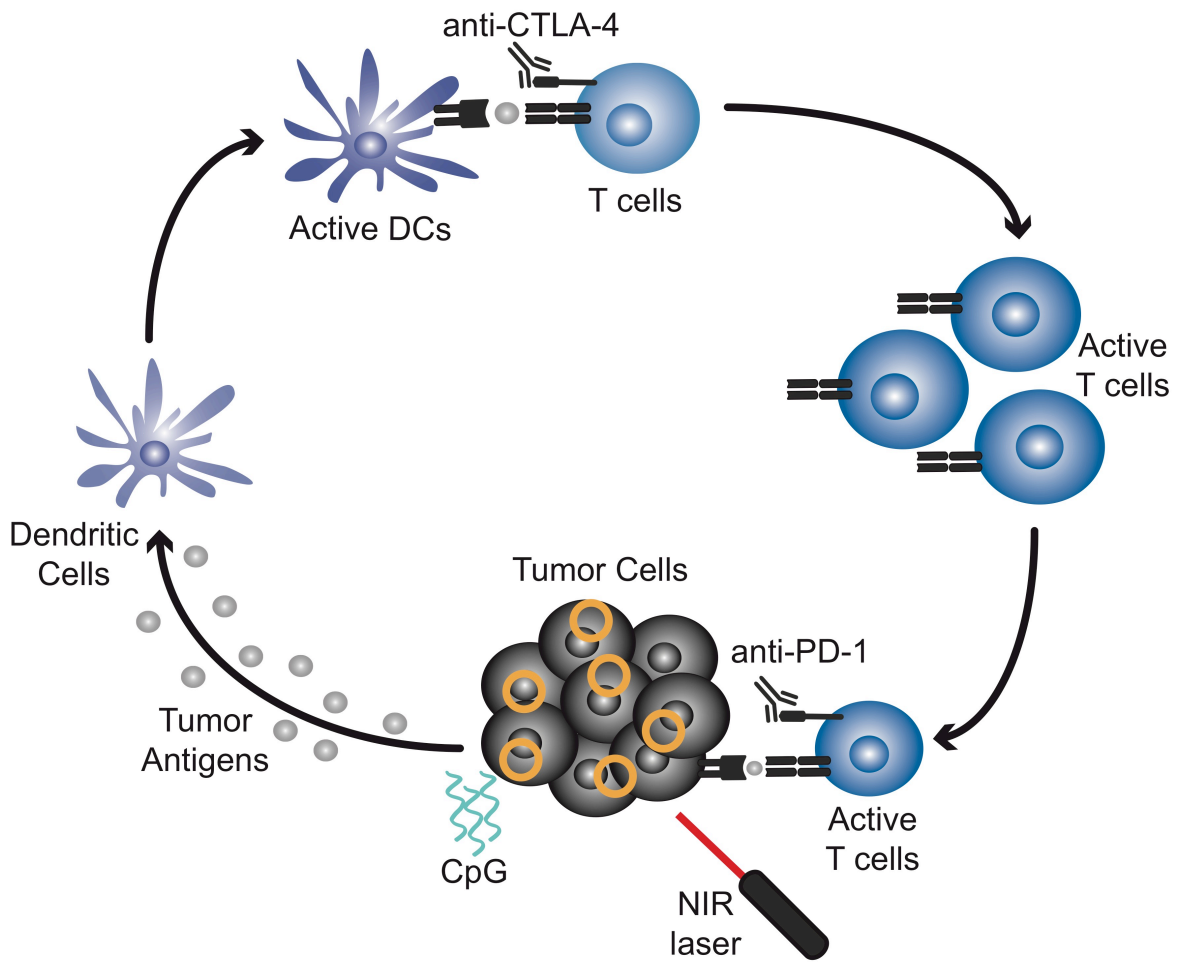

Figure 6. NIR laser light applied to the tumor is converted to ablative heat by hollow gold nanoshells. Tumor cells undergo cell death and release tumor antigens into circulation. Incorporating one or more immunotherapies can enhance the anti-tumor immune response and enable systemic immune monitoring.

Even without co-delivering immunotherapeutic agents, MNP-mediated tumor ablation has elicited systemic anti-tumor immunity. Fiering et al. used iron oxide nanoparticles and an alternating magnetic field to induce hyperthermia in a tumor and observed a subsequent induction of various cytokines and chemokines, activated DCs, and activated CD8+ T cells, providing resistance against rechallenge at both local and distant sites. Interestingly, the mechanisms initiated by hyperthermia do not rely on CD4+ T cell

expansion or IL-12 to support the propagation of the immune response[74]. This protective immunity effect against tumor rechallenge can also be observed following MNP-mediated ablation approaches including gold-nanoshell PTT, titanium oxide-mediated ultrasound, or MNP-enabled RF hyperthermia[76, 122-124].

To further enhance the immunogenic, anti-tumor potential of photothermal therapy, several groups have explored the effects of combining PTT with adjuvants, checkpoint inhibitors, and other immune stimulatory agents. For example, Lu et al. demonstrated that ablation using a metal organic framework combined with a small molecule inhibitor of indoleamine 2,3-dixoygenase (IDO) resulted in more antigen presentation to T cells, more T cells in the tumor microenvironment, and local and distal rejection of tumors[125]. They also observed abscopal effects and systemic, specific cytotoxic T cell expansion when combining a zinc-based particle with PD-L1 checkpoint inhibitors in a 4T1 breast carcinoma model[109].

Ablation of tumor tissue (including clinically with radiotherapy) not only facilitates antigen release but also improves vascular perfusion and chemotherapy penetration into the tumor[105]. The efficacy of combining metallic nanoparticle-induced ablation with chemotherapy and/or immunotherapy has been demonstrated using metallic nanoparticles in preclinical studies[70, 126-129]. In one study, gold nanorods conjugated with Y-shaped CpG facilitated ablation and were co-delivered with doxorubicin. The therapy induced production of IL-6 and TNF-α, resulting in a reduction of tumor volume in vivo[130]. In a separate study, the application of CpG and doxorubicin (Dox) in combination with copper ion-mediated ultrasound was found to improve systemic anti-

tumor immunity more than Dox alone[131]. In addition, mice treated with CuDox-CpG exhibited increased levels of leukocytes, CD4+ and CD8+ T cells as well as decreased levels of immune suppressive MDSCs[70]. These copper-based particles were further tested in combination with ultrasound ablation, CpG, and PD-1 successfully; notably the timing of the applied therapies is critical to their success due to the delicate interplay of activating immunity before releasing tumor antigens via hyperthermia[126]. Together, the evidence suggests that locally applied photothermal and ablative therapies enabled by metallic nanoparticles have the potential to initiate anti-tumor immunity, particularly if combined with immunotherapy and other complementary treatments to further promote systemic anti-tumor responses[132].

Beyond ablative therapies, some groups are leveraging the optical properties of MNPs to interrogate mechanisms of tumor biology and cancer immunotherapy. This mechanistic information can be used to design better therapies. For example, Yang et al. used gold nanoparticles and mass cytometry for single cell detection of immune cells, which illuminated the benefits of a MNP surface modification that improved particle uptake. AuNPs with this modification delivered OVA antigens to DCs, leading to vaccination and tumor reduction in vivo[133]. In addition, non-invasive, MNP-enabled in vivo immune cell tracking techniques have the potential for clinical translation to evaluate patient responses to immunotherapies. Several groups have used metallic nanoparticles with imaging modalities including CT and MRI to monitor immune cells in vivo[134-136]. Recent reviews have discussed metallic nanoparticles for diagnostic and monitoring applications including cancer immunotherapy and the opportunities and challenges for clinical translation[137-144].

Strategy: targeting the tumor immune microenvironment

The tumor microenvironment is often hostile to immune cell viability and function[145]. The local acidity, tumor signaling, and immune suppressive cytokines reduce the potency of cytotoxic T cells[3]. Metallic nanoparticles have been used to deliver agents that alter the microenvironment in order to make it more favorable for immune cell infiltration and subsequent tumor cell recognition and elimination[109].

Gold nanoshell-mediated PTT combined with gene therapy was found to downregulate NF-κβ signaling at the tumor site, reducing the pro-tumorigenic effects of the transcription factor and sensitizing the tumor to subsequent chemotherapy[146]. AuNPs delivering siRNA selectively silenced VEGF expression in tumor cells and tumor-associated macrophages, leading to tumor regression[147, 148]. Metallic nanoparticles have also demonstrated efficacy at targeting immune suppressive regulatory T cells (Tregs), downregulating the suppressive immune cell pathways. Cuprous oxide nanoparticles alter expression of drosophila transcription factor, leading to the induction of myeloid infiltration and subsequent systemic immunity[149].

Another way to alter the interaction of immune cells with tumor cells at the site of the tumor is through delivery of cytokines such as IFN-γ and TNF-α[150, 151]. AuNP-TNF-α particles in particular have progressed to clinical trials[151]. A different AuNP-TNF-α particle formulation has shown promise in combination with other therapies: their vascular disruption properties enable improved delivery of a secondary attack mechanism, such as T cells or chemotherapies[152]. Silver nanoparticles reduced tumor promoting cytokine (IL-1β) signaling resulting in inhibition of tumor growth *in vivo*[75]. In contrast to using signaling molecules to directly impact immunity, Shevtsov et al. attached recombinant heat shock protein 70 to iron

oxide nanoparticles and observed that the particle-delivered chaperone proteins improved tumor outcomes by facilitating antigen trafficking to APCs[73].

Strategy: enhancing cell-based therapies (ex-vivo)

Because the initiation of immunity *in vivo* is complex, some immunotherapy modalities use molecular biotechnology to manipulate immune cells *ex vivo* and reintroduce them to patients[153]. Two general strategies exist in this area. The first is to manipulate the dendritic cells *ex vivo*, and re-administer them to induce activation of T cells *in vivo*[154]. The second is to mature and expand T cells *ex vivo* and overwhelm the tumor's defenses with the sheer number of T cells in the system[37].

Nanoparticles can be used to improve the efficacy of *ex vivo* pulsed antigen-presenting cells including dendritic cells and macrophages. With a NanoAu-Cocktail comprised of AuNP-OVA and AuNP-CpG, pulsed DCs improved protection against foreign antigens[155]. Cho et al. demonstrated that DCs pulsed with iron-oxide zinc-oxide core-shell nanoparticles reduced tumor burden, improved survival, and had the added benefit of functioning as an imaging contrast agent[77]. Macrophages pulsed with cobalt oxide nanoparticles increased antigen-specific T cell responses *in vivo*[69].

Nanoparticles also have the potential to address some of the limitations of adoptive T cell therapy by delivering material *ex vivo*. In one study, iron oxide nanoparticles improved T cell expansion and stimulated T cell activity by spatially bringing together CD3 T cell receptors[156]. In another, Schutz et al. conjugated their magnetic nanoparticles with MHC-IgG and T cell receptors to activate T cells *ex vivo*, enabling a reduction in tumor burden in immunocompromised mice when the modified T cells were administered *in vivo*[157].

1.5. Status of Clinical Translation of Metallic Nanoimmunotherapy

There are currently several ongoing and completed clinical trials that utilize metallic nanoparticles for therapeutic applications. Of these, only one formulation actively employs a component of the immune system, of which we will focus in detail here. Aurimune, also known as CYT-6091, is a 27 nm gold nanoparticle functionalized with thiolated PEG and recombinant human tumor necrosis factor α (rhTNF-α). In 2010, CYT-6091 completed Phase I dose escalation trials in 29 advanced stage cancer patients with very promising results[151]. Phase II studies are planned for pancreatic cancer patients in combination with second line therapies; however, further details have yet to be announced[158]. TNF-α, a well-known inflammatory cytokine, targets tumor-associated vasculature and induces hyperpermeability of the tumor neovasculature as well as massive hemorrhagic necrosis of the tumor[159, 160]. Though TNF-α has not been sufficient in inducing remission on its own, it has been shown to generate a significantly more pronounced anti-tumor response when administered following chemotherapy, compared to chemotherapy alone. This effect is believed to be due in part to the enhanced delivery of the chemotherapeutic agent through the more permeabilized (via TNF-α) tumor vasculature. Unfortunately, a sufficient TNF-α dose often cannot be reached at the tumor site due to dose-limiting toxicities including hypotension, hepatotoxicity, malaise, and fatigue[161, 162].

Hyperthermic limb perfusion has arisen as a promising option to increase the local concentration of TNF-α while limiting systemic side effects, by locally perfusing only the target limb with a high dose of drug[163, 164]. In studies investigating the delivery of TNF-α and melphalan using isolation perfusion, the overall response rate for several cancers –

including carcinoma, sarcoma, and melanoma – ranged from 75% to 100%[163, 165, 166]. CYT-6091 seeks to mimic the success of hyperthermic limb perfusion by preferentially extravasating into the tumor site via the EPR effect, effectively increasing the local concentration of TNF-α while simultaneously limiting its systemic biodistribution. The presence of surface functionalized PEG is thought to help improve delivery to the tumor site by increasing nanoparticle stability and preventing phagocytic clearance via the reticuloendothelial system, all of which contribute to improved circulation times[167, 168].

In the first clinical trial using nanoparticles to systemically deliver TNF-α, CYT-6091 was well tolerated with no maximum tolerable dose reached. Predictable side effects associated with TNF-α (such as fever), were treated with antipyretics or H2 blockers, while hematologic changes such as lymphopenia and a redistribution in circulating lymphocytes resolved on their own after 24 hours. Dose-limiting side effects typically observed with TNF-α alone, including hypotension and hepatotoxicity, were not seen even at doses of up to 600 μg/m2 of CYT-6091 (which exceeds the target dosage of 1 mg of TNF-α per treatment). Area under the curve (AUC) analysis reveals that this is 4-fold higher than the maximum tolerable dose established for TNF-α alone[151].

Ultimately, out of 29 patients, only one patient showed a partial response, with four displaying stable disease. However, these results should be interpreted in light of the studies aims. As a Phase I trial, the purpose of this study was to establish a maximum tolerable dose. In addition, TNF-α treatment should be followed by chemotherapy in order to produce a robust response. From this Phase I trial however, several notable findings were made. Biopsied tissue samples viewed using transmission electron microscopy

suggest preferential accumulation of particle complexes in target tumor tissue but not corresponding healthy tissue or liver, the latter of which serves as the clearance site of the CYT-6091 complexes. In addition, pharmacokinetic data demonstrates that the circulating half-life of TNF-α was approximately 5-fold longer with CYT-6091 than with TNF-α alone (130 minutes vs. 28 minutes respectively). Lastly, immunogenicity data indicate that no anti-TNF-α antibodies were generated against the exogenous recombinant TNF-α protein.

The authors of the study theorize that the strong localization of the CYT-6091 nanoparticle complexes to the tumor site is the result of both the passive EPR effect and active TNF-α targeting to the tumor vasculature. Fenestrations of the tumor neovasculature, which are typically 200 to 400 nm in size, allow for the 27 nm CYT-6091 particles to passively extravasate into the tumor[159, 160, 167, 169, 170]. At the same time, active TNF-α binding to the tumor neovasculature has been shown to dramatically reduce tumor targeting times. In one study, TNF-α reduced the time it took for colloidal gold nanoparticles to localize to the tumor site from 24 hours down to 30 minutes[171]. The state of the tumor vasculature may also play an important role in nanoparticle targeting. In the CYT-6091 study, two patients who did not have their primary tumors surgically removed prior to CYT-6091 administration appeared to have the largest number of nanoparticles aggregates in their biopsied tumor samples. This suggests that an intact tumor neovasculature may improve nanoparticle tumor targeting, in which case CYT-6091 should be administered together with chemotherapy as a neoadjuvant prior to surgical resection of the tumor.

As part of a Phase II trial, the authors would like to test CYT-6091 using a protocol that more closely mimics the isolated limb perfusion protocol that has demonstrated such a robust response. This would involve administering CYT-6091 systemically first, followed 30 to 60 minutes later by chemotherapy[163, 164]. While Phase II trials have not yet begun for their lead therapy CYT-6091, CytImmune has developed several other nanoparticle formulations based on gold. These include an interferon-conjugated nanoparticle (CYT-61000), a gemcitabine-conjugated nanoparticle (CYT-71000), and a second generation Aurimune platform which carries both TNF-α and paclitaxel (CYT-21000)[158].

Other metallic nanoparticles that have advanced to clinical trials for the treatment of cancer but do not directly utilize the immune system include NU-0129, AuroLase, Magnablate, and NBTXR3. NU-0129 is a spherical gold nanoparticle coated with nucleic acids intended to modulate Bcl2L12 gene expression levels in glioblastoma. It entered first-in-human phase 0 safety evaluations earlier in 2017[172]. Though not explicitly an immunotherapy, this platform has demonstrated preclinical efficacy when incorporating immunotherapeutic materials[92]. AuroShell, the therapeutic nanocomplex of AuroLase, is a silica-gold nanoshell coated with PEG designed to thermally ablate solid tumors following exposure to a near-infrared laser[173-177]. Eleven patients with refractory and/or recurrent head and neck cancer were separated into treatment groups and were given increasing doses of AuroShell, increasing 808 nm laser wattage exposure, or both as part of a Phase I trial. Although the study was completed in 2014, the results have not yet been published[178]. Magnablate is an iron nanoparticle complex that operates similarly to AuroLase. A magnet is used to heat the nanoparticle formulation, inducing thermal ablation of the tumor site. As part of an early Phase I trial, the study enrolled twelve patients with

prostate cancer and assessed the anatomical distribution of particle complexes injected directly into the prostate. The study was completed in 2015, however the results for this trial have also not yet been published[179]. Another metallic nanoparticle in clinical development is NBTXR3, a radiosensitizer designed to accumulate in the tumor. Nanobiotix, the company translating the compound, is pursuing Phase I trials in the US for soft tissue sarcomas and head and neck cancer[180]. It should be noted that while ablation induced by these particles is not necessarily a type of immunotherapy, recent studies suggest that the release of antigens from thermally ablated tumor tissue can prime the immune system to induce a systemic and prolonged anti-tumor response[181]. Indeed, this effect has been seen clinically following radiotherapy ablation combined with immunotherapy[115, 116, 182]. Accordingly, a thorough investigation into the role of the immune system with these ablative therapies is warranted.

1.6. Challenges for Translating Metallic Nanoparticle Therapeutics

Inorganic nanoparticles for cancer therapeutic indications face significant hurdles to FDA approval that have yet to be surmounted despite the preclinical progress outlined previously[183]. The FDA has not provided comprehensive guidance on the translation of metallic nanoparticles because so few candidates have entered the clinic for therapeutic applications. Regulation of nanoparticles requires each component to be evaluated for safety, resulting in more expensive trials than those carried out for traditional small molecule therapeutics. Partnerships between investigators and the FDA mediated by the Nanoparticle Characterization Lab aim to lower the barriers to clinical advancement for the companies pursuing these trials and offer preclinical toxicology evaluations to accepted

applicants at no cost to the investigator[184]. However, the expense required to develop these formulations and the lack of an approved metallic nanoparticle precedent have discouraged investigators from pursuing clinical translation. Even if investigators want to pursue clinical translation of MNPs, there are few funding mechanisms and research rewards available for these pursuits. Despite decades of research and billions of federal dollars spent, the first metallic nanoparticle therapeutic has yet to achieve FDA approval[185]. In light of these trends, it has become particularly difficult to justify the pursuit of metallic nanoparticle therapies over biodegradable (polymeric/liposomal) nanoparticle delivery methods. Indeed, many prominent groups that focus on clinical translation have shifted to non-metallic particles when developing translational therapies[86, 92].

Recent evidence about the long term in vivo biocompatibility of metallic nanoparticles compounded with the persistent lack of progress of MNP therapies in clinical trials have contributed to a lack of confidence in the translatability of metallic nanoparticle therapeutics. Aurolase's gold-silica nanoshells have demonstrated clinical safety in Phase I trials[186]. Yet, concerns remain for other gold nanotherapeutic formulations because it is difficult to compare results of biodistribution and toxicity studies of particles across different sizes, shapes, charges, preparations, or delivery routes[187, 188]. In addition, in vitro studies do not always correlate with in vivo data, making proper characterization for toxicity expensive and time consuming to repeat for each new particle[189, 190]. In general, the surface coatings (such as PEG) used to protect engineered MNPs are thought to be degraded in vivo[191]. In regards to the core nanoparticles themselves, most inorganic nanomaterials comprised of silver, zinc, and iron are degraded in vivo; gold, on the other

hand, is traditionally considered to resist degradation and is thus often characterized as bioinert[192]. However, recent long term studies have demonstrated evidence that gold is degraded over long time scales and breaks down into smaller, potentially toxic components[193, 194].

In light of the hurdles facing clinical translation of MNPs, strong justification for using MNPs instead of polymeric and liposomal formulations is necessary for investigators aiming to make a clinical impact. Examples in which MNPs offer unique advantages include therapies that leverage the optical properties of MNPs for ablation or utilize the innate immune stimulation properties of MNPs for cancer immunotherapy applications. Studies examining nanoparticle interactions with the immune system have gained renewed focus due to the recent successes of cancer immunotherapy[28, 195-198]. Preliminary evidence suggests that nanoparticles can elicit humoral and cellular immunity without the assistance of other immune stimulating agents, warranting further evaluation of the processes by which they initiate immune stimulation[65, 82, 97]. In order to improve the uses of nanoparticles for immunotherapeutic applications, further studies are required to better understand how metallic nanoparticles interact with immune environments.

1.7. Conclusion

Metallic nanoparticles have demonstrated success in a variety of immunotherapeutic applications, ranging from delivery of immunomodulating materials (antigens, adjuvants, cytokines, checkpoint inhibitors) to induction of tumor antigen release upon local ablation. Yet, most of this work remains in preclinical stages. The lack of clear regulatory guidance for MNPs,

minimal opportunities for funding translational safety investigations, and few incentives for investigators to pursue these challenging paths have resulted in a void of MNPs in clinical trials. However, evaluating therapies that leverage the uniquely beneficial properties of metallic nanoparticles is an area of opportunity for developing clinically translational metallic nanoparticles for cancer immunotherapy.

Chapter 2

Previous Data & Project Aims

2.1. Introduction

The work compiled in this thesis builds upon previous work performed in the lab since 2010. This section will illustrate the work outlined by previous lab members, which laid the foundation for my work.

2.2. Gold nanoparticles as immunotherapy carriers

Gold nanoparticles improve adjuvant immunotherapy delivery

Gold nanoparticles are excellent carriers of immunotherapies because they naturally biodistribute to the spleen and lymph organs, which contain many of the immune cell populations that initiate immunity. Lin et al demonstrated that 15 nm gold nanoparticles improve delivery of CpG adjuvants, which leads to improved helper T cell activation. The addition of a flexible spacer led to the particles with the best immunity,

likely due to improved availability of the CpG molecules to interact with the TLR-9 receptors upon endocytosis. The AuNP-CpG particles elicited anti-tumor immunity and improved survival compared to PBS and free CpG treatments[85].

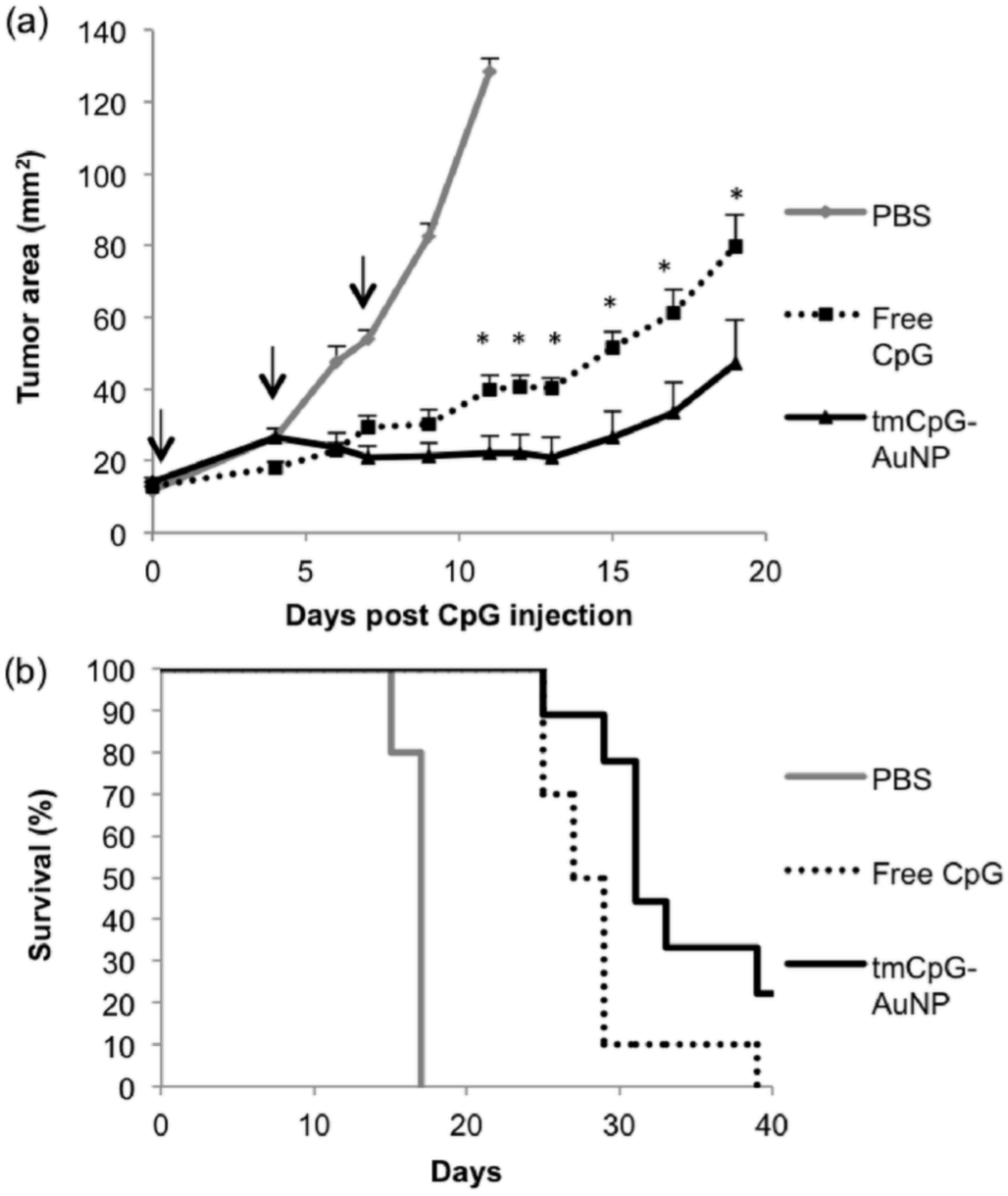

Figure 7. Anti-tumor outcomes following AuNP-CpG, free CpG, or PBS administration. Treatment conditions were injected intratumorally into B16-OVA tumors on days 0,4, and 7[85].

In addition to improving the delivery of CpG adjuvants which boost anti-tumor immunity, our group has previously demonstrated that AuNPs can deliver antigens to initiate a vaccine response.

Synthesis and Characterization of Gold Nanovaccines (AuNVs)

Lin et al demonstrated successful conjugation of peptides to AuNPs resulting in gold nanovaccines of optimal size that elicited immune responses *in vitro*. AuNVs were designed to be smaller than 100 nm for desired mononuclear phagocyte system uptake, and cellular endocytosis properties. 30 nm AuNP cores were first coated with a self-assembled monolayer of 5000 MW COOH-PEG-SH. Peptides were then conjugated to the particles using EDC/Sulfo-NHS chemistry. DLS indicated the particles were smaller than 80 nm, UV-vis spectra showed a 5 nm peak shift following peptide conjugation, and TEM imaging illustrated and altered surface compared to the AuNP-PEG particles (**Figure 8**). When delivered to dendritic cells (DCs) in vitro, the DCs stimulated cytotoxic T lymphocytes and did not suffer from cytotoxicity[58]. Our group then explored the immune modulating properties of AuNVs *in vivo*.

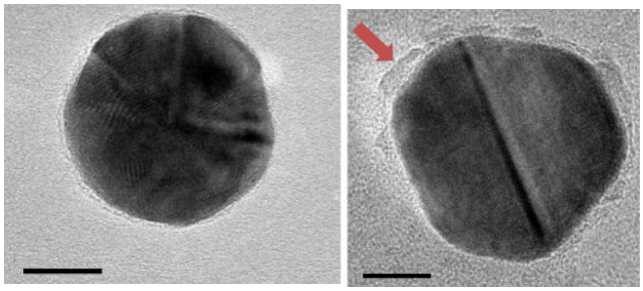

Figure 8. TEM confirms peptide conjugation to AuNVs. The surface of the peptide-coated AuNV appears rougher and thicker (red arrow) than the PEG-coated AuNP above, (scale bar = 10 nm) [58].

AuNP-OVA elicits an antigen specific immune response in vivo

After conjugating OVA to the AuNPs as illustrated above, Almeida et al. subcutaneously (s.q.) injected 2 x 10^{11} AuNV into both flanks of C57/BL6 mice. A boost was administered 10 days later. One week after the boost, the spleens were harvested to perform an ELISPOT assay to determine the antigen specific CD8+ CTL ability to secrete interferon gamma (IFN-γ). High IFN-γ levels correspond to anti-tumor immunity. The group observed that AuNP-OVA elicited an enhanced antigen-specific immune response over all free OVA conditions (**Figure 9**). Co-delivery with the AuNP-CpG adjuvant improves the response with free OVA but not with AuNP-OVA[199]. This result demonstrated that the AuNV system successfully elicited an antigen-specific immune response *in vivo.*

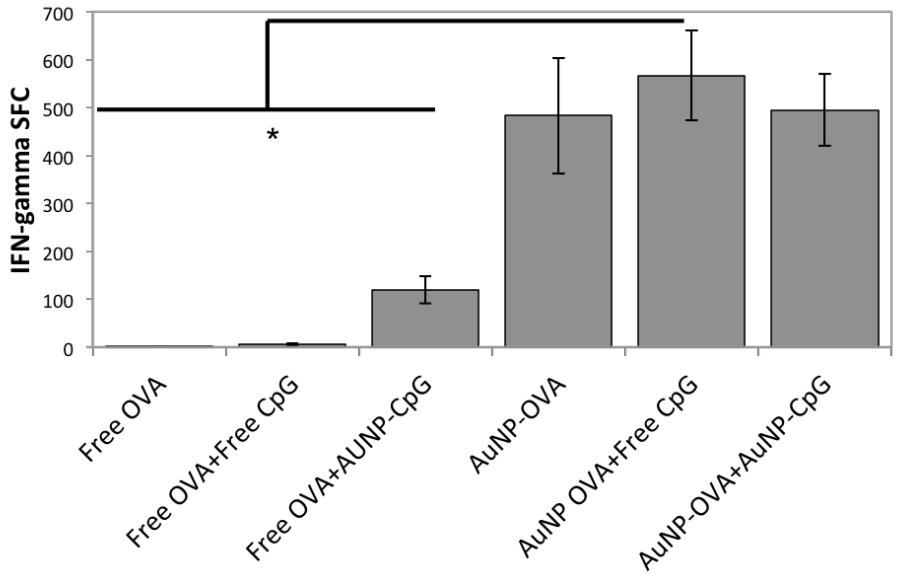

Figure 9. AuNVs enhance antigen-specific immunity *in vivo*. Following subcutaneous treatment with various conditions (doses of 2 × 10^{11} AuNP-OVA, 10^{12} AuNP-CpG, 50 μg OVA and 4.7 μg CpG), spleens were harvested to determine the number of IFN-γ spot forming splenocytes as measured in the ELISPOT assay. *, $p<0.02$ [199].

AuNP-OVA prevents tumor occurrence and extends survival in prophylactic model

Based on the *in vivo* antigen-specific immunity elicited by AuNP-OVA, Almeida et al. then examined if these AuNVs could prevent tumor formation in a prophylactic model. Prime and boost doses of various conditions (PBS, OVA, OVA + AuNP-CpG, AuNP-OVA, AuNP-OVA + AuNP-CpG) were administered s.q. 10 days apart as above with each dose consisting of 2 × 1011 AuNP-OVA, 1012 AuNP-CpG, 50 μg OVA, or 4.7 μg CpG. On day 17 in this study, mice were injected with 105 B16-OVA cells subcutaneously. AuNP-OVA vaccination prevented tumor growth and prolonged survival for the duration of the study (Figure 3). In contrast, free OVA did not perform better than control, and free OVA + adjuvant delayed but did not prevent tumor growth[199]. These results demonstrate the success of AuNP-OVA in a prophylactic tumor challenge model, illustrating the vaccine attributes of AuNVs.

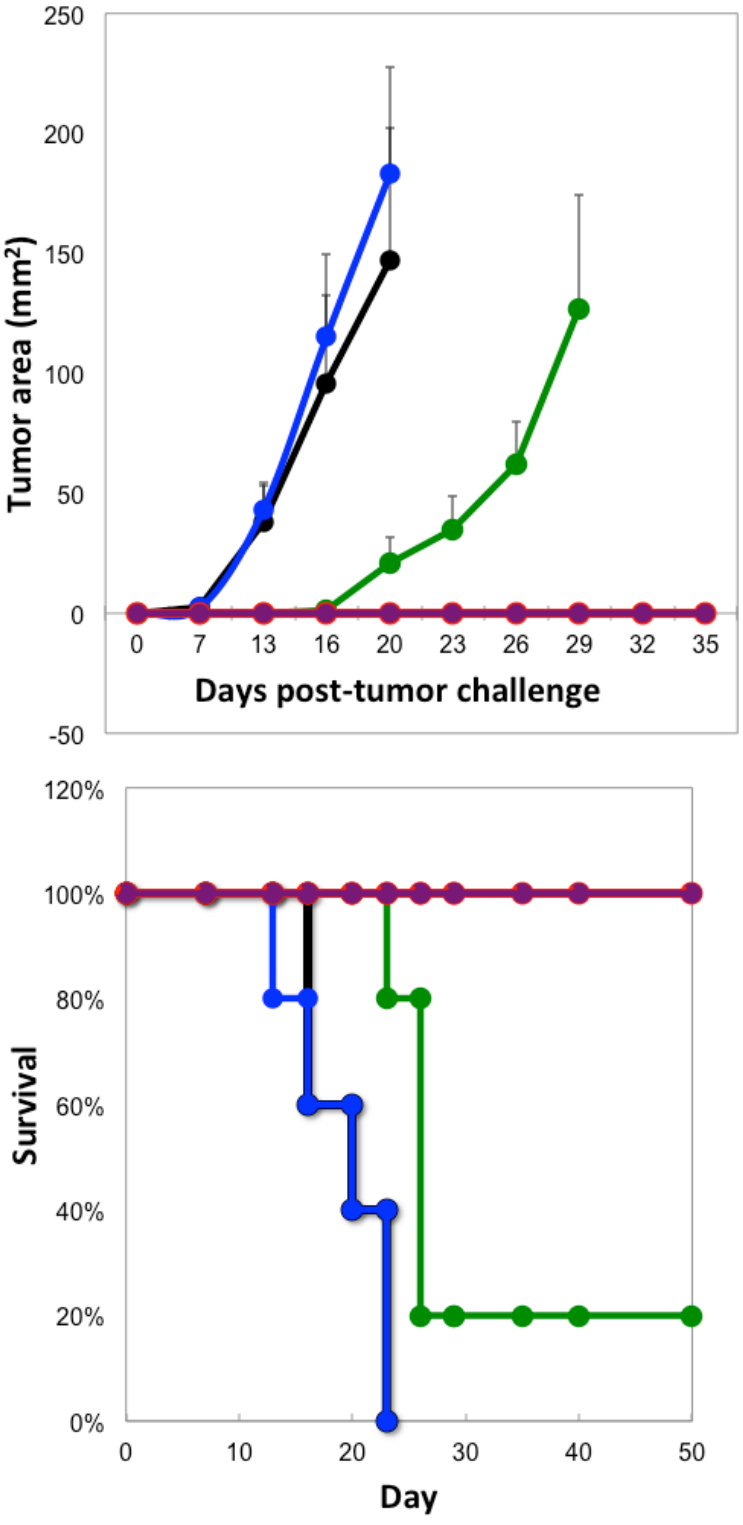

Figure 10. AuNP-OVA prevents tumor occurrence and extends survival in prophylactic model.
*, $p<0.02$ [199]

AuNP-OVA prevents tumor growth, extends survival in therapeutic model

After observing that AuNP-OVA had demonstrated the capacity of AuNVs to function as a melanoma vaccine, we then explored the ability of AuNVs to treat established tumors. C57/BL6 mice were first implanted s.q. with B16-OVA tumors that were grown to 5 mm2. After tumors were established, mice were then treated s.q. with priming and boosting dosages of various conditions (PBS, OVA, OVA + AuNP-CpG, AuNP-OVA, AuNP-OVA + AuNP-CpG). AuNP-OVA treatment conditions prevented further tumor growth and extended survival (**Figure 11**). Again, our group observed that AuNP-OVA did not benefit from codelivery with adjuvant. Collectively, these experiments demonstrated that the AuNV system elicited an immune response in vivo, prevented tumor growth in a prophylactic and therapeutic applications, and extended survival in the B16-OVA melanoma model system[199].

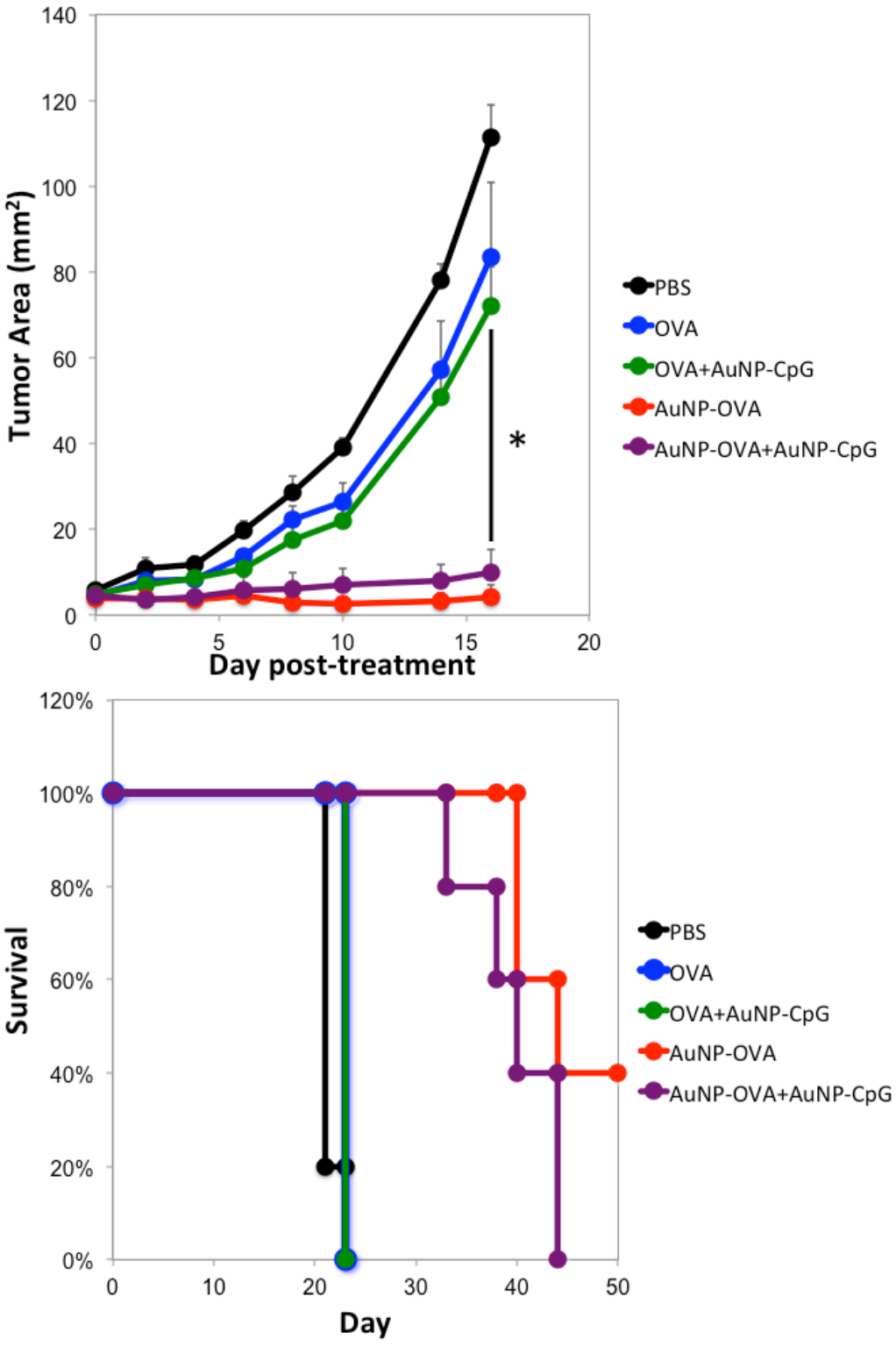

Figure 11. AuNP-OVA limits tumor progression and extends survival. Mice were injected subcutaneously with B16-OVA tumor cells and then were given two doses of various conditions. Tumor size and survival were measured. *, $p<0.02$ [199].

These previous results demonstrated that gold nanovaccines have the potential to elicit immunity against a tumor in both a prophylactic and therapeutic model system. However, these results are limited by the fact that ovalbumin, the antigen trained for recognition, is not normally expressed in murine tumors and was artificially incorporated into the tumor cell lines in order to test the efficacy of the vaccine. The next step of this work was to evaluate the ability of gold nanovaccines to elicit immunity against an endogenous tumor antigen. These studies can be found in Chapter 3.

2.3. Gold-nanoshell ablative photothermal therapy

Another way to leverage gold nanoparticles for vaccination against tumor antigens is to use ablative therapy to release the tumor antigens and danger signals for recognition and uptake by immune cells. In 2013, Bear et al reported the effects of PTT on local and metastatic tumors and interrogated how the coadministration of adoptive T cell immunotherapy impacted tumor outcomes[124].

PTT eliminates primary tumors

Gold nanoparticle-mediated photothermal therapy (PTT) destroys B16-Ovalbumin (OVA) tumors in mice. $5x10^5$ B16-OVA tumor cells were injected subcutaneously into the flanks of C57BL/6 albino mice. Hollow gold nanoshells were injected intratumorally when the tumors reached 5x5 mm. A near infrared laser was applied to the HGN-containing tumors, resulting in elimination of the primary tumor (**Figure 12**)[124].

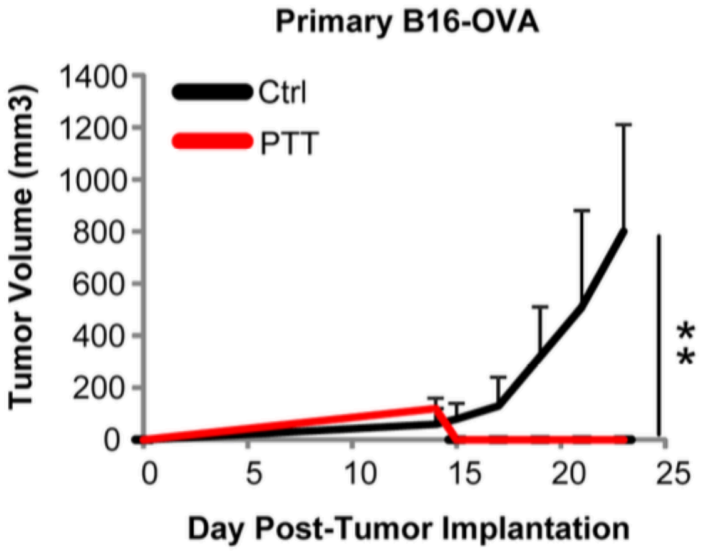

Figure 12. PTT Ablation elimunates primary tumor. *p<0.01 [124].

PTT elicits anti-tumor immunity

Photothermal therapy (PTT) exhibits weak, systemic anti-tumor immunity. $5x10^5$ B16-OVA tumor cells were injected subcutaneously into both flanks of C57BL/6 albino mice. PTT treatment was applied to only one tumor. The untreated contralateral tumors grew more slowly in the mice that received PTT than in the mice that received a PBS injection ($p<0.05$, **Figure 13**), indicating that PTT elicits anti-tumor immunity[124].

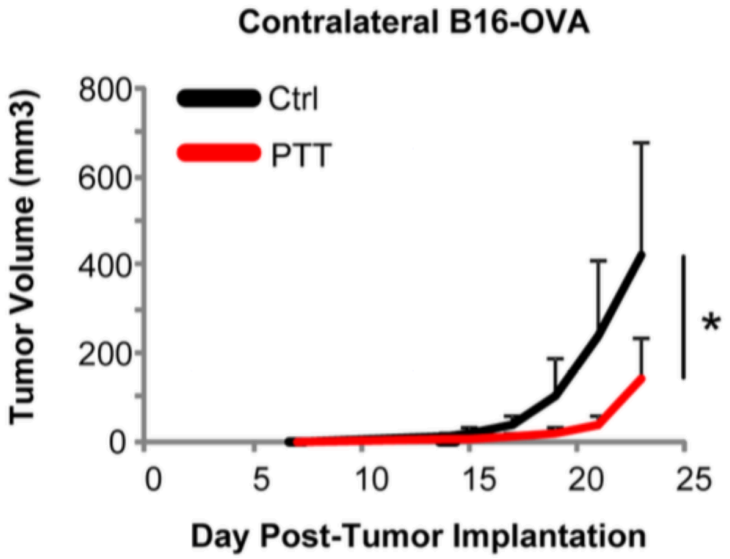

Figure 13. PTT slows growth of untreated tumor. The contralateral B16-OVA tumor has slower growth than the contralateral tumor of an untreated mouse. *p<0.05. Interestingly, this result is not observed wth B16-F10 tumors[124].

PTT also elicits pro-tumor immune responses.

To model metastases, C57BL/6 albino mice were injected with B16F10 cells subcutaneously (s.q.) on the flank on day 0 to establish the primary tumor and intravenously (i.v.) on day 6 to establish lung metastases. Mice treated with PTT developed exacerbated lung metastases compared to PBS controls (**Figure 14**) [124].

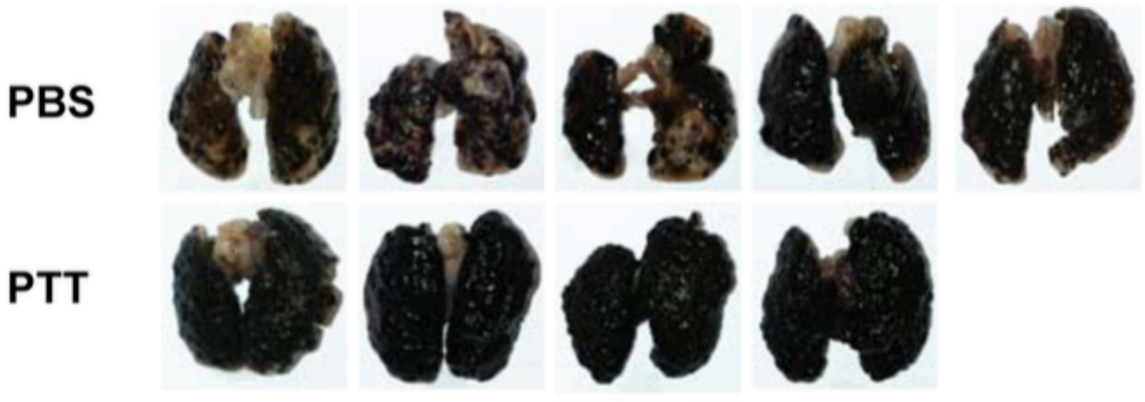

Figure 14. PTT induces lung metastases. Lungs of mice treated with PTT have more tumors (a) quantified by increased weight (b) compared to PBS control[124].

The proposed mechanism of exacerbated metastatic tumor burden following PTT is the expansion of suppressive immunes such as MDSCs cells. Following PTT, more suppressive MDSCs are found in metastatic tumors than in PBS controls (**Figure 15**) as measured by flow cytometry[124].

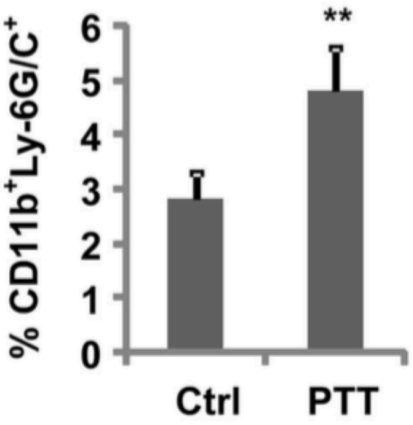

Figure 15. MDSC cells are expanded following PTT. **p<0.01 [124].

The incorporation of adoptive T cell immunotherapy mitigates some of the exacerbation of lung metastases seen following PTT. When PTT was combined with adoptive T cell therapy, the contralateral tumors grew more slowly on average than ATCT alone. This outcome suggests PTT is releasing tumor antigens and ATCT is promoting an anti-tumor immune response against those released antigens.

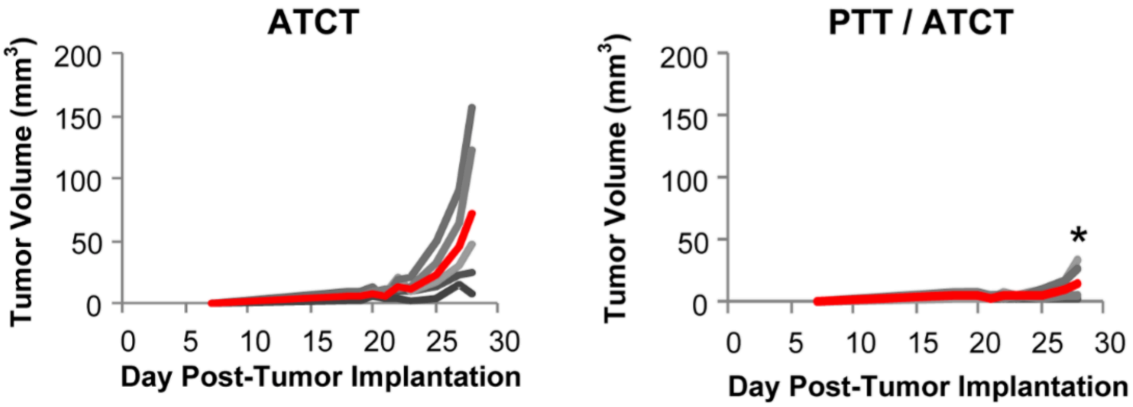

Figure 16. Adoptive T cell thearpy combined with PTT slows tumor growth of contralateral tumors. The PTT treatment enabled by hollow gold nanoshells initiates systemic anti-tumor immunity which is supplemented by Adoptive Transfer Cell Thearpy such that contralateral tumors have slower growth than ATCT alone . *p<0,05 [124].

2.4. Conclusion

Previous members of the Drezek Lab laid a strong foundation exploring the use of gold nanoparticles for initiating cancer immunotherapy. AuNPs deliver adjuvants, antigens, and release tumor antigens via photothermal ablation. Though the projects approach the immunotherapeutic strategy from a slightly different perspective, each relies on the unique cancer immunotherapeutic offered by combining gold nanoparticles with immunotherapeutics. Each of these projects began the critical work

of demonstrating proof-of-concept efficacy of gold nanoparticles for initiating immunity. However, these studies were limited by the use of B16-OVA tumors throughout these studies, which are not a clinically relevant model due to the transgenic antigens induced in the tumor cells which make vaccination easier. My goal was to evaluate these gold nanoimmunotherapy platforms using more clinically relevant model systems in order to better demonstrate the potential of gold nanoimmunotherapy platforms toward clinical translation.

2.5. Project Aims

In order to demonstrate clinical translatability of gold nanovaccine platforms, I pursued two specific aims:

1. Demonstrate gold nanoparticle delivery of a clinically relevant melanoma peptide antigen and illustrate improvement of antigen-specific immunity (**Chapter 3**)
2. Combine gold-nanoshell mediated photothermal tumor ablation with CpG, an easy to administer immunotherapy currently undergoing clinical trials (**Chapter 4**)

Upon successful completion of these aims, we will demonstrate the potential of gold nanoparticles to elicit *in situ* vaccination outcomes across several platforms and immunotherapeutic approaches.

Chapter 3

Gold Nanovaccines

3.1. Abstract

Cancer vaccines may become an affordable, off-the-shelf therapy for immunogenic cancers. However, one limitation of these peptide antigen vaccines is their poor delivery to relevant immune organs, such as the spleen or lymph tissues. Sufficient delivery of immunotherapeutic vaccines to immune organs is necessary for initiation of a strong anti-tumor immune response. We previously demonstrated that gold nanoparticles (AuNPs) improve antigen delivery, enable anti-tumor immune responses and extend survival in a mouse melanoma B16-OVA model system. To evaluate if AuNPs initiate anti-tumor immunity in a more clinically relevant B16-F10 model, we assessed the ability of AuNPs to deliver an endogenous, tumor-associated antigen, TRP-2. We first modified our synthesis approach to accommodate the hydrophobic peptide. After administering the AuNP-TRP-2 vaccine *in vivo*, we observed an increase in activated T cells in the spleen, indicating that

gold nanovaccines can initiate immunity *in vivo* in a more clinically relevant mouse melanoma tumor model. Future work combining gold nanovaccines (AuNVs) with other complementary immunotherapies could boost this AuNV-initiated response toward therapeutic outcomes. To our knowledge this result is the first and only example of gold nanoparticle-enabled T cell activation against an endogenous tumor antigen *in vivo*.

3.2. Introduction

The goal of cancer immunotherapy is to reactivate the immune system to recognize and treat tumor cells[4]. Cancer vaccines provide antigen presenting cells (APCs) with a relevant tumor antigen, leading to activation and expansion of immune cells that carry out cytotoxic mechanisms against cells expressing that antigen[9]. The initiation of an immune response originates with APCs such as Dendritic Cells (DCs), the primary target cells of cancer vaccination strategies[41]. DCs uptake antigens and present them on their surface, where CD8+ cytotoxic T cell receptors interact with them[43, 200]. Subsequently, the T cells activate, differentiate and expand such that millions of antigen-specific cytotoxic T lymphocytes (CTLs) survey the body. When they recognize a cell expressing the antigen, CTLs release cytotoxic material into the target cells to initiate cell death[6].

There are several interactions along this pathway where a vaccine strategy can be hindered or boosted. During antigen presentation in the spleen or lymph tissues, receptors such as the immune checkpoint CTLA-4, can interfere with the ability of APCs to activate T cells[33]. Such inhibition could be prevented through administration of α-CTLA-4 or similar checkpoint inhibitor therapies, which support T cell activity[16, 201]. Most cancer

vaccine strategies require an adjuvant to boost the response initiated by the antigen[14]. Adjuvants support anti-tumor immunity by promoting Th1 directed responses as well as cytokine and interferon production, which together support activation of dendritic cells, CD4+ helper T cells, natural killer cells, and cytotoxic T cells[14]. Once the activated CTLs migrate out of the lymph tissues, additional hurdles can hinder the CTLs from performing their intended cytotoxic function. Some of these hurdles include migration to the tumor site, accessing tumor cells, and surviving the harsh conditions in the tumor microenvironment[3]. Tumors may physically limit T cell access when insulated by thick extracellular matrix. In addition, tumor microenvironments often threaten T cell viability when characterized by a depletion of nutrients or low pH[15]. Even if activated CTLs successfully reach the tumor cells, individual tumor cells can deactivate T cells through the expression of immunosuppressive protein ligands such as PD-L1 on their surface[18]. Existing clinical therapies such as α-PD-1/PD-L1 checkpoint inhibitors interfere with these interactions and restore T cell function[202]. Other therapies are under development to reduce the hurdles placed on T cells by the tumor microenvironment and could be complementary to cancer vaccination strategies[25].

An immune response must be initiated using an antigen that is specific to the tumor to avoid autoimmunity[7, 203]. Thus, the selection of this tumor antigen is an important step in the development of clinically relevant cancer vaccines[78]. Tumor antigen selection is based on the tumor profile, and most are historically characterized by tissue of origin. Melanoma is a common model for studying cancer vaccines because it is a very immunogenic tumor with several decades of literature describing the tumor biology and other therapeutic strategies[204]. Notably, the first checkpoint inhibitors were developed

and first indicated for melanoma[16]. Several tumor-associated antigens have been previously identified in melanoma[204, 205]. Some antigens such as Tryptophanase-related proteins (TRP), MART-1, tyrosinase, and gp100 are upregulated in both mice and human melanomas and are often used to evaluate the efficacy of novel cancer vaccine strategies [206-213].

Previous attempts to administer peptide antigen cancer vaccines to patients have yielded extremely low response rates because the small peptides degrade soon after systemic administration and cannot reach the immune tissue targets to initiate a sufficiently strong immune response[22, 44]. Nanoparticles offer a solution to this hurdle because they can transport antigenic peptides to the relevant APCs in the spleen[60, 214]. Gold nanoparticles from 20-80 nm have been shown to exhibit such delivery to the spleen in several studies[60, 80, 191, 215, 216]. Gold nanoparticles are also advantageous carriers of cancer vaccines due to the range of facile chemistries available to conjugate therapeutic molecules to their surface[28].

Previously, we showed that gold nanoparticles improved delivery of antigens as illustrated with a proof-of-concept antigen, ovalbumin (OVA), which led to a therapeutic reduction of tumor growth *in vivo*[83]. The OVA antigen was transduced into the B16 melanoma cell line prior to implantation of the tumor cells in mice. The AuNP-OVA therapy initiated a stronger cytotoxic T cell response than OVA alone, slowed tumor growth, and extended survival of mice with B16-OVA melanoma tumors. These results showed that improved delivery of antigens to antigen presenting cells led to the production of robust cytotoxic T lymphocytes that reduced tumor growth and improved survival following both

prophylactic and therapeutic administration. However, since OVA is not naturally expressed in mice or mice tumors, training the immune system to recognize this exogenous antigen is easier than training immunity against an endogenous antigen because the immune system has tolerance mechanisms to prevent autoimmunity against self-antigens[203, 217]. We sought to further the development of gold nanovaccines by evaluating their ability to initiate an immune response when incorporating an endogenous, melanoma-associated antigen, such as Tryptophanase-related protein 2, or TRP-2[218].

The transition from OVA to TRP-2 represents an important step toward the goal of clinical translation of gold nanoparticle vaccines for cancer immunotherapy. For the studies described herein, we evaluated the efficacy of AuNVs to induce an immune response when delivering an antigen that is overexpressed clinically in melanoma. Because some healthy melanocytes express TRP-2, immune memory mechanisms may dampen attempts to initiate strong cytotoxic immunity against TRP-2 expressing-cells[219]. While some polymeric nanoparticle formulations and other vaccine strategies have successfully elicited anti-tumor immunity upon delivery of TRP-2, to our knowledge no gold nanoparticle cancer vaccines delivering tumor-associated antigens have been reported[211, 220].

3.3. Methods

Particle synthesis

AuNP-TRP-2 particles were initially synthesized using the same protocol as previously reported[83]. After observing particle aggregation and instability with this method, we adjusted the protocol to the following '2 Step with 1% Tween' protocol, which

is nearly identical to our previously reported protocol with the exception of the bolded sections:

First, 10800 uL of 30 nm AuNP colloid (Ted Pella, Redding CA; 2x10^11 particles/mL) was combined with 1080 uL of autoclaved Mili-Q water and 120 uL of 0.5 mM HS-PEG5000-COOH (Nanocs, Woburn, MA) in a 50 mL tube (Corning, Corning NY). This mixture was incubated overnight on a nutator. The next day, the solution was slowly aged with 1500 uL 0.1 M Sodium Phosphate (Teknova, Hollister, CA) containing 1%Tween 20 (Sigma, St. Louis, MO) and 1500 uL of 1M Sodium Chloride (Sigma, St Louis, MO) by adding increasing amounts of salt (200 uL, 200 uL, 400 ulL 700 uL of each salt solution with at least 30 minutes in between additions). After a second overnight incubation on the nutator, the particles were aliquotted in 2 mL round bottom tubes (Eppendorf, Hamburg, Germany) and washed twice with PBS at 7,000g for 20 minutes. The particles were reconstituted together in a new 50 mL tube containing 10 mL of 0.1 M MES at pH6 (Thermo, Waltham, MA). Next, 4.25 mg of EDC (Thermo, Waltham, MA) in 500 uL of MES followed by 6.4 mg of Sulfo-NHS (Thermo, Waltham, MA) in 500 uL of MES were added to the particles. The mixture was incubated for 15 minutes on the nutator. **The particles were again washed twice** in 2 mL tubes and then resuspended in 10 mLs of DPBS (Life Technologies, Carlsbad, CA). The TRP-2 peptide (1 ug/uL; Genemed Synthesis, San Antonio) was suspended in 500 uL of autoclaved Milli-Q water **with 1% Tween 20** and added to the particles. Following a one hour incubation period, 500 uL of 10 mM NH2OH (Aldrich, St. Louis, MO) was added to quench the reaction. Following another one hour incubation period, the particles were washed three times in 2 mL tubes and reconstituted in DPBS.

Another particle synthesized was AuNP-TRP2-DNP, which included an immune adjuvant, dinitrophenol (DNP). The DNP was covalently linked to the C terminus of the TRP-2 peptide (Genemed Synthesis, San Antonio, TX). The TRP-2-DNP was incubated with the AuNP-PEG-COOH in lieu of TRP-2 using the same syntheses method as listed above.

Particle characterization

After synthesis, we confirmed particle conjugation, concentration, and size. The sample was diluted 1:10 and analyzed with an Agilent Cary 60 UV-vis spectrophotometer. Comparing the peak of the sample to the peak and known concentration of the bare particles, we determined the concentration of the sample. The sample was further diluted 1:100 for evaluation of particle size using Dynamic Light Scattering (Malvern Zetasizer Nano ZS at 25°C).

Evaluating ability of gold nanovaccines to elicit immunity in vivo

C57BL/6J albino mice were purchased from Jackson Labs and housed at Rice University's pathogen free facility. All studies were approved and conducted in accordance with the Institutional Animal Care and Use Committee at Rice University. 50 ug of free TRP-2 peptides, 2 x 1011 AuNP-TRP-2 particles, or 2 x 1011 AuNP-TRP-2-DNP particles were injected subcutaneously into mice on Day 0. The same concentration was then repeated on day 10 to boost the response. On Day 21, the mice were sacrificed and spleens extracted for ELISpot analysis[83]. Spleens were first harvested and homogenized with a 70 um strainer (Falcon, Corning, NY). After removal of red blood cells (Sigma RBC lysis buffer), the remaining immune cells were counted (Nexcelom, Lawrence, MA). Cells from each mouse were plated at an equal density into several rows in a plate per spleen. Cells

were then incubated with TRP-2, RPMI complete media (negative control), or phorbol myristate acetate and ionomycin (positive control) overnight. The following day, the plates were washed then incubated with the detection antibody followed by the reporter antibody. After a final wash, the plates were dried, punched, and sent for reading of the number of spot forming cells observed in each well (ZellNet, New York, NY).

We also evaluated the ability of AuNP-TRP-2 to act as a therapeutic cancer vaccine. B16F10 tumor cells (ATCC, Manassas, VA) were cultured in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Life Tech) and 1% penicillin/streptomycin (Thermo, Waltham, MA). Mice were implanted with 5 x 106 B16F10 tumors on day 0. A dose of 2 x 1011 AuNP-TRP-2 gold nanovaccines were administered on days 7 and 17. Tumor size was evaluated with digital caliper until the mice reached humane endpoints based on tumor size (>1cm2).

3.4. Results

Establishing AuNV synthesis protocol compatible for conjugation of TRP-2

The first hurdle to overcome when transitioning from AuNP-OVA to AuNP-TRP-2 was adjusting the synthesis to be compatible with the TRP-2 peptide. Changing the nanoparticle surface directly affects how the particles interact with their environment[54]. OVA has a neutral net charge with few reactive side chains to disrupt the shape of the particle or impact the chemical interactions with the environment surrounding the AuNP-OVA particle (**Table 2**). On the other hand, the terminal amino acids of the TRP-2 are

hydrophobic, which reduces overall particle stability such that the AuNP-Trp2 particles aggregate readily in solution (**Figure 7**).

Table 2. Peptide characteristics of ovalbumin and TRP-2.

Name	Sequence	Length	Charge	Isoelectric Point
OVA	SIINFEKL	8	0	6.81
TRP-2	SVDFFVWL	9	- 1	3.05

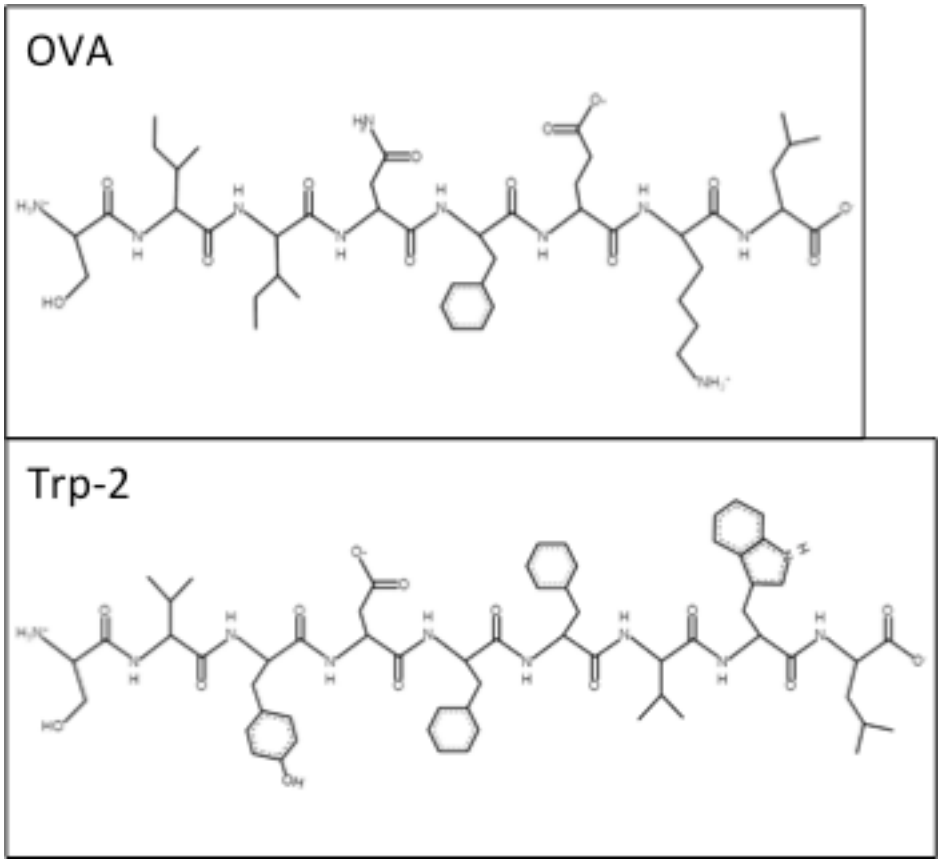

Figure 17. Amino acid sequence of OVA and TRP-2. The OVA and TRP-2 peptides are illustrated from N-terminus to C-terminus. The characteristics of the amino acids near the C-terminus of the peptide drive the particle characteristics upon conjugation. TRP-2 has more hydrophobic side chains and has net charge of -1.

We began by synthesizing the particles using the same method used for AuNP-OVA synthesis (**Figure 8**). Briefly, thiolated PEG-COOH spontaneously forms a monolayer on the surface of AuNPs when incubated overnight. The COOH terminus is then available for EDC/Sulfo-NHS chemistry to link the N-terminus of the peptide to the intermediate PEG layer. However, using this previously reported method resulted in AuNP-TRP-2 particles that were too unstable for further analysis or application. The hydrophobic interactions on the surface of the particles cause the particles to aggregate such that they precipitate out of solution.

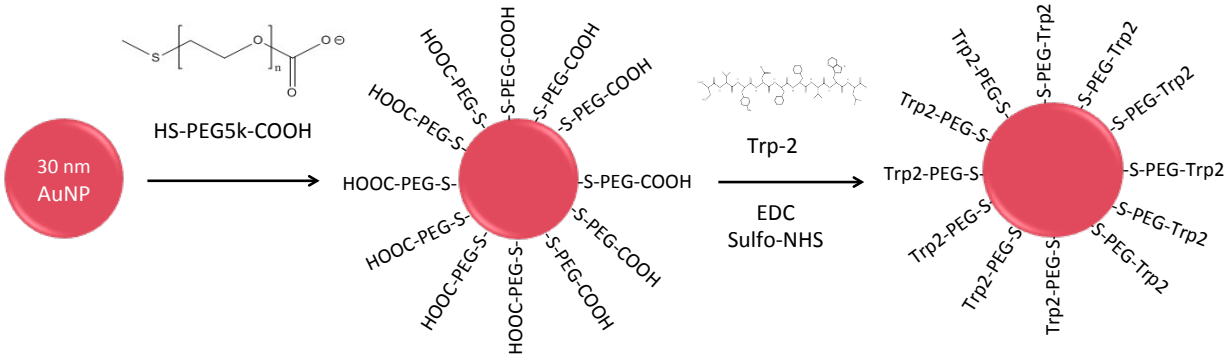

Figure 18. Gold Nanovaccine Synthesis. Thiol-functionalized PEG with a carboxyl terminus spontaneously assembles a monolayer on the 30 nm gold nanoparticles. The addition of EDC and Sulfo-NHS enables the conjugation of the N-terminus of the TRP-2 peptide to the carboxyl group exposed on the AuNP-PEG particles.

To address the observed particle instability, we introduced Tween 20 into the synthesis upon addition of the peptides to the AuNP-PEG-COOH. Acting as a detergent, the Tween made it more difficult for hydrophobic interactions between individual TRP-2 peptides to cause particle aggregation (**Figure 3**). We acknowledge that some studies suggest that Tween 20 may possess adjuvant properties capable of enhancing cytotoxic T lymphocyte activity; though, much of the detergent would be washed out in the subsequent

synthesis steps[221]. We also evaluated how reducing the concentration of Tween 20 added with TRP-2 affected particle stability visually (**Figure 9**) and with characterization methods (**Figure 10**), but maintained 1% Tween for subsequent studies.

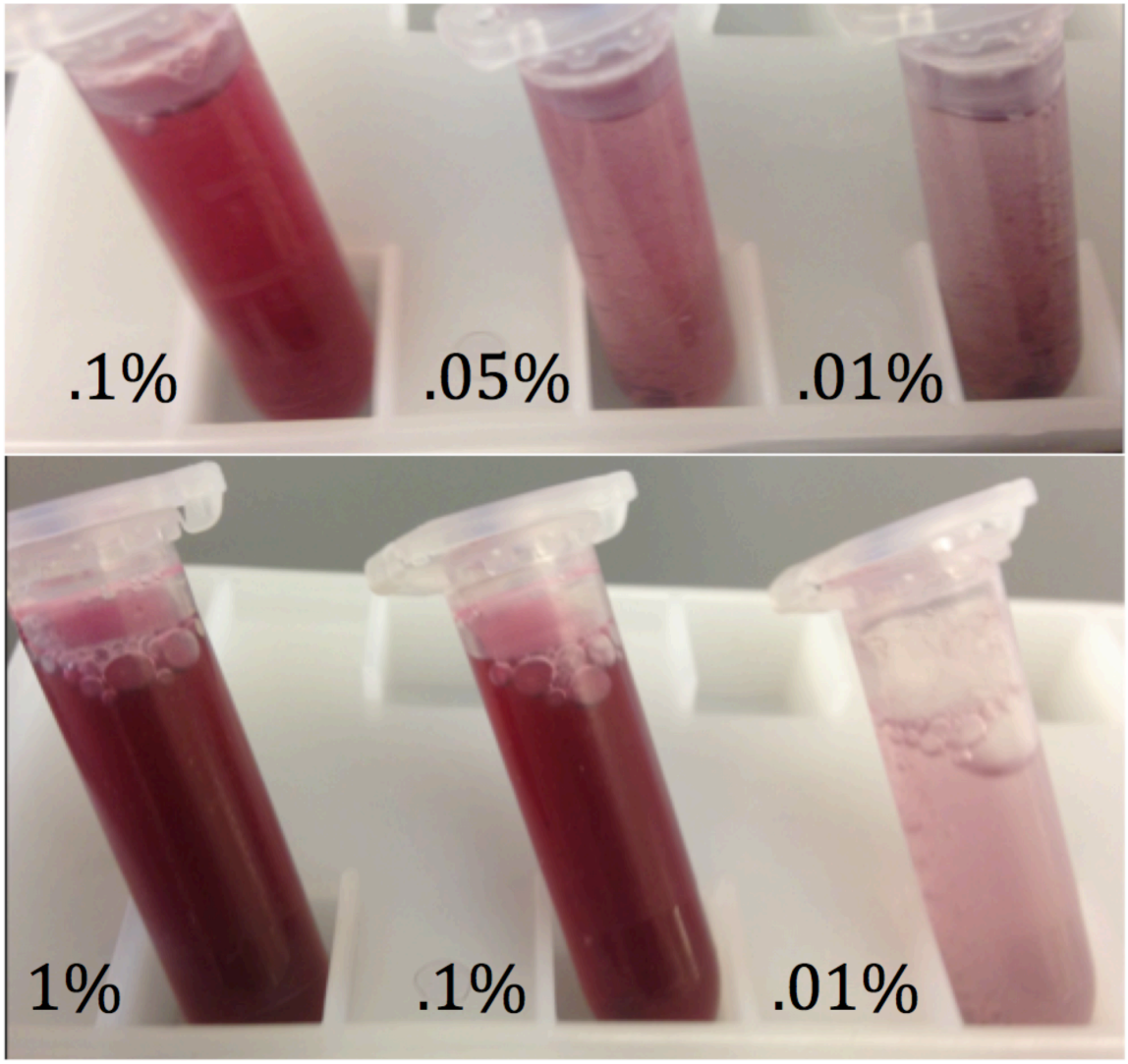

Figure 19. Visualization of particles with decreasing amounts of Tween 20 when synthesized with 1 Step (top) or 2 Step (bottom) methods. The amount of Tween included in the volume of Trp-2 added to the AuNP-Trp-2 solution (after activation with EDC/Sulfo-NHS) was decreased by a factor of 10 and particle stability was evaluated visually. The particles synthesized by the 1 Step method were only stable at 1% Tween; at 0.1% Tween the particles began to become unstable and aggregate. The particles synthesized following the 2 Step method were stable at 1% and 0.1%. Particles remained in solution at 0.01% Tween though the yield was too low to be usable.

Another important consideration for the conjugation of TRP-2 versus OVA was the presence of arginine side chains in TRP-2 that are absent in OVA (**Table 2**). These side chains have a carboxyl group that is susceptible to activation during the EDC/Sulfo-NHS reaction, which could lead to more unpredictable branched peptide structures instead of a linear addition and could cause more batch-to-batch variations. We added a wash step after the addition of EDC/Sulfo-NHS to attempt to mitigate this branched chemistry and called this protocol the '2 Step' method.

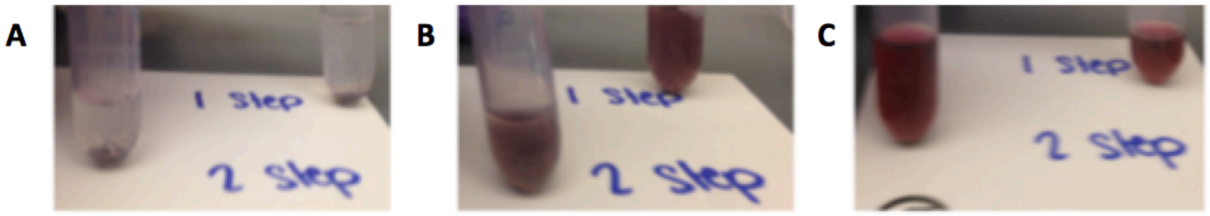

Figure 20. Images of AuNP-TRP-2 following addition of Tween. Under the previously reported synthesis protocol, particles aggregated after addition of TRP-2 to the particle surface (A) and still appeared grainy and prone to aggregate immediately after sonication (B). With the addition of Tween 20 into the synthesis (C), the particles were more monodisperse and stable over time.

We evaluated the particle stability and immunogenicity of two reactions: '1 Step' in which the particles were not washed after the addition of EDC/Sulfo-NHS, thus leaving any carboxyl groups available to react (at the end of the PEG, at the end of the linearly added peptides, or at any side chain with a carboxyl or amine side chain), and '2 Step' in which the particles were washed after adding EDC/Sulfo-NHS, thus limiting the reaction to the addition of a single peptide on the PEG-carboxyl terminus of the particles. We characterized the particles synthesized with each method using UV-vis and DLS (**Table 3**).

The 2 Step method in which TRP-2 was diluted in deionized water with 1% Tween yielded the most monodisperse solution with a small size increase compared to AuNP-PEG-COOH.

Table 3. AuNP-TRP-2 characteristics following different synthesis methods.

Sample	Size (nm)	Polydispersity
Bare 30	34	10%
AuNP-PEG-COOH	78	17%
AuNP-TRP-2 2 Step 1% Tween	84	18%
AuNP-TRP-2 1 Step 1% Tween	98	25%
AuNP-TRP-2 2 Step PBS	895	39%
AuNP-TRP-2 1 Step PBS	863	34%

The 2 Step method in which TRP-2 was diluted in deionized water with 1% Tween yielded the most monodisperse solution with a small size increase compared to AuNP-PEG-COOH. Further particle characterization can be seen in **Figure 11**.

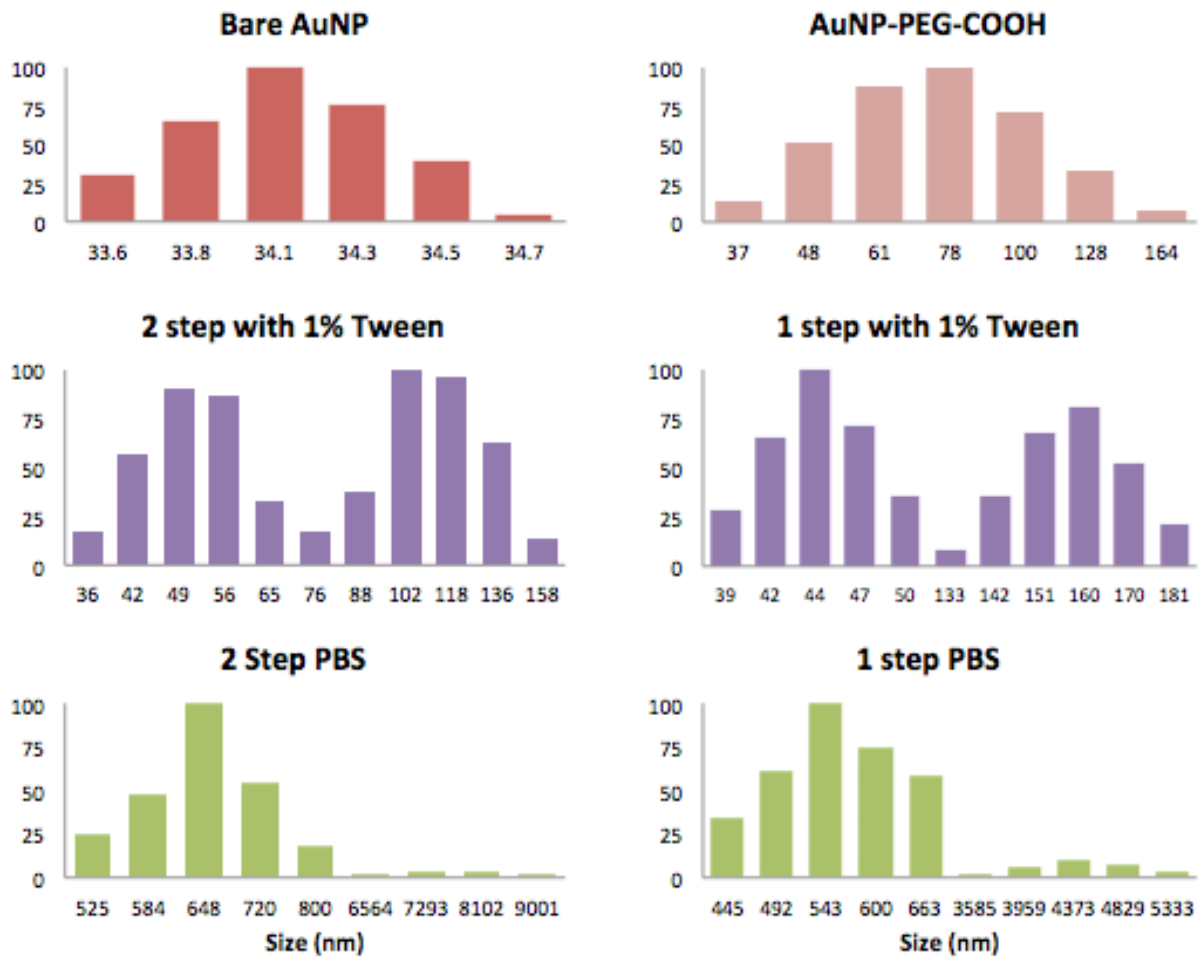

Figure 21. Particle sizes of AuNP-Trp-2 with different synthesis methods. The distribution of the amount of particles represented across a range of sizes is displayed. Bare AuNPs were around 30 nm as expected. PEGylation increased the particle sizes to approximately 78 nm. The addition of Trp-2 with Tween to PEGylated AuNPs illustrated two particle species: some conjugated successfully and others too small. The particles synthesized without Tween, however, had extremely large particle readings suggesting high levels of particle aggregation.

This 2 Step method with Tween has been used to synthesize AuNVs with a range of peptide properties and is more universal than the method previously reported (**Table 4**)[58].

Table 4. Amino acid characteristics of melanoma-associated peptides. Amino acid sequences, length, net charge, and isoelectric point listed for melanoma-associated peptides that were successfully conjugated to AuNPs using the 2 Step with Tween method.

Name	Sequence	Length	Charge	Isoelectric Point
OVA	SIINFEKL	8	0	6.81
Trp2	SVDFFVWL	9	- 1	3.05
Gp100	KVPRNQDWL	9	+1	10.14
Tubb3	FRRKAFLHWYTGEAMDEMEFTEAESNM	27	-3	4.35
Kif18b	PSKPSFQEFVDWEKVSPELNSTDQPFL	27	-3	3.83

We have conjugated longer peptide sequences of neoantigens (nAg) found in B16.F10 melanoma tumor cells to AuNVs and have characterized their properties. Upon conjugation of Kif18b or tubb3 neoantigens to AuNP-PEG-COOH, we observe stable particle formation, a 2 nm red shift on uv-vis, and a population of particles larger than those observed in AuNP-PEG-COOH prior to conjugation (**Figure 12**).

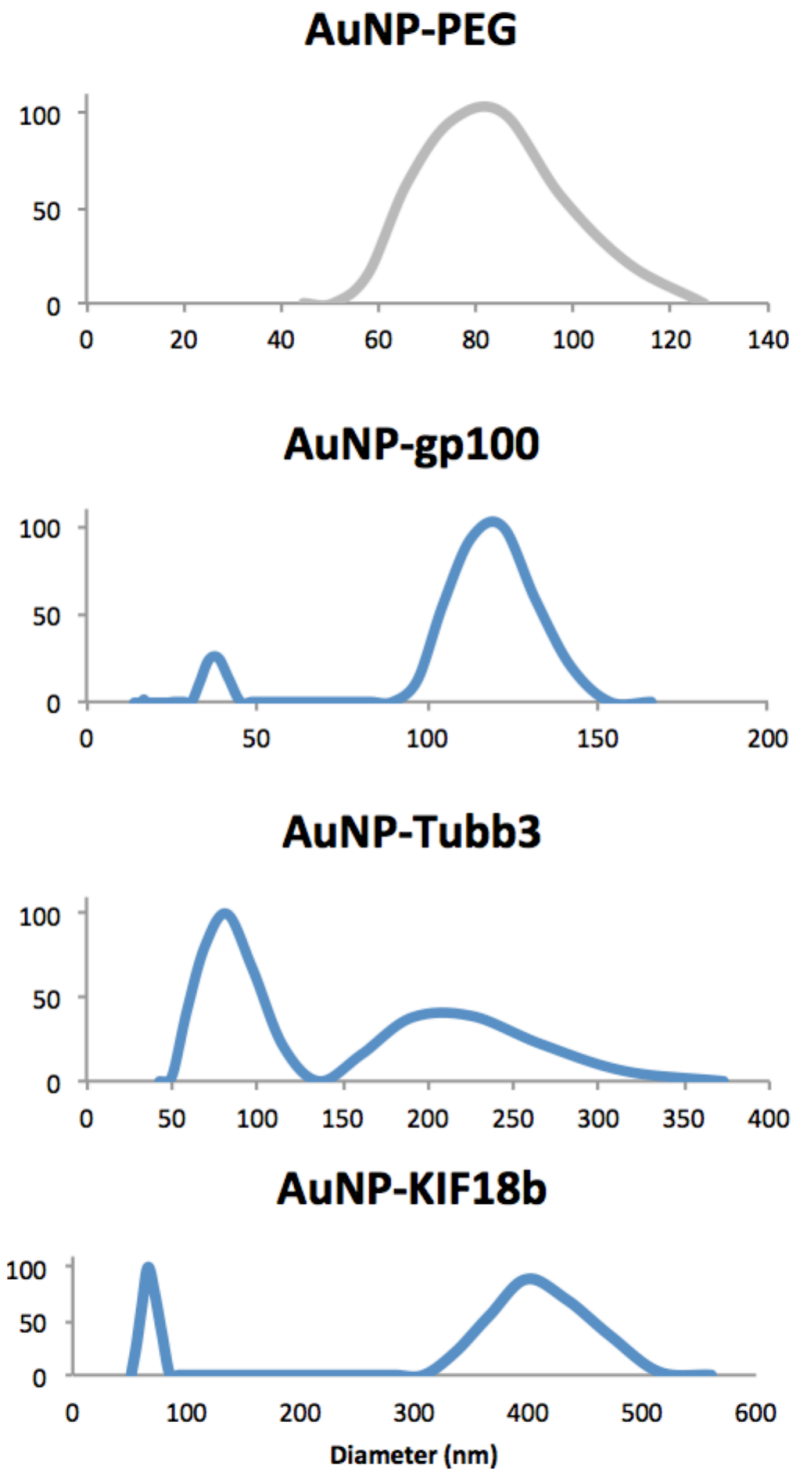

Figure 22. Dynamic Light Scattering results of AuNP-antigen particles compared to AuNP-PEG-COOH. The population of larger particles (>50 nm) in the various AuNVs indicates that some of the particles were successfully conjugated.

This synthesis method described above has been implemented to conjugate AuNP-OVA, AuNP-gp100, AuNP-Trp2, AuNP-kif18b, and AuNP-Tubb3. The two-step method improved AuNV mediated immunity *in vivo*, and diluting the peptides in 1% tween prior to adding them to the conjugation solution improves particle stability across a range of peptides.

We then compared the efficacy of the particle synthesis methods *in vivo*. We injected the AuNP-TRP-2 particles subcutaneously into C57BL/6J mice. Their spleens were harvested for analysis via ELISpot. The particles prepared with the 2 Step method induced significantly higher IFN-γ production in their splenocytes (indicating improved T cell activity) than the particles prepared with the 1 Step method(**Figure 13**, p=0.010). We hypothesize that the 2 step method resulted in a more linear layer of peptides that benefited particle uptake or antigen processing and presentation and thus led to a stronger TRP-2 specific immune response.

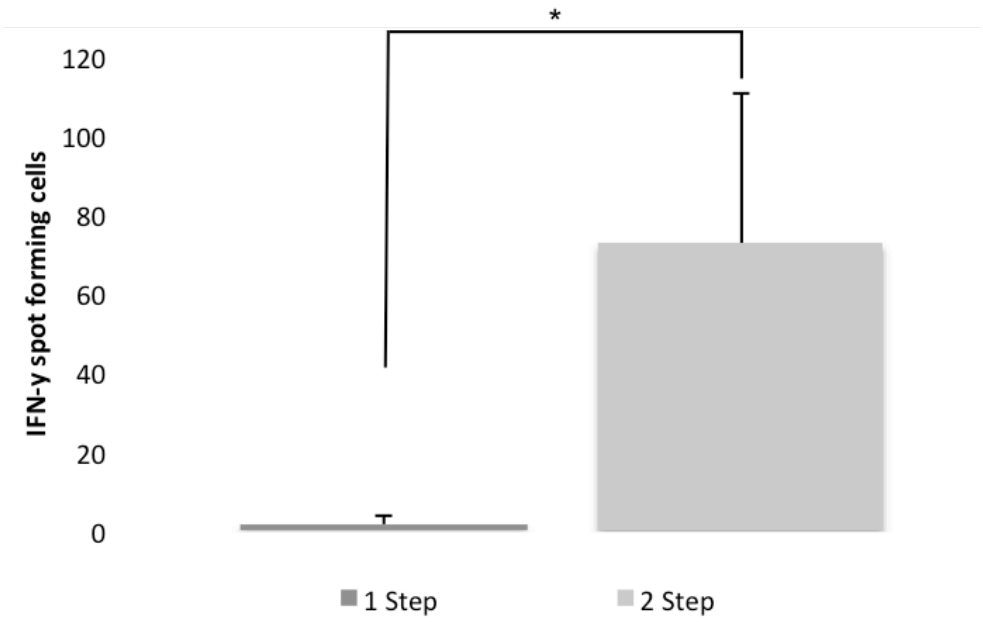

Figure 23. Evaluation of immunogenicity of AuNP-TRP-2 prepared with 1 Step or 2 Step synthesis protocols. The AuNP-TRP-2 particles prepared with a wash step removing the EDC/Sulfo-NHS before the addition of TRP-2 peptides (2 Step method) resulted in particles that elicited stronger activation of TRP-2 specific T cells in the spleen (*p=0.01).

Next, we evaluated if the particles could improve delivery of the TRP-2 peptide to the spleen and thus improve the TRP-2 specific T cell response *in vivo*. In addition, we investigated whether or not the coadministration of the adjuvant dinitrophenol (DNP) boosted the immune response. Following two doses of TRP-2, AuNP-TRP-2, or AuNP-TRP-2-DNP, the mouse spleens were collected for ELISpot analysis. Both the AuNP-TRP-2 and the AuNP-TRP-2-DNP conditions significantly improved antigen-specific immunity compared to TRP-2 alone (**Figure 14**, $p<0.01$).

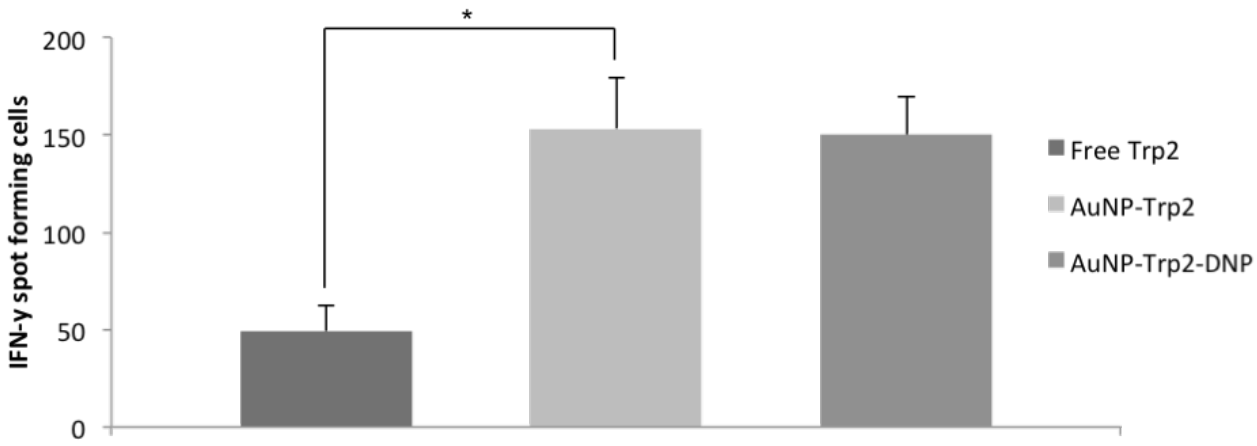

Figure 24. Evaluation of gold nanovaccines incorporating the endogenous antigen, TRP-2. Free TRP-2, AuNP-TRP-2, and AuNP-TRP-2-DNP particles were injected twice, 10 days apart and the spleens were harvested 7 days after the second administration. ELISpot of the splenocytes showed higher TRP-2 specific T cell responses following AuNP-mediated delivery compared to free delivery (*p=0.01). DNP did not improve the immune response compared to AuNP-TRP-2 alone.

This result demonstrates the first time that an endogenous tumor associated antigen has shown improved immunity when delivered on an inorganic nanoparticle formulation. We have illustrated the potential of gold nanoparticles to deliver endogenous antigens to the relevant antigen presenting cells in the spleen that lead to a strong, specific, T-cell response.

Although the values of IFN-γ Spot Forming Cells were lower than those observed when delivering AuNP-OVA, this was expected because TRP-2 is an endogenous antigen. Surprisingly, DNP did not further boost immunity as would be expected following adjuvant co-administration. Though this observation is consistent with our previous work that showed that an adjuvant did not improve T-cell activation, tumor reduction, or survival outcomes in the AuNP-OVA studies using the CpG adjuvant, it is surprising that the presence of an adjuvant did not enhance immunogenicity when delivered with an

endogenous antigen, which is comparatively weaker than OVA and should require an adjuvant to elicit T cell activation. We and others have demonstrated inherent immunogenicity of gold nanoparticles in our previous AuNV work and elsewhere in the literature[65, 82, 83, 96, 97, 222]. Even bare gold nanoparticles have been used in combination with other immunotherapies to treat several tumor models[94, 95]. Perhaps the CpG and DNP adjuvants appear ineffective because the gold nanovaccines have inherent adjuvant properties that are not further improved by the delivery of additional adjuvants; but further study is warranted.

We then evaluated the anti-tumor activity of AuNP-TRP-2 in vivo. Because we had observed CTL activation in the splenocytes, we expected to see some of those T cells migrate to the tumor and kill TRP-2 expressing tumor cells, slowing tumor growth. We implanted B16F10 tumors subcutaneously on Day 0 and injected two doses of AuNP-TRP-2 subcutaneously on Days 7 and 14. Surprisingly, AuNP-TRP-2 administration failed to decrease tumor sizes compared to PBS control. Therefore, despite the fact that TRP-2-specific T cells are active and upregulated in the spleen, the response is insufficient to cause a decrease in tumor volume. The lack of therapeutic efficacy could be due to an inability of T cells to traffic to the tumor site or inhibitory signals deactivating the T-cells in the microenvironment[15]. AuNP-OVA vaccination against B16-OVA cells may have been able to overcome such hurdles due to the stronger CTL response afforded by the nature of the exogenous peptide vaccination or the antigen specificity artificially modeled by the B16-OVA melanoma tumor cells used in those studies. Other work evaluating TRP-2 vaccines observed similarly weak responses to those observed in our study[223].

Further studies are warranted to boost the antigen-specific immune response initiated in the spleen, which should translate into a more robust therapeutic anti-tumor response. Co-administration with checkpoint inhibitor therapies already in the clinic or currently in development may improve therapeutic efficacy of gold nanovaccine administration by improving T cell activity. Other therapies that sensitize the microenvironment to be more favorable to CTL activity could also boost the response initiated by AuNP-TRP-2. Combinations with ablative therapies or immunotherapy-compatible chemotherapies could further boost the immune response initiated herein. The administration of cancer vaccines in combination with such therapies would provide immune memory against metastatic disease progression or remission of tumors with those antigen signatures. As the cancer immunotherapy field continues to expand, new combination approaches may emerge that could leverage the initiated immune response described here.

3.5. Conclusion

We demonstrated that gold nanoparticles improve delivery of the TRP-2 tumor-associated peptide antigen and activated TRP-2 specific cytotoxic T lymphocytes. This critical first step of generating active TRP-2-specific T cells in the spleen is required for cancer vaccination therapies to be effective. This result is the first known report of gold nanoparticle formulation inducing T-cell activation with an endogenous tumor antigen. While the AuNP-TRP-2 particles were unable to elicit a therapeutic anti-tumor effect, further evaluation in combination with checkpoint inhibitors and other immunotherapies could improve this weakly activated immune response.

Chapter 4

Ablative Photothermal Therapy

4.1. Abstract

Gold nanoparticle-mediated photothermal therapy (PTT) locally ablates a primary tumor by using the surface plasmon resonance properties of hollow gold nanoshells to convert near infrared light into cytotoxic heat. Following local ablation, tumor antigens and inflammatory signals are released and can be detected by the immune system, providing an opportunity to leverage this response for systemic anti-tumor immunity. We have previously shown that PTT can result in both pro-tumor and anti-tumor immune responses, and that the tumor-promoting immune response is driven by an increase in myeloid-derived suppressor cells (MDSCs). Here, we evaluated the ability of CpG immunotherapy, known to reduce MDSC activity, to mitigate the pro-tumor outcomes of photothermal therapy. Our results indicate that the combination of PTT and CpG improves treatment of the PTT-treated tumor and prevents exacerbation of metastatic growth

following PTT. Thus, the locally applied PTT + CpG reduces the primary tumor volume and enables systemic anti-tumor immunity.

4.2. Introduction

Cancer immunotherapy aims to reactivate the body's immune system to recognize and kill tumor cells. Therapies are under development to initiate, boost, or enable the immune system to be aware of and treat its own tumor cells. Recent clinical studies suggest that immunotherapies enhance traditional therapeutic strategies including chemotherapy and radiation [181]. These studies have reported the *in situ* vaccination effect of radiotherapy combined with immunotherapy, in which tumor antigens released by the radiotherapy are incorporated into a systemic immune effect boosted by a co-administered immunotherapy[110, 116, 182, 224].

In a process similar to radiotherapy, ablative hyperthermia induced by other techniques including radiofrequency ablation, focused ultrasound, and photothermal therapy also increase blood flow in tumors, induce cytotoxicity, and disrupt tumor vasculature [102-104]. As a result, the immune system is alerted of the tumor due to the tumor-specific antigens and danger signals that are released from the tumor environment [105, 106]. Dendritic cells (DCs) uptake these antigens and interface with T cells in draining lymph nodes, leading to an activation of the anti-tumor immune response[43]. We and other groups have evaluated combination of ablative therapies in combination with immunotherapies[70, 74, 107, 108, 110, 122, 124, 126, 132, 225-227].

Previously, our group studied the effect of gold nanoshell-mediated photothermal therapy on tumor outcomes and interrogated how the immune response following ablative therapy can impact tumor burden and survival outcomes[124]. In addition to releasing tumor antigens, gold nanoshell-mediated photothermal therapy also stimulates a cascade of pro-inflammatory cytokines and chemokines including IL-6, IL-1β, TNF-α, C-CSF, GM-CSF, and CCL2 that can promote the expansion of both pro-inflammatory (CD4+ and CD8+) and suppressive (MDSC) immune cells. While some studies have demonstrated systemic anti-tumor immunity following PTT, most illustrate that PTT alone is not sufficient to eliminate systemic tumors [74, 107, 109, 112, 124, 125, 132, 228]. In fact, our studies indicated that PTT exacerbates metastases due to an upregulation of MDSCs, a pro-tumor immune cell associated with advanced stage progression in cancers including breast, colon, lung, melanoma, and pancreatic [124, 229-232]. Thus, therapies that are known to reduce the amount or activity of MDSCs are of particular interest to evaluate in combination with photothermal therapy.

One candidate immunotherapy that is known to reduce the activity of MDSCs is CpG oligodeoxynucleotides[233-235]. Synthetic nucleotides containing unmethylated CpG motifs stimulate dendritic cells (DCs) via the Toll-Like 9 Receptor ligand and induce the secretion of pro-inflammatory cytokines (i.e. IL-12, IFN-γ)[236]. CpG ODN 1826 (5'-TCCATGACGTTCCTGACGTT-3') has been used as a vaccine adjuvant in several pre-clinical models and is in clinical trials for advanced melanoma [48, 84, 237, 238]. Because CpG stimulates anti-tumor immunity and also prevents the maturation of immune-suppressive MDSCs, CpG is an excellent immunotherapy candidate to combine with PTT in order to downregulate undesired immune responses following ablation[235].

Since observing that gold nanoshell-mediated PTT had the potential to induce both anti-tumor (increase CD4+ and CD8+ T cells and prevent tumor rechallenge) and pro-tumor (increase in MDSCs and metastatic burden) immune responses, our goal was to direct the immune response against the tumor by co-delivering an immunotherapeutic agent to promote antitumor immunity and mitigate the expansion of pro-tumor immune pathways. Thus, the locally applied ablative therapy would have the potential to act systemically. We interrogated how the addition of CpG immunotherapy would impact the ability to treat the primary, PTT-treated tumor and the untreated metastases.

4.3. Methods

Gold nanoshell synthesis

Hollow gold nanoshells were synthesized as previously described[124]. Gold tetrachloroauric acid ($HAuCl_4$) was reduced onto sacrificial silver nanoparticle templates via galvanic replacement and tuned to 808 nm. An aqueous solution of 0.2 mM silver nitrate ($AgNO_3$) was aged with 0.5 mM sodium citrate. In Erlenmeyer flasks, 50 uL aliquots were heated to 60C. 1 mL of 100 mM sodium borohydride was injected into the heated solution and stirred for one hour. Upon cooling to room temperature, 1 mL of 200 mM hydroxylamine hydrochloride was added to activate the solution so that when 1 mL of 200 mM $AgNO_3$ was added, the silver seed grew to small silver cores. After aging, the solution was again heated and 100 uL of 1% $HAuCl_4$ was added to the solution until the gold etched the particles such that their peak plasmon resonance was around 850 nm. Following

washing and PEGylation, the particles blue-shifted toward 808 nm. All chemicals were purchased from Sigma Aldrich.

Photothermal Therapy

Hollow gold nanoshells (HGNs) were coated with SH-PEG (5000 kDa) overnight then washed and reconstituted in PBS. Tumors were intratumorally injected with 40ul of HGN (OD 20). Glycerol was applied liberally to the skin to minimize discomfort to the animal. The mice were treated with a near-infrared laser (3 W/cm2, λ = 808 nm, spot diameter = 8 mm) for 3 minutes. For the PTT + CpG group, this treatment was immediately followed by an intratumoral injection of 16.4 μg CpG 1826.

Cytokine and Chemokine Assay

Blood was collected from the saphenous vein on day 12 and 15, which was the equivalent of 24 and 96 hours following ablation for the PTT and PTT+ CpG groups. Serum was isolated and frozen until analysis by the Millipore Miliplex system per the manufacturer's instructions. The assay measures the amount of 32 cytokines, growth factors, and chemokines simultaneously.

Cell Culture

B16F10 tumor cells (ATCC) were cultured in DMEM (Life Tech) with 10% FBS (Life Tech) and 1% penicillin/streptomycin (Thermo). Tumor cells were collected for implantation when in their growth phase (between 40-60% plate confluency).

Animal Studies

C57BL/6J Albino (C57BL/6J-Tyr-2J/J) mice from Jackson Labs were housed at Bellicum Pharmaceuticals in their pathogen free facility. The study was approved by the Institutional Animal Care and Use Committee at Bellicum and conducted in accordance with the approved protocols.

Lung Metastases (Survival Study)

A concentration of 5 x 10^6 B16F10 tumor cells were implanted subcutaneously in the right flank of the mouse on day 0. On day 3, 5 x 10^6 B16F10 tumor cells were injected intravenously to mimic lung metastases. Tumor volume was evaluated with digital caliper until the mice reached humane endpoints based on tumor size (>1cm^2) or reduced activity.

Primary and Contralateral Flank Tumors (21 Day Study)

A concentration of 5 x 10^6 B16F10 tumor cells were implanted subcutaneously in the right flank of the mouse on day 0. On day 5, a concentration of 5 x 10^6 B16F10 tumor cells were injected subcutaneously in the left flank. Tumor volume was evaluated with digital caliper.

4.4. Results

Lung Metastases Study

Mice were given a primary subcutaneous B16F10 tumor on Day 0 followed by an intravenous tumor injection on Day 3 to mimic lung metastases. The primary flank tumor was treated with PTT or PTT + CpG on Day 11 after which the mice were evaluated for

primary tumor size and survival (**Figure 15**). Each group contained 5 mice.

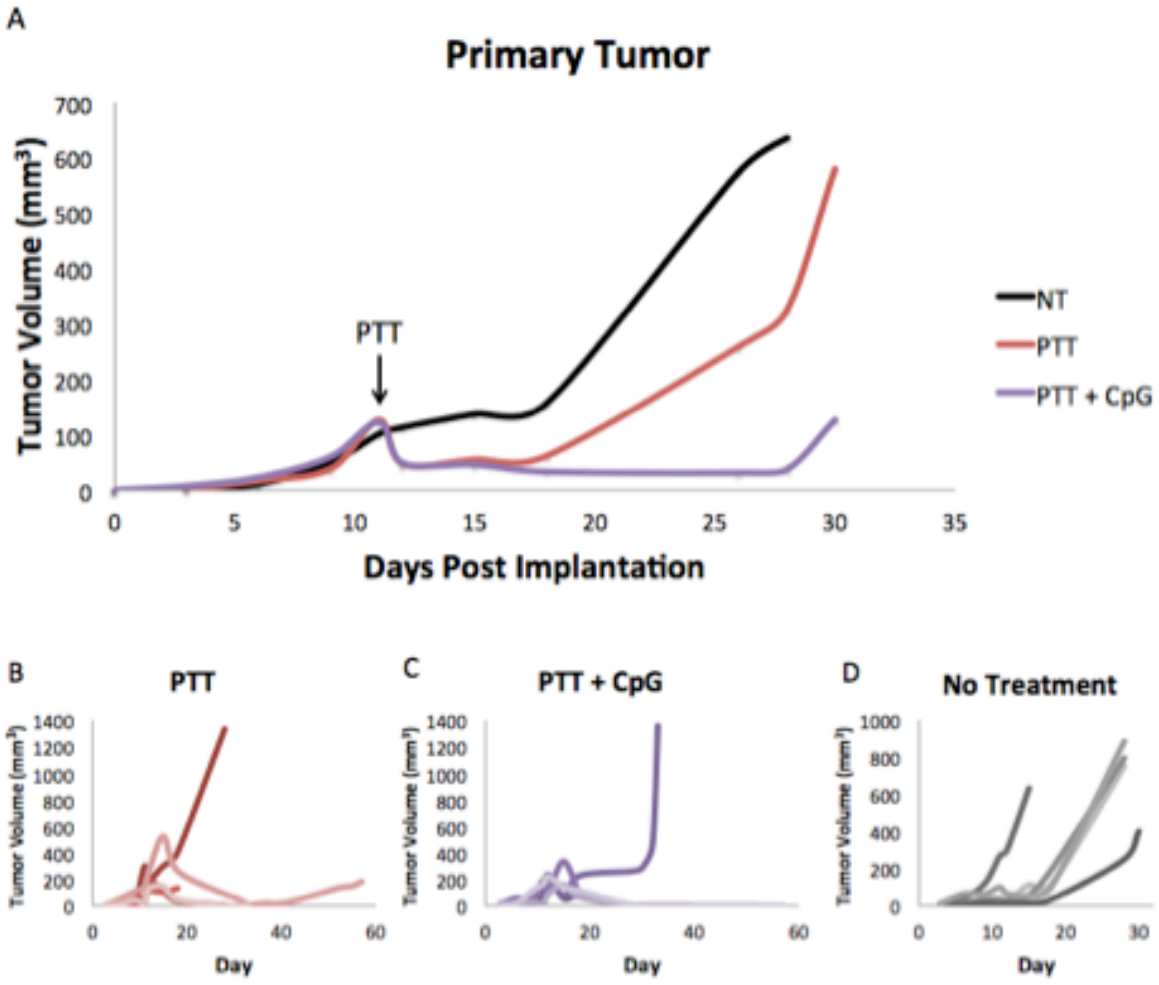

Figure 25. Growth of primary tumors. (A) Average tumor volume across 5 mice per condition. Mice treated with PTT + CpG had reduced tumor volume and delayed tumor growth compared to those treated with PTT alone, which showed reduction in tumor size after ablation but eventually recurred to nearly approach the untreated group by day 30. (B,C,D) Individual tumor volume tracking for each mouse. (B) One PTT-treated mouse rapidly regrew its primary tumor and another began to recur 30 days after PTT (B). One PTT + CpG mouse recurred it's tumor suddenly and had exponential growth in just two days, while three other mice in this group survived past day 30 and not recur their tumors (C). All untreated mice reached their primary tumor endpoints by day 30 (D).

The untreated mice grew tumors rapidly and all had reached humane endpoints for tumor size by day 30. The PTT-only mice were characterized by delayed tumor growth as a result of the ablation treatment but most had a recurrence of their primary tumor or were humanely sacrificed due to complications from their lung metastases by day 33. The PTT + CpG group delayed tumor growth for a longer period of time and fewer mice recurred their tumors compared to PTT alone. Thus, while PTT did reduce tumor volume due to tissue ablation, most of the mice did not see sustained improvement compared to the no treatment group. CpG slowed the rate of primary tumor growth and reduced the likelihood of primary tumor recurrence (80% of primary tumors recurred in PTT condition versus 20 % in PTT + CpG condition). Mice treated with PTT + CpG survived longer than untreated mice (**Figure 16**).

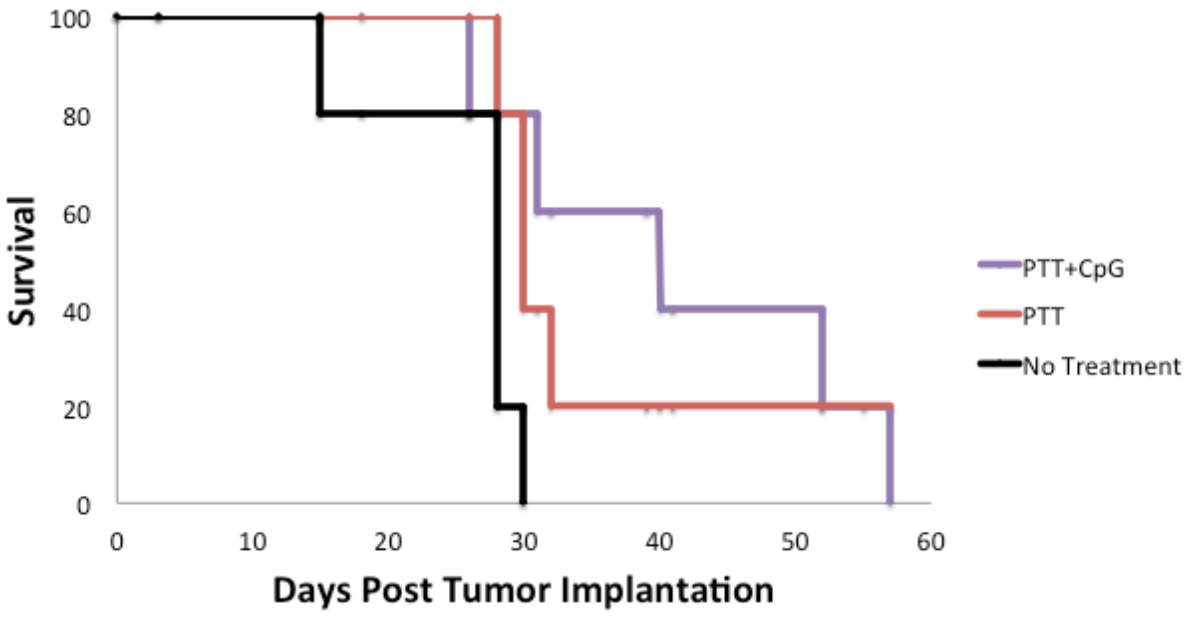

Figure 26. Survival in the lung metastases study. Mice treated with PTT + CpG survived longer than PTT-treated mice, which more closely resembled the no treatment condition with the exception of one mouse who survived through day 60.

Though PTT reduced the primary tumor volumes following ablation, all but one mouse in the PTT-treated group reached their humane endpoint soon after the non-treated group. One PTT-treated mouse recurred its primary tumor rapidly by day 30. A second mouse appeared to have a small primary tumor but upon post-mortem evaluation (also day 30), had developed subcutaneous metastases all across both flanks and around to the abdomen. Two other mice in this group reached their endpoints on days 18 and 33 respectively due to breathing and activity complications. With the exception of one mouse that survived the 60-day study, there was no survival advantage conferred by the PTT treatment.

Administering CpG following PTT extended survival of the mice compared to the no-treatment control. Growth of the primary tumor was slowed and the mice trended toward improved survival compared to the PTT only group seemingly due to the fact that CpG mitigated worsening of lung metastatic burden.

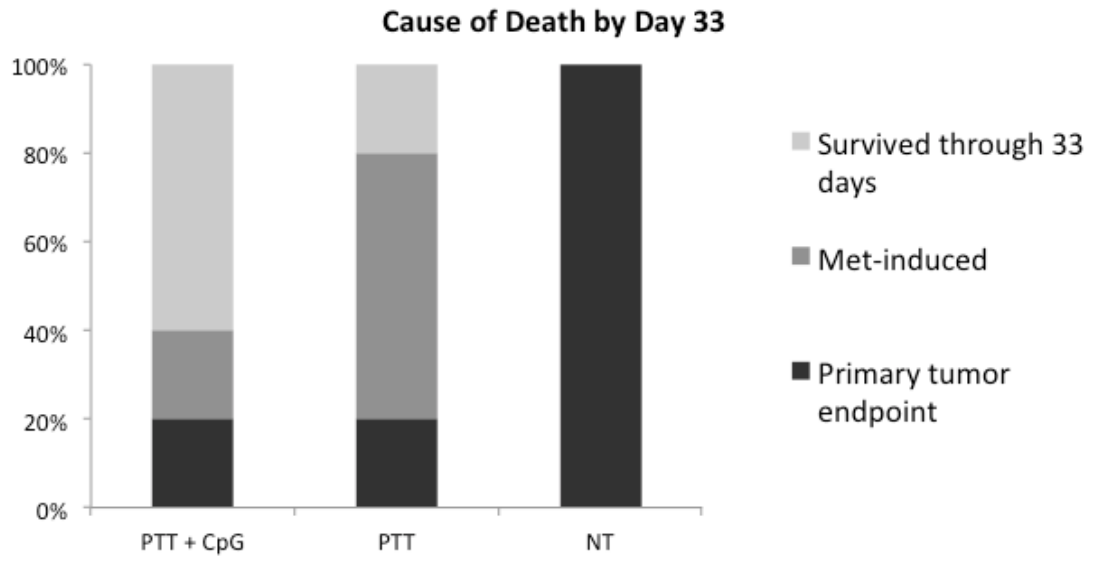

Figure 27. Cause of death after 33 days. All untreated mice (n=5) reached their humane endpoints for tumor size by day 33. Most of the mice in the PTT group (3 of 5 mice) reached their humane endpoints due to complications from their lung metastases. The majority of the PTT + CpG group (3 of 5 mice) was still active through day 33.

Thirty-three days after primary tumors were implanted, all mice in the untreated group had reached the humane endpoint due to size of primary tumor. Only 20% of PTT and PTT + CpG reached primary tumor endpoints. At day 33, 60% of mice in the PTT group had died from breathing complications due to lung tumor burden but only 20% of PTT + CpG mice reached this endpoint (**Figure 17**). 60% of the mice in the PTT + CpG group survived through day 33. Each would die from metastases complications by day 60.

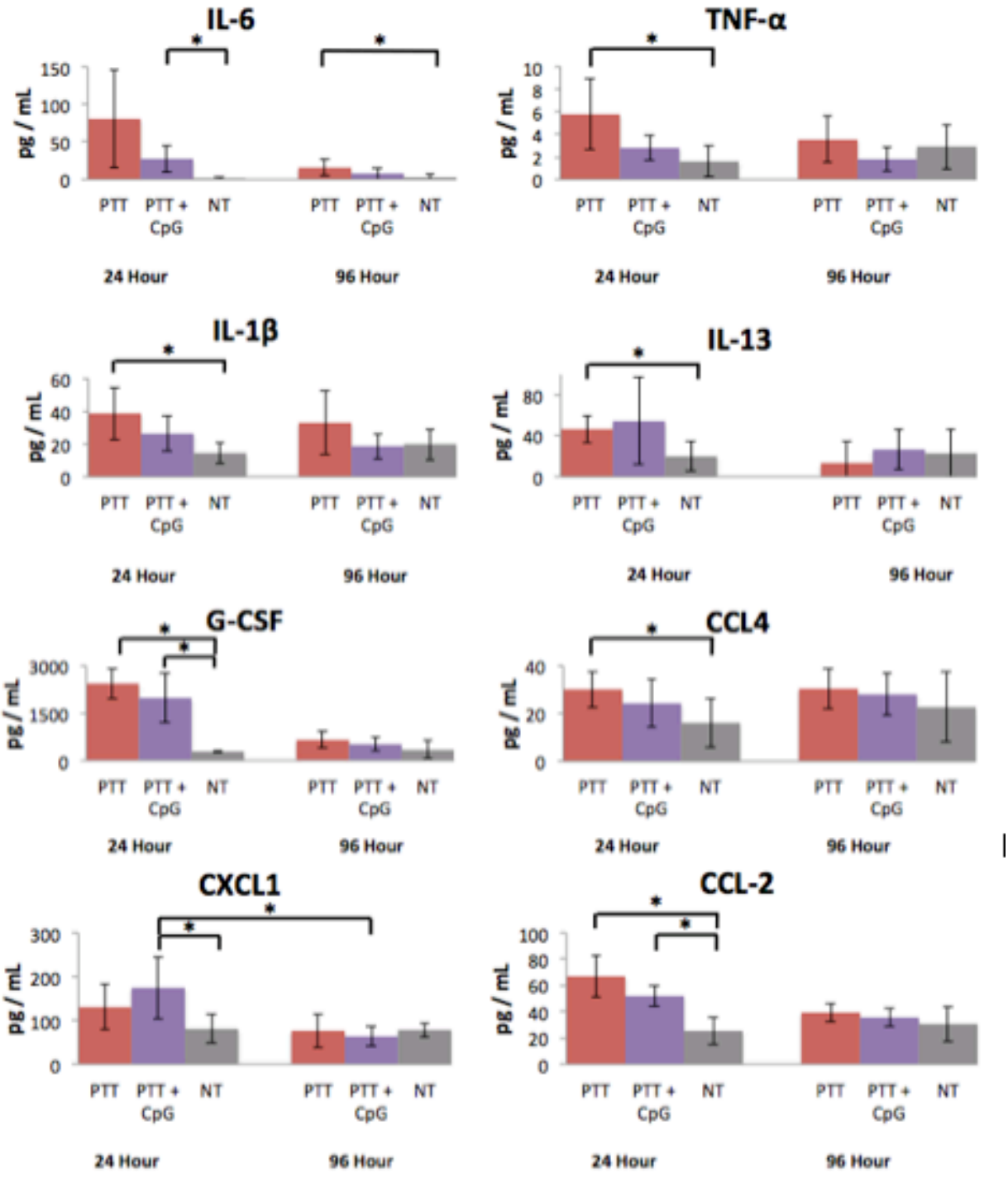

Figure 28. Cytokines increased in lung metastasis study. PTT treatment resulted in upregulation of several cytokines and chemokines, many of which were less elevated in the PTT + CpG treated mice. Signifiance between groups was evaluated by a Students T Test. *p<0.05.

Upon evaluating the cytokine and chemokine levels for each of the mice 24 and 96 hours after treatment, we observed several factors that were upregulated in PTT-only compared to the no treatment groups (**Figure 18**). Many of the observed cytokine changes were consistent with our previous results[124]. Pro-inflammatory cytokines (TNF-a, interleukins), stem cell differentiation factors (G-CSF), and chemokines that attract immune cells to sites of inflammation (CXCL-1, CCL-2, CCL-4) were upregulated in the PTT and PTT + CpG groups compared to the untreated controls. The addition of CpG often reduced the cytokine levels observed, though IL-6, G-CSF, and CCL-2 were significantly elevated in both PTT and PTT + CpG treatment conditions. These results are consistent with the hypothesis that CpG would impact the immune response compared to PTT-only treatment.

Contralateral tumor study

We then used a flank metastasis model system to better observe the impact of CpG on the contralateral, non-treated tumor. We also chose a fixed endpoint for cellular analysis but were unable to complete the flow cytometry analysis due to unforeseen circumstances. Therefore, the study stops at 21 days instead of survival. There were five mice per group.

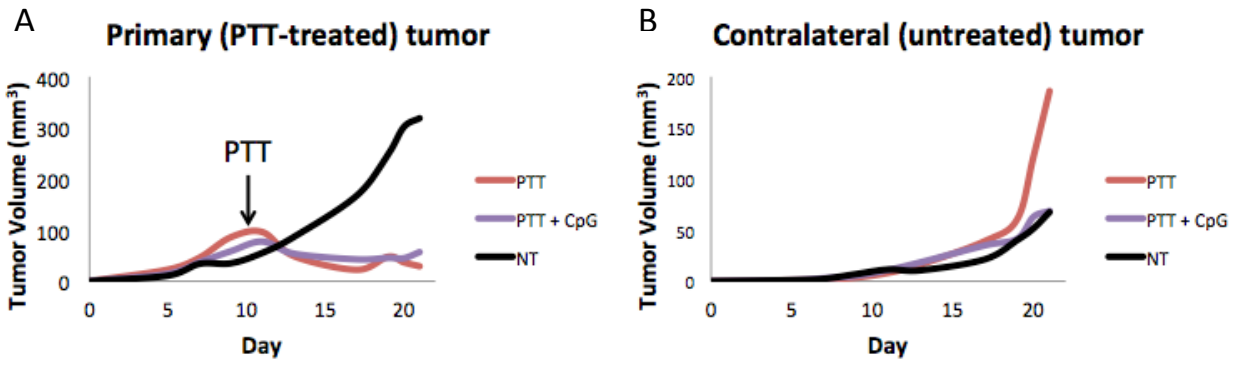

Figure 29. Tracking the growth of primary and contralateral tumors over time. (A) Application of laser ablation in the PTT and PTT + CpG groups reduced the volume of the treated tumor while the untreated tumors continued to grow over time. (B) The contralateral tumors grew much faster in the PTT-treated mice compared with the untreated mice, which is consistent with our previous studies. The addition of CpG with PTT mitigated the expansion of the contralateral tumor such that their growth resembled the untreated controls.

PTT and PTT + CpG groups both saw a reduction in primary tumor volume after ablation (day 11). Consistent with our lung metastases studies, the contralateral flank tumor grew exponentially in the PTT group compared to the untreated group. The CpG prevented this growth such that the PTT + CpG treated mice had similar contralateral tumor growth rates to the untreated controls.

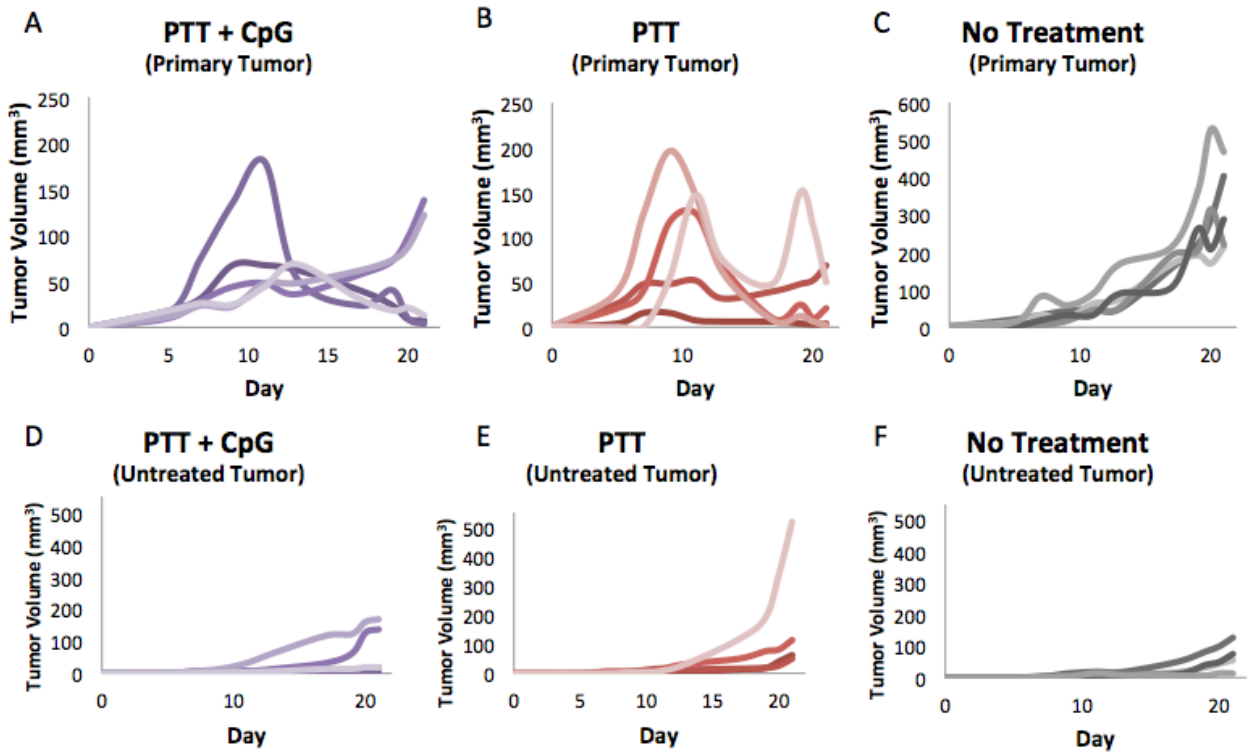

Figure 30. Growth curves for individual mice. The growth of each mice for both primary tumors (A,B,C) and contralateral tumors (D, E, F) are illustrated.

Together, these data suggest that PTT tends to exacerbate lung metastases but that this effect can be dampened by administration of CpG immunotherapy. Future studies evaluating the changes in immune cell activity (CD8+, CD4+, MDSCs) in PTT + CpG conditions may provide a better understanding of the molecular mechanisms driving the mitigation of the exacerbated metastatic tumor growth seen in the PTT-only treatments. In addition, confirmation of activated T cells against tumor antigens (as measured by ELISpot) would also confirm the *in situ* vaccination effect predicted to protect against future tumor challenge

4.5. Conclusion

We evaluated the tumor burden and survival outcomes of combining PTT with the immunotherapy CpG. As expected, the CpG immunotherapy mitigated the pro-tumor effects following photothermal therapy and led to slower tumor growth and improved survival in a lung and subcutaneous metastatic melanoma model. These results support the growing body of evidence that ablative therapies can induce anti-tumor immunity upon release of tumor antigens, and that combination with immunotherapies can help the immune system be alerted to these tumor antigens. Combinations of ablative therapies with immunotherapies enable the immune cells to more effectively mount a systemic anti-tumor response following the locally applied therapy. Though our work was performed in pre-clinical mouse models with currently non-FDA approved treatment strategies, these results match clinical observations observed with combinations of radiotherapy and approved immunotherapies.

Chapter 5

Future Directions

As illustrated in Chapter 1, gold nanoparticles have substantial hurdles remaining before they can reach clinical adoption. To date, no gold nanoparticle has been FDA approved for clinical use. There are some gold nanoparticles in trials but they are early phase or very slow to progress[49]. Despite billions of dollars, several excellent research teams working toward improving outcomes, and institutions like the Nanoparticle Characterization Lab built to enable nanoparticle successes in clinical trials, the path to clinical adoption remains long and arduous[183]. Even with the inherent advantages of gold nanoparticles including facile surface chemistry and likely inherent adjuvant properties, the advantages still may not be sufficient to overcome the extreme time and cost investment necessary for the first gold nanoparticle approval to meet the FDA requirements. Despite this bleak outlook, the studies outlined in this thesis demonstrate progress of gold nanoparticles toward translation into the clinic. For example, we demonstrated for the first time that gold nanovaccines elicit anti-tumor immunity when

delivering an endogenous, tumor-associated antigen. Future work combining such therapies with upcoming immunotherapies and tumor metabolic therapies may expand this anti-tumor immunity from a splenic response to a systemic anti-tumor therapeutic response.

For our gold nanoparticle ablation studies, these particles more closely resemble the gold nanoshells that are undergoing Phase II clinical evaluation and may seem to be on a more clear path to clinical translation[178]. However, the hollow gold nanoshells, while advantageous for tuning to NIR lasers, may have undesired toxicity upon long term breakdown of the gold shell, which could release any toxic silver ions remaining in the particle interior[193]. Though this particle will also face difficulty on the path to clinical translation, from a broader perspective these ablative immunotherapy combination approaches are further evidence of a phenomenon observed clinically: the abscopal effect. The abscopal effect describes the systemic anti-tumor activity resulting from a locally applied therapy[181]. Since the approval of checkpoint inhibitors, several clinicians have observed that combinations of radiotherapy with immunotherapy produce strong, systemic anti-tumor immune responses[116, 117, 121].

A more radical idea challenges the need for toxic methods of ablation – including radiotherapy. Light-based ablative therapy that heats the tumor, but does not ablate it, still releases danger signals and initiates recruitment of anti-tumor immune cells to the site of the tumor, enabling recognition and *in situ* vaccination[106]. Hyperthermia as an approach for treating cancer is not common in the United States but has been demonstrated in Korea and other countries[239]. Perhaps, a less toxic approach to releasing tumor antigens and

recruiting immune cells is worth exploring. Indeed, when we evaluated a heat-only control (no hollow gold nanoshells but 3 min laser therapy), we observed excellent tumor burden reduction and survival outcomes compared to no treatment and even without combining with immunotherapy. This unexpected result warrants further study.

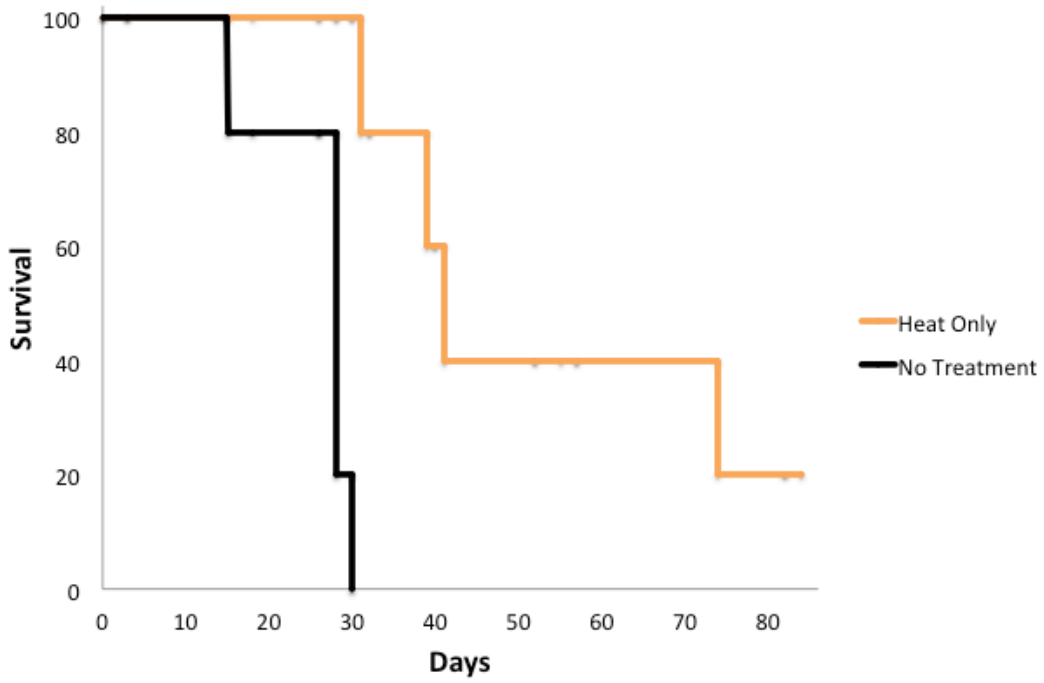

Figure 31. Heat only (application of laser at 3W for 3 minutes without hollow gold nanoshells) improves survival without the addition of immunotherapy.

Furthermore, the combinations enabled by tumor ablation and immunotherapy can compound well beyond CpG or adoptive T cell therapy. Some immunotherapy-compatible chemotherapies, such as sunitinib, may be excellent candidates for triple combinations of ablation, immunotherapy, and chemotherapy to further reduce the immune suppressive cell activity and to boost systemic anti-tumor outcomes[240]. I performed pilot studies

with ablation combined with PD-1 checkpoint inhibitors or sunitinib that suggested that these combinations would further improve systemic immunity.

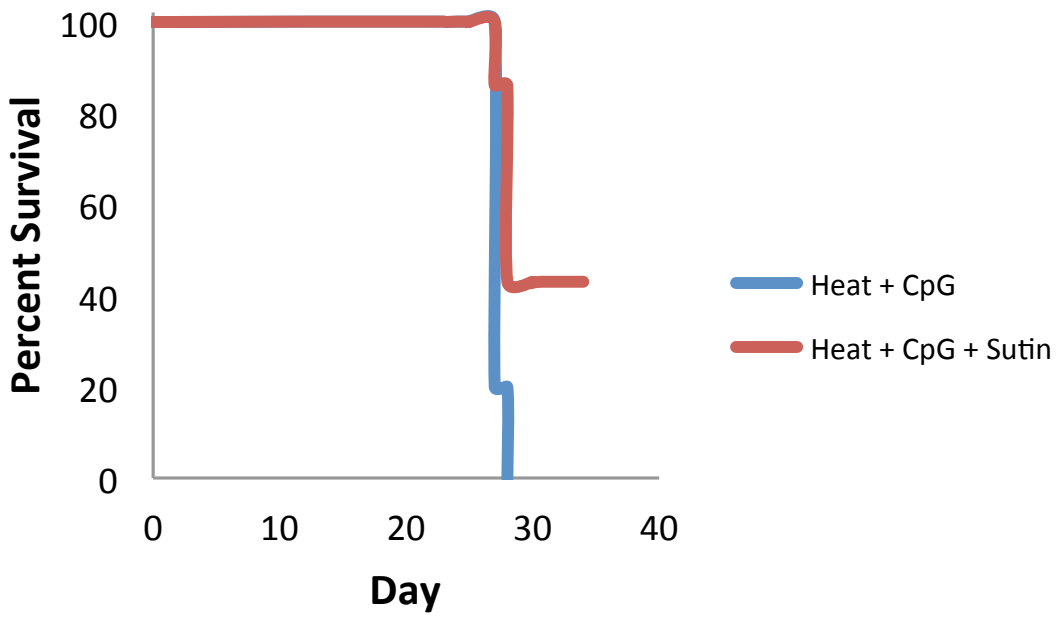

Figure 32. Sunitinib chemotherapy improves survival oucomes. Heat treatment involved applying laser to tumor at 3W for 3 minutes without hollow gold nanoshells. Combining this therapy with CpG and sunitinib chemotherapy improved survival outocmes compared to heat + CpG without sunitinib.

Future work could explore these combinations and others (as new immunotherapies will continue to be approved in the next decade) to discover the combination that would be strong enough to warrant the investment in clinical development of gold nanoimmuotherapeutics.

Chapter 6

Conclusion

Gold nanoparticles are excellent compounds to deliver and enable cancer immunotherapies. They are simple to make and have properties that can be used for enabling therapy including natural biodistribution to immune cells and organs, optical properties for ablation, and perhaps inherent adjuvant properties. Although their path to FDA approval is steep and many hurdles remain, preclinical studies demonstrated in this thesis support the growing body of evidence that gold nanoparticles have the potential to be powerful platforms for enabling cancer immunotherapies. We demonstrated that gold nanovaccines can elicit anti-tumor immunity against a tumor-associated antigen and that gold nanoparticle photothermal therapy combined with CpG immunotherapy can elicit systemic, anti-tumor effects. Future work may bring these technologies toward clinical translation by combining the strategies demonstrated here with clinical therapies including checkpoint inhibitors, adjuvants, monoclonal antibodies, and chemotherapies.

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