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Metallic Nanoparticles for Cancer Immunotherapy

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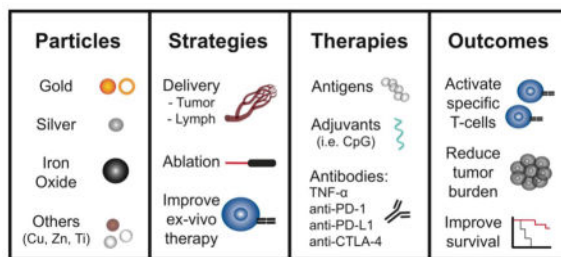
Abstract

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 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 review, 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.

Graphical Abstract

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Keywords

Cancer; Nanotechnology; Immunotherapy

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 1 illustrates how activation of specific T cells can be initiated *in vivo*.

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 2[14].

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].

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 3, 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].

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].

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].

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[28, 65, 66]. 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.

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, 77]. 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, 78]. 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[79]. 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[80]. 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[81]. 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[82].

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[83]. Several groups have shown that delivery of CpG using AuNPs improves CD4+ helper T cell and cytokine activation, leading to improved CD8+ responses downstream[84–86]. 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[87].

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[88]. Lee et al. demonstrated that AuNPs and ferritin nanoparticles induced a CTL response against the model RFP antigen when co-administered with CpG[89, 90]. This effect was abscopal in that the local treatment provided systemic immune protection and prevented RFP-expressing melanoma growth *in vivo*[90]. 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[91].

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*[81]. 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* [82]. 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[92]. 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[93–95]. Bare gold nanorods elicited innate immune signaling pathways including toll like receptors, NOD-like receptors, and MAP kinases *in vivo* [96]. Bare silver nanoparticles have demonstrated anti-tumor activity *in vivo* in a lymphoma model by inducing apoptosis and slowing angiogenesis[97–99]. Other particles comprised of a silver core and gold shell have also shown preliminary anti-tumor activity[100]. 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[101–103]. As a result, tumor-specific antigens and danger signals are released from the tumor environment, alerting the immune system as illustrated in Figure 5[104, 105]. 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 [106–116]. 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[117–120].

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[73]. 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[75, 112, 121, 122].

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-dioxygenase (IDO) resulted in more antigen presentation to T cells, more T cells in the tumor microenvironment, and local and distal rejection of tumors[123]. 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[108].

Ablation of tumor tissue (including clinically with radiotherapy) not only facilitates antigen release but also improves vascular perfusion and chemotherapy penetration into the tumor[104]. The efficacy of combining metallic nanoparticle-induced ablation with chemotherapy and/or immunotherapy has been demonstrated using metallic nanoparticles in preclinical studies[69, 124–127]. 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*[128]. 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[129]. 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[69]. 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[124]. 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[130].

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*[131]. 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*[132–134]. Recent reviews have discussed metallic nanoparticles for diagnostic and monitoring applications including cancer immunotherapy and the opportunities and challenges for clinical translation[135–142].

Strategy: targeting the tumor immune microenvironment

The tumor microenvironment is often hostile to immune cell viability and function[143]. 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[108].

Gold nanoshell-mediated PTT combined with gene therapy was found to downregulate NF- κ B signaling at the tumor site, reducing the pro-tumorigenic effects of the transcription factor and sensitizing the tumor to subsequent chemotherapy[144]. AuNPs delivering siRNA selectively silenced VEGF expression in tumor cells and tumor-associated macrophages,

leading to tumor regression[145, 146]. 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[147].

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 [148, 149]. AuNP-TNF- α particles in particular have progressed to clinical trials[149]. 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[150]. Silver nanoparticles reduced tumor promoting cytokine (IL-1 β) signaling resulting in inhibition of tumor growth *in vivo*[74]. 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[72].

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[151]. 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*[152]. 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[153]. 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[76]. Macrophages pulsed with cobalt oxide nanoparticles increased antigen-specific T cell responses *in vivo*[68].

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[154]. 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*[155].

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[149]. Phase II studies are planned for pancreatic cancer patients in combination with second line therapies; however, further details have yet to be announced[156]. 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[157, 158]. 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[159–162].

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%[164–166]. 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 $\mu\text{g}/\text{m}^2$ 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[149].

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[157, 158, 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 nanoparticle 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 Aurimmune platform which carries both TNF- α and paclitaxel (CYT-21000)[172].

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[173]. Though not explicitly an immunotherapy, this platform has demonstrated preclinical efficacy when incorporating immunotherapeutic materials[91]. 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[174–178]. 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[179]. 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[180]. 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[181]. 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[182]. Indeed, this effect has been seen clinically following radiotherapy ablation combined with immunotherapy[114, 115, 183]. Accordingly, a thorough investigation into the role of the immune system with these ablative therapies is warranted.

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[184]. 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[185]. 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[186]. 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[85, 91].

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[187]. 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[188, 189]. 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[190, 191]. In general, the surface coatings (such as PEG) used to protect engineered MNPs are thought to be degraded *in vivo*[192]. 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[193].

However, recent long term studies have demonstrated evidence that gold is degraded over long time scales and breaks down into smaller, potentially toxic components[194, 195].

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[196–200]. 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, 81, 96]. 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.

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

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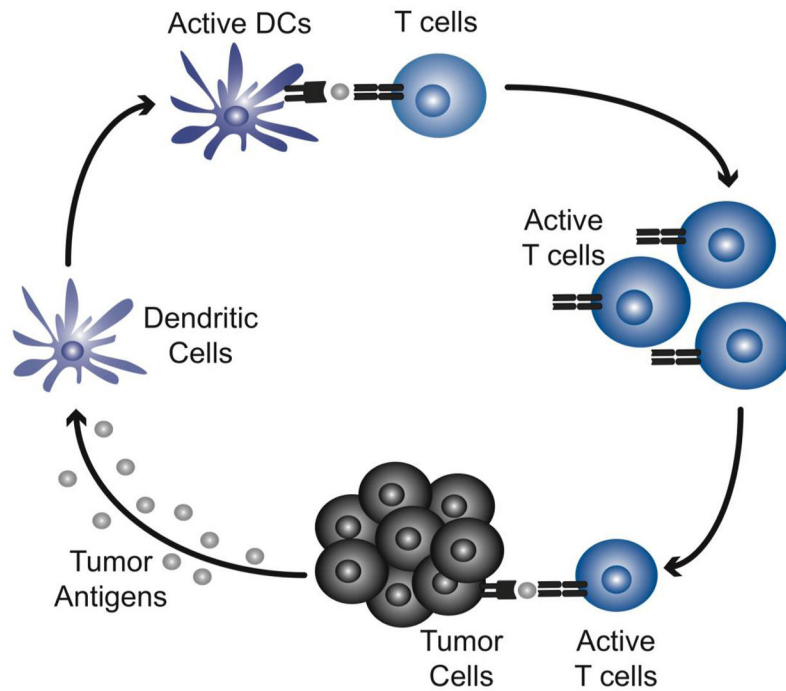


FIGURE 1. Dendritic cells (DCs) uptake, process, and present tumor-associated antigens to T cells in lymphoid tissues. T cells are activated, differentiate, and expand before entering systemic circulation. When T cells identify tumor cells with the corresponding antigen, they release cytotoxic material into the cell, inducing apoptosis.

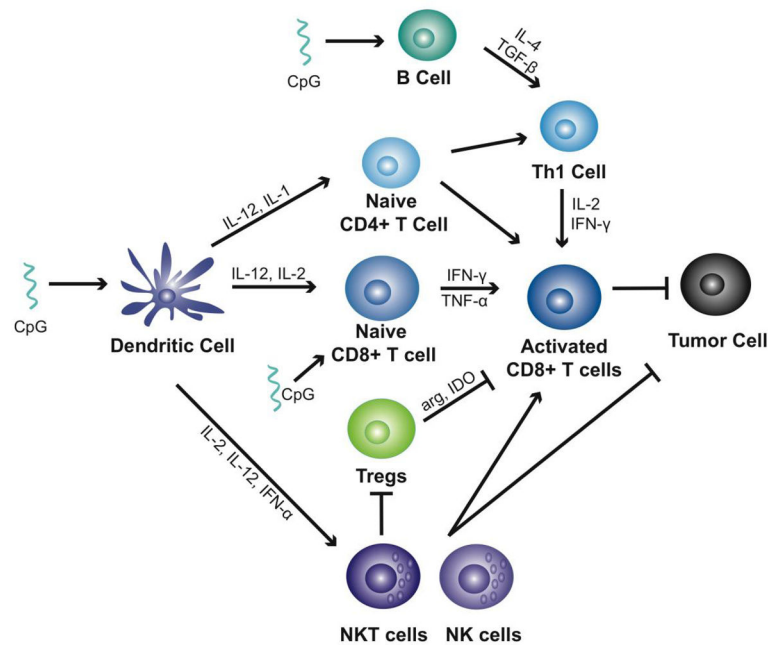


FIGURE 2.

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.

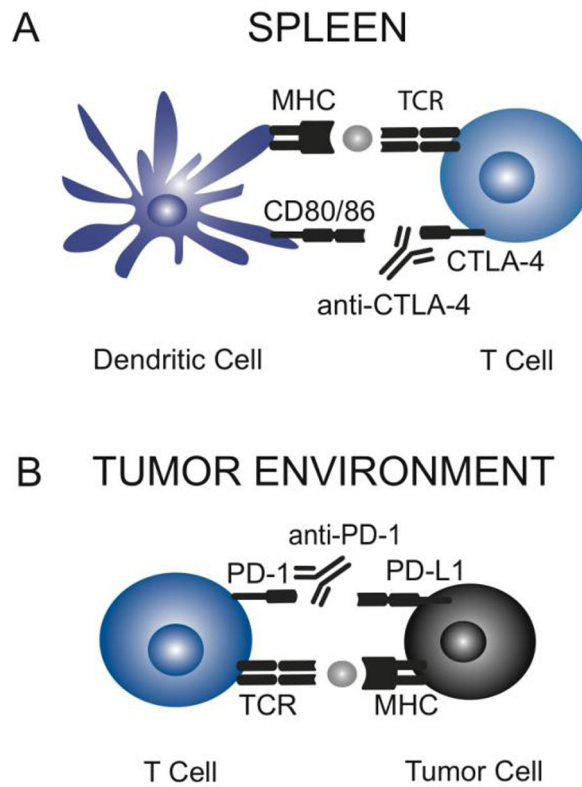


FIGURE 3. Clinically approved checkpoint inhibitors enable T cells to perform their cytotoxic activity by A) enabling T-cell activation by antigen-presenting cells or B) preventing tumors from deactivating T-cells via pathways including PD-1 and PD-L1.

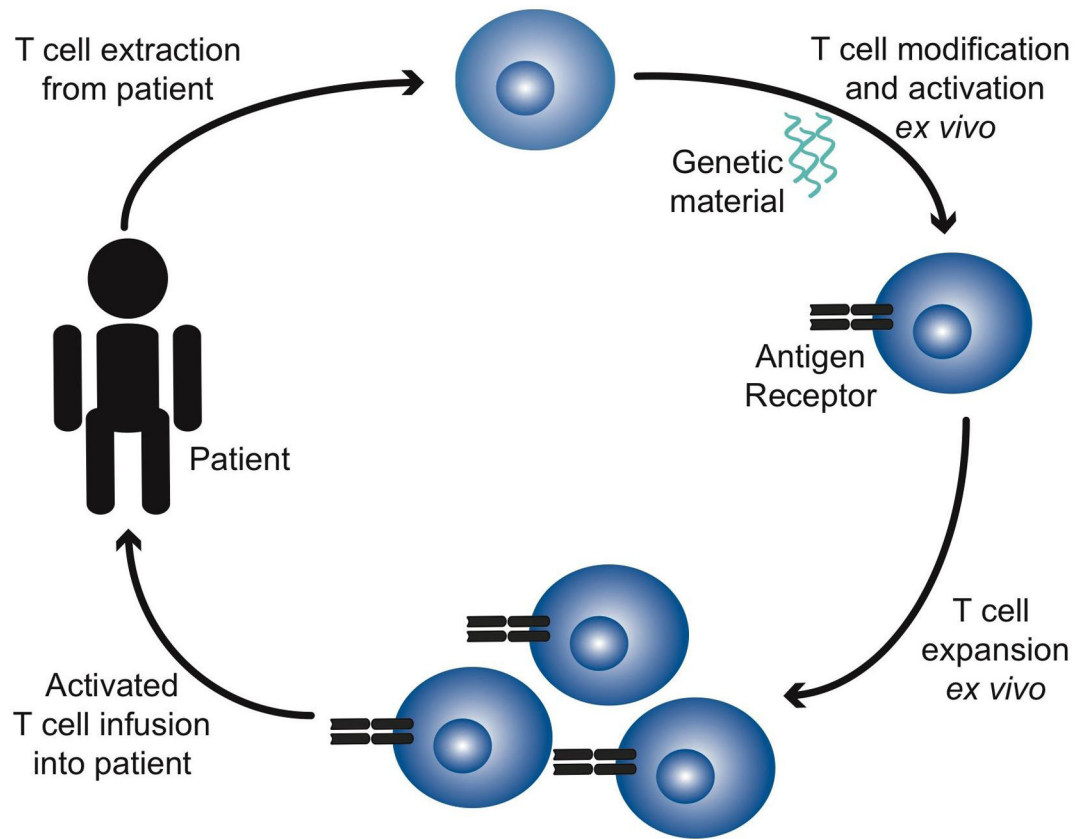


FIGURE 4.

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

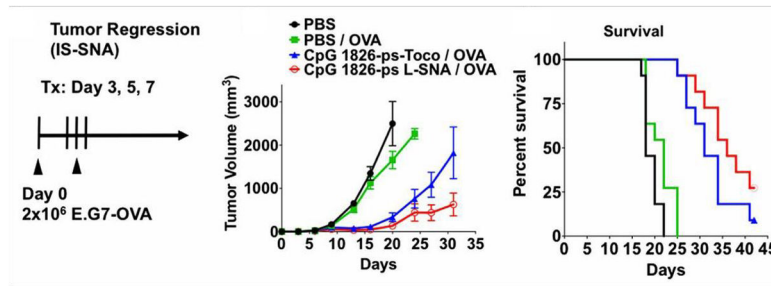


FIGURE 5. Gold nanoparticles delivering OVA antigen and CpG adjuvant reduced tumor volume and improved survival in a therapeutic E.G7-OVA tumor model system [91]. Reprinted from *PNAS* 112(13):3892–7 (2016) Radovic-Moreno et al. with permission from *PNAS*.

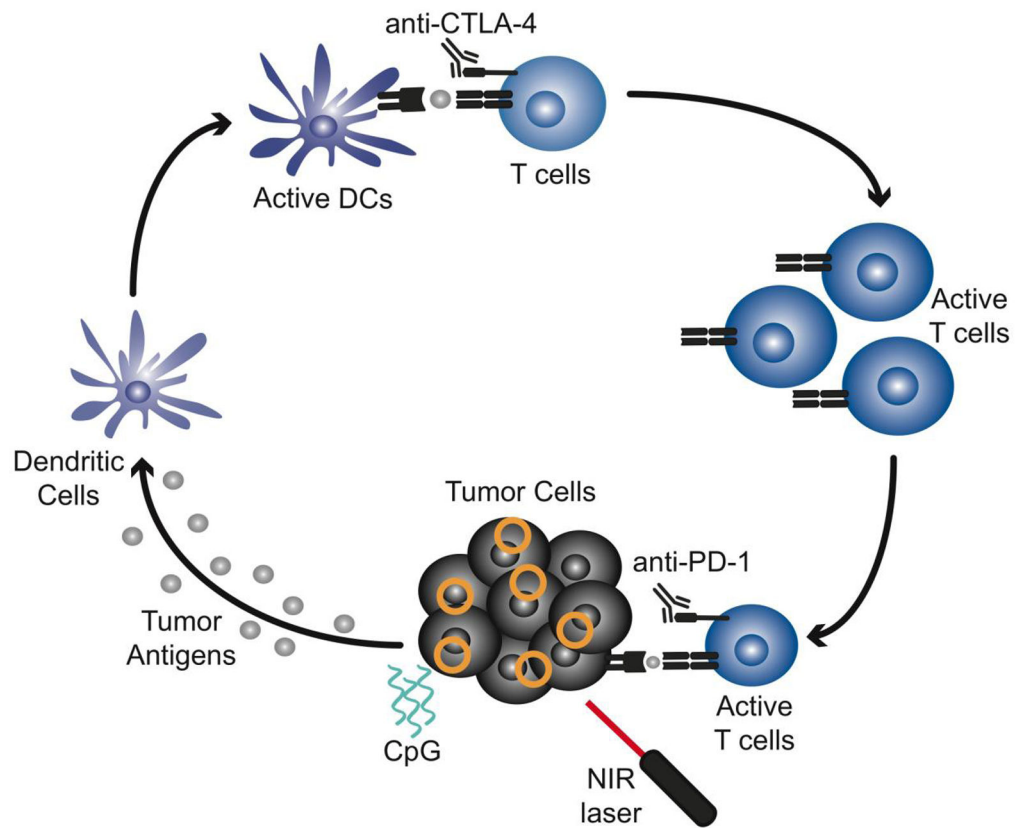


FIGURE 6.

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.

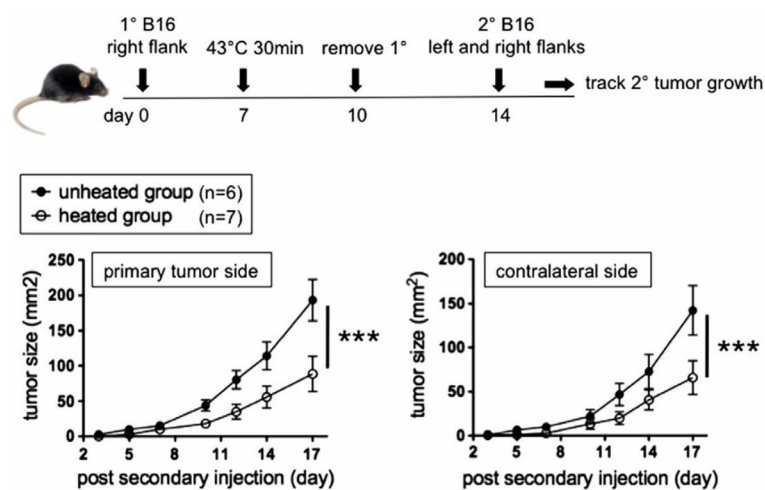
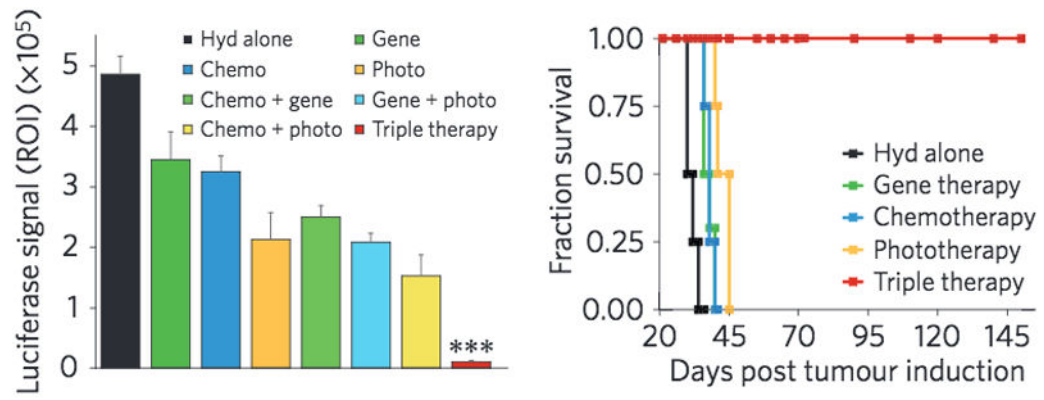


FIGURE 7.

Iron oxide nanoparticles and an alternating magnetic field induced hyperthermia when applied to primary melanoma tumors. The treatment elicited systemic immunity by slowing tumor growth of the contralateral, non-treated tumors. This effect was observed without administration of an additional immunotherapeutic agent, indicating that tumor ablation has the potential to induce systemic immunity [73]. Reprinted from *Nanomedicine: Nanotechnology, Biology, and Medicine*, vol 10(6): 1273–1285 (2014), Toraya-Brown et al., with permission from *Nanomedicine*.

**FIGURE 8.**

Triple combination of chemotherapy, siRNA, and photothermal therapy reduced tumor burden and improved survival compared to each therapy alone[145]. Reprinted from *Nature Materials*, vol 15, 1128–1138 (2016) Conde et al., with permission from *Nature Materials*.

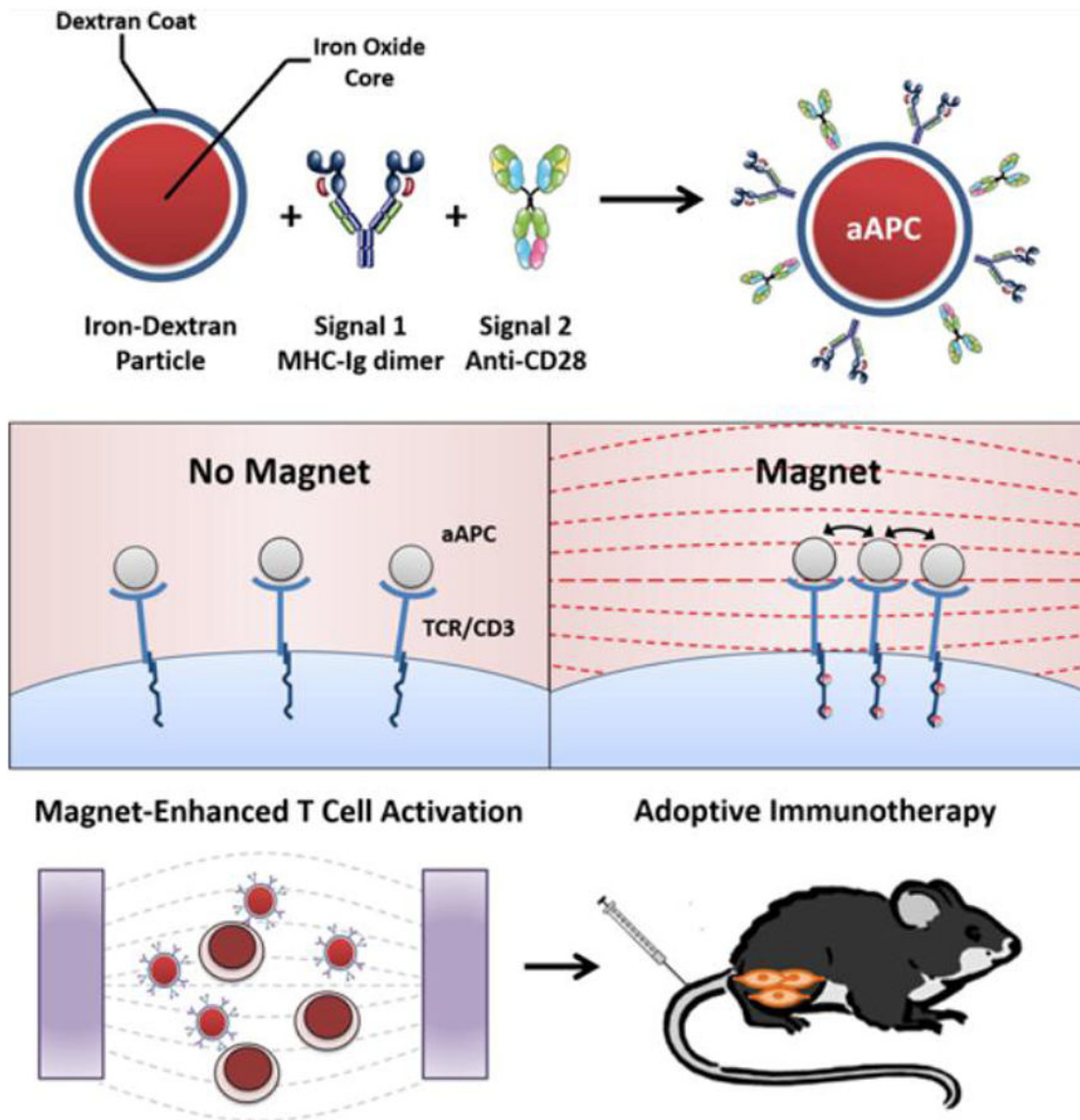


FIGURE 9. Paramagnetic nanoparticles clustered TCRs, which increased T cell expansion *ex vivo* and improved adoptive T-cell treatment of melanoma tumors. [154]. Reprinted from *ACS Nano*, 8(3):2252–2260 (2014) Perica et al. with permission from *ACS Nano*.

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	[67]
Cobalt oxide	Antigen delivery	Induce macrophage activation	Increased antigen-specific CTLs <i>in vivo</i>	[68]
Cuprous oxide	Alter tumor microenvironment	Alter expression of drosophila transcription factor	Induced myeloid infiltration and systemic immunity	[69]
Gold	Antigen/adjuvant delivery; Photothermal therapy	Increased CTL responses; tumor ablation released tumor antigens	Reduced tumor growth <i>in vivo</i> ; prevented tumor growth <i>in vivo</i>	[28, 70]
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	[71–73]
Silver	Reduce tumor-promoting cytokines	Decreased IL-1 β signaling in tumor microenvironment	Inhibited fibrosarcoma tumor growth <i>in vivo</i>	[74]
Titanium dioxide	Immune stimulation induced by ultrasound	ROS generation increased pro-inflammatory cytokines and interleukins in the tumor	Suppressed tumor growth <i>in vivo</i>	[75]
Zinc oxide	Antigen delivery (pulsed DCs)	Improved antigen-specific CTL responses	Delayed tumor growth <i>in vivo</i>	[76]

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.