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Cancer immunotherapy with immunoadjuvants, nanoparticles, and checkpoint inhibitors: Recent progress and challenges in treatment and tracking response to immunotherapy

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Abstract

Chemotherapy, surgery, and radiation are accepted as the preferred treatment modalities against cancer, but in recent years the use of immunotherapeutic approaches has gained prominence as the fourth treatment modality in cancer patients. In this approach, a patient's innate and adaptive immune systems are activated to achieve clearance of occult cancerous cells. In this review, we discuss the preclinical and clinical immunotherapeutic (e.g., immunoadjuvants (in-situ vaccines, oncolytic viruses, CXC antagonists, device activated agents), organic and inorganic nanoparticles, checkpoint blockade etc.) that are under investigation for cancer therapy and diagnostics. Additionally, the innovations in imaging of immune cells for tracking therapeutic responses and limitations (e.g., toxicity, inefficient immunomodulation, etc.) are described. Existing data suggest that if immune therapy is optimized, it can be a real and potentially paradigm-shifting cancer treatment frontier.

Keywords

Cancer immunology; Immunoadjuvant; Nanoparticles; Checkpoint inhibitor; Immunotherapy

1. Introduction

Advanced stage malignant tumors are typically resistant to chemo- and radiotherapy and are difficult to target, resulting in modest patient survival despite aggressive treatments. To improve clinical outcomes, current cancer research is focused on finding a more efficient and selective cancer therapeutic that overcomes the limitations of tumor evasive factors (Fouad & Aanei, 2017; Vinay, et al., 2015). In this context, immunotherapies are increasingly emerging as an interesting alternative approach due to their superior specificity and efficiency of targeting malignant tumors (Geering & Fussenegger, 2015).

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8. Conflict of Interest Statement

The authors declare that they have no competing interests.

Immunotherapies alter the tumor microenvironments by selectively recognizing and binding to immunosuppressive proteins that are expressed on tumor cells, activated T and B cells, macrophages, and regulatory T cells to activate local and systemic anti-tumor immune responses against cancerous cells (Farkona, Diamandis, & Blasutig, 2016; Gong, Chehrazi-Raffle, Reddi, & Salgia, 2018; Kruger, et al., 2019). While both innate and adaptive immunity play key roles in proper immune function, their key differences are important to distinguish. Innate immunity primarily responds to immediate and acute insults such as foreign pathogens, and it is mainly mediated by phagocytic cells such as natural killer cells, monocytes, macrophages, dendritic cells, and neutrophils (Platt & Wetzler, 2013). In contrast, foreign pathogens/agents are processed and presented by the major histocompatibilty complex (MHC) as antigens by innate cells to then activate the adaptive response, which includes cells of humoral and cell-mediated immunity (i.e., helper, cytotoxic, and suppressor-T cells and B cells). Other major differences between innate and adaptive immunity include efficacy, response time, and specificity. Innate response sacrifices specificity and effectiveness for a more immediate response that is always active. In contrast, adaptive response sacrifices speed for high specificity and extreme efficacy to specified antigens built over previous exposure (Boehm & Swann, 2014).

Recent research on immunomodulation as a possible contributor to a targeted design aimed against cancer has resulted in many different methodologies, including (i) immune-targeted nanoparticles (NPs), (ii) in situ vaccinations, (iii) checkpoint inhibitors such as anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) or anti programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1), and (iv) various combinations of these in combination with other known modalities. For example, many recent studies have evaluated the effect of NPs on both innate and adaptive immunity and their mode of action in tumor models. Together, (i) inorganic NPs, (ii) organic NPs, and (iii) combination NPs dominate this newly created niche. Likewise, the mode of action of in-situ vaccinations includes harnessing the ideal immune activation without the need for the labor-intensive biopsy for every individual patient (Hammerich, Bhardwaj, Kohrt, & Brody, 2016). The effectiveness of an in-situ vaccination (ISV) on a per patient basis can be high (Hammerich, et al., 2019). In situ vaccinations utilize the killing of tumor cells, the release of tumor-associated antigens (TAA), and the capturing of those antigens to activate T effector cells to elicit the systemic immune response and recognition of the tumor microenvironment (Pierce, Campbell, Pai, Brody, & Kohrt, 2015). The use of the local tumor as the antigen source in the context of the patients means that all relevant antigens, whether tumor-associated or neo-antigens released by ISV can be included in the vaccine, obviating the need to identify the antigens (Hammerich, Binder, & Brody, 2015). They are used most effectively in combination with other therapies, and extensive research has been focused on combinations with therapies previously listed (Hammerich, et al., 2016; Hammerich, et al., 2015). Beyond this, another goal to push immunomodulation to the forefront of all cancer therapies has been to observe immunotherapy through an imaging system in real time. A common pitfall of immunotherapy is the inability to monitor an improvement in immune responses in real time longitudinally and beyond the treatment window. Noninvasive macrophage or T cell imaging would not only provide peace of mind to the patient by showing that the therapy is effective and active, but it also would lend itself to filling many gaps in knowledge- such as the

elusive mechanism of the abscopal effect of some therapies to secondary tumors that were not directly targeted or treated. It would also provide a systematic and mechanistic link to explain how immune cells are directly related to any desired phenomena.

Herein, we review the recent pre-clinical and clinical utilization of immunomodulator therapies using any of the above listed approaches (NPs, ISVs, checkpoint inhibitors and/or immune cell imaging) alone or in combination, and we discuss both their beneficial aspects and their inevitable pitfalls in the broad context of innate versus adaptive immunity within cancer immunotherapeutic modalities (Fig. 1). We also discuss the next-generation combination therapeutic approaches, their associated pathways, and imaging methods to track immune cells for the assessment of therapeutic responses.

2. Immuno-Adjuvants

Immune adjuvants are based on modern vaccinations with a slight modification. Modern vaccinations commonly use an antigen and an adjuvant in conjunction to cause and boost an immune response and build antibodies against the chosen antigen. In contrast, immunoadjuvants (e.g., ISVs) use the tumor as the antigen source to elicit a patient-specific immune response (Hammerich, et al., 2015; Lizotte, et al., 2016). In particular, ISVs have the potential to greatly reduce the cost of therapy due to the simplicity of treatment on a per patient basis. A unique feature of the ISV regimen is that it alters an immunosuppressive tumor microenvironment from a low intratumoral T cell infiltration type to a T cell inflamed phenotype (Hammerich, et al., 2015; P. Sharma & Allison, 2015). ISVs also do not preclude other immunotherapies, and with modern image-guidance systems enabling precise visualization and injection into a tumor almost anywhere within the body, this approach can be translatable for treatment of a variety of tumors types.

Among the immunoadjuvant approaches actively under investigation, using oncolytic viruses (OVs) that target cancer themselves (Chiocca & Rabkin, 2014), and using novel antibodies/physical treatments appear to be important. These avenues have been pursued as stand-alone treatments and in a combinatory setting. Examples such as using oncolytic viruses to display TAAs to dendritic cells are described below.

2.1 Oncolytic Viruses

OVs utilize the altered immunogenicity of tumors to evade phagocytic death and replicate exclusively in the cancerous cells (Chiocca & Rabkin, 2014). Both natural non-pathogenic autonomous OVs (e.g., parvoviruses, myxoma virus (MYXV; poxvirus), Newcastle disease virus (NDV; paramyxovirus), reovirus, and Seneca valley virus (SVV; picornavirus); and genetically engineered OVs (e.g., measles virus (MV; paramyxovirus), poliovirus (PV; picornavirus), vaccinia virus (VV; poxvirus), adenovirus (Ad), herpes simplex virus (HSV), VV, and vesicular stomatitis virus (VSV; rhabdovirus)) have been investigated for tumor immune modulation (Chiocca & Rabkin, 2014). The microenvironment of a solid tumor is characterized by the presence of M2 macrophages (Hobson-Gutierrez & Carmona-Fontaine, 2018), release of cytokines such as IL-10 (Mannino, et al., 2015), and downregulation of type-I IFNs that normally promote an inflammatory response against foreign pathogens (Gajewski & Corrales, 2015). OVs can prey on the mutated machinery of solid tumor cells

and induce the release of TAAs and pro-inflammatory cytokines to re-activate systemic antitumor immunity. Here we discuss the main contributors in OV class for tumor immune modulations: adenoviruses, poxviruses (Breitbach, Thorne, Bell, & Kirn, 2012), herpes simplex virus (HSV) (Delwar, et al., 2016), coxsackieviruses, poliovirus, and measles virus (Brown, et al., 2014).

Adenoviruses are versatile and useful because they can be modified and infect many different cancer cell types (Uusi-Kerttula, Hulin-Curtis, Davies, & Parker, 2015). In addition, adenoviruses are typically replication incompetent (dies in the infected cells), achieve high gene delivery and expression, and their viral genome do not incorporate with the host genetic modules preventing genotoxicity (Sato-Dahlman & Yamamoto, 2018). Adenoviruses have been widely investigated against a variety of tumor types for oncolytic disruption of immunosuppressive environment (Gallo, Dharmapuri, Cipriani, & Monaci, 2005). For instance, adenovirus overexpressing the immune co-stimulator OX40 ligand (OX40L) was shown to induce immunogenic cell death (ICD) and recruit lymphocytes to inhibit growth of murine glioma (H. Jiang, et al., 2017). Despite significant promises, the challenges of transient expression, clearance, and non-specific targeting remain a key bottleneck in the clinical translation of adenovirus vectors. This has been addressed to some extent by development of targeted adenovirus, paving the way for clinical use of strain H101 in china (Yokoda, Nagalo, & Borad, 2018). Currently, several adenovirus-based gene delivery systems are in clinical trials for treatment of lung cancers (), cutaneous and uveal melanoma (Garcia, et al., 2019), and (pancreatic, biliary, colorectal or ovarian;). Early Phase I data from uveal melanoma studies in 12 patients indicates effective infection, but demonstrate a lack of tumor regression. Therefore, additional works are needed such that improved viral capsid and tumor cell interaction is attained to promote uptake and replications in the cancerous cells; details of which were covered in a recent review by *Baker et al.* (Baker, Aguirre-Hernandez, Hallden, & Parker, 2018).

Among the poxviruses, vaccinia viruses can effectively engage the T cells to promote oncolytic activity and bystander effect against secondary metastatic tumor cells without significant toxicities (F. Yu, et al., 2014). For example, a western reserve strain of vaccinia tested via intratumoral injection in a first-in-human phase I clinical trial was found to be non-toxic in the dose-escalation study (Zeh, et al., 2015). Other virus strains in phase I dose-escalation in advanced cancer cases have also shown superior outcomes with reduced toxicities to healthy organs. For instance, a mutant of HSV-1 replicated in tumors without damaging the surrounding normal tissue (Kasuya, et al., 2014). More recently, a promising OV called JX-594, a modified, replication-incompetent vaccinia virus encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) and LacZ, was shown to inhibit tumor vasculature among other multiple pathways (Merrick, Ilett, & Melcher, 2009). Additionally, when combined with sunitinib, a multitargeted kinase inhibitor, JX-594 amplified antitumor activity (M. Kim, et al., 2018). The detailed review of viral properties of vaccinia, herpes simplex virus (HSV), and coxsackievirus was reviewed recently (Z. S. Guo, et al., 2019). Notable findings from the review suggested that the viruses induce immunogenic tumor cell death, enhance STING and Batf3-dependent dendritic cell activation, and synergize with immunotherapies including checkpoint blockages. The main hurdles for these viruses included the sub-optimal propagation in the entire tumor cell population, inadequate

systemic antitumor effects against metastatic cells, and interactions with gut microbiota influencing the oncolysis of tumor cells and therapeutic outcomes.

2.2 Focused Ultrasound, and radiation based physical treatments

Adjuvants used as a part of a cancer therapy utilize a similar concept like OVs to create a more efficient systemic response by changing chemokine profiles, TLR modifications, and acting as a receptor agonist/antagonist to promote a certain pathway, thereby enabling immune detection of cancerous cells (Hammerich, et al., 2016). Adjuvancy to tumors can also be provided by physical treatments such as focused ultrasound (FUS) heating, which causes cell stress and tumor antigen release (Maloney & Hwang, 2015), thus promoting cancer recognition by the immune system. Unlike ionizing radiation, which damages collateral tissues and induces oncogenic proteins, FUS generates protein coagulation and non-lethal thermal stress in the tumors less aggressively (Silvestrini, et al., 2017; van den Bijgaart, et al., 2017). We and others have shown that FUS-induced local heating and stress modify the tumor microenvironment to impart several benefits including enhanced solid cancer chemotherapy, tumor antigen release, expression of heat-shock proteins, upregulation of pro-phagocytic signals such as calreticulin (CRT), and overall tumor immunity compared to conventional treatment (T. Chen, Guo, Han, Yang, & Cao, 2009; T. Chen, Guo, Yang, Zhu, & Cao, 2011; de Smet, et al., 2013; Formenti & Demaria, 2013; Z. Hu, et al., 2007; X. Huang, et al., 2012; Kang, Demaria, & Formenti, 2016; F. Liu, et al., 2010; Manzoor, et al., 2012; Ranjan, et al., 2012; Y. Zhang, Deng, Feng, & Wu, 2010). A tumor microenvironment modified with focused ultrasound can also improve combinatorial immunotherapy regimens. In murine models with bilateral B16 flank melanoma, we performed intratumoral injections of anti-CD40 in combination with FUS heating (M. Singh, et al., 2019). CD-40 is a member of the tumor necrosis factor receptor family that is highly expressed in antigen presenting cells (APCs) including macrophages, monocytes, dendritic cells, and B cells (Afreen & Dermime, 2014; Clark, et al., 1988; van Kooten & Banchereau, 2000). The administration of CD-40 agonists can reprogram macrophages and T-cells. We hypothesized that in situ vaccination with a combination of intratumoral CD-40 agonist antibody and local FUS heating (FUS40) will improve T-cell effector function and trigger tumor suppressive M1 macrophage phenotype in murine melanoma to result in superior anti-tumor effects. FUS heating was applied for ~15 min in right flank tumor, and intratumoral injections of CD-40 were performed sequentially within 4h. A total of 3 FUS and 4 anti-CD-40 treatments were administered unilaterally 3 days apart. Mice sacrificed 30 days post-inoculation showed increased population of tumor-specific CD-4+ and CD-8+T cells rich in Granzyme B+, interleukin-2 (IL-2) and IFN-γ production and with poor PD-1 expression upon the FUS40 combinatorial therapy. The resultant immune-enhancing effects of the combinatorial treatment suppressed B16 melanoma growth at the treated site by 2–3-fold compared to control, FUS, and CD-40, and achieved significant regression in untreated tumors relative to CD40 alone. In addition to CD40 agonists, FUS has also been combined with other therapies (e.g., checkpoint blockade (Silvestrini, et al., 2017), TLR agonist (Chavez, et al., 2018). Early data suggests an increased expression of genes associated with T-cell activation and stimulation (Eomes, Prf1 and Icos was 27, 56 and 89-fold higher) with CpG TLRs and curative treatments upon combination of checkpoint inhibitors with FUS ablation in mice

with high melanoma burden. This thereby suggests a need to further investigate the FUS-based combinatorial approaches in clinical settings.

Like FUS, radiation exposure of tumors can enhance immune detection by promoting immunogenic cell death, ATP and HMGB1 secretion and the translocation of calreticulin protein; all of which can result in enhanced tumor cell phagocytosis (Gameiro, et al., 2014). Mechanistically, radiation induced calreticulin in tumor cells is processed by dendritic cells facilitating antigen presentation and T-cell cytotoxicity, whereas the HMGB1 trigger the production of the cytokines TNF, IL-1, IL-6, and IL-8 from monocytes to enhance antigen production from the DCs by binding to toll like receptors, and the released ATPs bind to the purine receptors of DCs causing inflammasome release and IL1β (Z. I. Hu, McArthur, & Ho, 2017). An interesting feature of local radiation immune modulations is its ability to induce systemic antitumor effect. Several preclinical and some clinical case studies support the feasibility of this idea (Azami, et al., 2018; Leung, Wang, Jin-Jhih, & Chan, 2018), although such findings are rare and often inconsistent. Most likely, the immunoactivity of radiation is counterbalanced by suppressive cytokines, and cells (e.g. myeloid derived suppressive cells), thereby suggesting the need for combining them with checkpoint blockage, and other immune modulators. The interrelationship of radiation dose and checkpoint blockade, and outcomes against secondary untreated tumors have been covered in detail by other reviews (Buchwald, et al., 2018; Manukian, Bar-Ad, Lu, Argiris, & Johnson, 2019). In general, the reviews propose an overall optimal immune-stimulating radiation dose in the range of 8 and 10 Gy per fraction in 1–3 fractions, however, additional studies may be needed to verify these findings. It also appears that introducing immunotherapy immediately after radiation induced antigen released could be crucial for more durable response rates in patients.

2.3 Toll like, GM-CSF and CXC-chemokine receptors antagonists

The TLRs recognize different kinds of ligands based on their foreign characteristics. TLRs 1, 2, 4, 6, and 10 recognize specific lipid composition, TLRs 5 and 11 recognize foreign proteins, and TLRs 3, 7, 8, and 9 recognize foreign nucleic acids (Seledtsov, Goncharov, & Seledtsova, 2015). Additionally, TLR 2 recognizes Gram positive bacteria, TLR 4 recognizes Gram negative bacteria, and viruses are recognized by TLR 3 (by dsRNA), 7 (by ssRNA), and 9 (by CpG-DNA). TLR agonists of all kinds can be used as potential immunomodulators. For instance, targeted TLR 9/CpG-DNA using a TLR 9 agonist was used to disrupt the tumor microenvironment of a TC-1 cell line with HPV-16 E6 and E7 oncogenes in C57BL/6 mice containing myeloid-derived suppressor cells, tumor-associated macrophages, and T regulatory cells (Chang, et al., 2014). Stimulation of TLR 2 was found to cause colorectal cancer proliferation and growth whereas suppression by blockade or CRISPR modification induced the opposite effect (Y. D. Liu, et al., 2018). Furthermore, recent studies have shown a role for the TLRs in metabolic reprogramming of immune cells, directly impacting immunotherapy outcomes (L. Huang, Xu, & Peng, 2018). Although a detailed mechanistic discussion of TLR for cancer immunotherapy is beyond the scope of this paper, broadly TLRs act by influencing the differentiation and function of different T-cell subsets, including Th1, Th2 and Th17 cell, and through reprogramming of the suppressive function of tumor-derived $CD4^+$, $CD8^+$ and γ^δ Treg cells. Thus, the

combination of TLRs with FUS (and other therapies e.g., hypo-fractionated radiotherapy, checkpoints, etc.) can be a promising approach to cytotoxic therapy of solid tumors.

Other cancer immunotherapy approaches include incorporation of GM-CSF, CXCR antagonism, and heat shock protein modulations in tumors. GM-CSF is a chemokine that derives its antitumor effect through dendritic cell maturation. Colorectal cells that overexpress GM-CSF do not develop tumors in immunocompetent mice. Interestingly, 100% of patients diagnosed with these specific cancers survived at least 5 years after initial diagnosis (Urdinguio, et al., 2013). This finding suggested that immunomodulator therapies that target GM-CSF expression as a biomarker and a therapeutic can be highly promising. However, further investigation in other cancer models, including non-small cell lung cancer, glioma, human bladder cancer cell line 5637, colorectal cancer, melanoma, and bone metastasis, revealed that the prevalence of GMCSF is, in fact, a negative biomarker linked to a poor survival rate (Aliper, Frieden-Korovkina, Buzdin, Roumiantsev, & Zhavoronkov, 2014). Thus, using GM-CSF as a therapeutic or a biomarker may dependent on the type of cancer and the patient status, and needs detailed investigations prior to clinical introduction and use.

CXC-chemokine receptors are transmembrane G protein-coupled receptors that are known to bind cytokines of the CXC-family (e.g., CXCR2 and CXCR4) and are primarily responsible for growth and movement of cells during angiogenesis and neovascularization. Overexpression of CXCR2 and CXCR7 is a predictive indicator of poor outcome and is associated with metastasis and drug-resistance in human colorectal cancers (Desurmont, Skrypek et al. 2015). In one study, colorectal cancer patients (>50) who had CXCR7 expression above 1 had a survival rate of 28.0 ± 4.67 months compared to 64.4 ± 3.0 months for those with expression levels <1. Likewise, esophageal cancer expressing CXCR2 was linked to significantly higher incidences of distant metastasis ($p = 0.0052$; 57.6%) compared to those with lower CXCR2 expression (36.7%, $p = 0.063$) (Nishi, Takeuchi et al. 2015). In another study, CXCR4 and CXCR7 expression in breast cancer cells of 100 breast cancer patients showed a positive correlation with progressive worsening condition associated with many roles- primarily metastasis (Wu, Qian et al. 2015). Further, oral cells expression of CXCR1 and CXCR2 was typically higher in cancerous cells. When exposed to IL-8 and GROα, an increase in Matrix Metallopeptidase 7 (MMP-7) was noticed in oral cancer cell lines compared to normal cells. Thus, while still not fully understood, CXCRs seem to play a role in cancer biology (Khurram, Bingle et al. 2014).

Based on the aforementioned study results, researchers have tried to develop antagonists that would counter the effects of CXCRs. The main effect of such therapies is limiting the migration and metastasis of an existing tumor and improving immunotherapy outcomes (S. B. Peng, et al., 2015; Xiang, et al., 2015). For example, *Gil et al.* blocked CXCR4 using an oncolytic vaccinia virus to inhibit ID8 mouse ovarian epithelial cancer cell growth and metastasis (Gil, Komorowski et al. 2014). Currently, cancers such as osteosarcoma (K. Jiang, et al., 2019), prostate carcinoma (Goltz, et al., 2016) and hepatocellular carcinoma (Y. Chen, et al., 2015) show significant resistance to checkpoint inhibition. While the exact mechanism is not yet fully understood, it is hypothesized that CXCR4/CXCL12 axis and cancer associated fibroblasts may be responsible for checkpoint resistance (Scala, 2015). For

example, in murine pancreatic cancer, *Fearon et al.* showed that the resistance to checkpoints is mediated via carcinoma-associated fibroblasts (CAF) that also influences the CXCR4/CXCL12 axis (Fearon, 2014). In general, an inverse relationship between CXCR4 expression and immune activation have been established in several studies. For instance, in a K7M2 mouse osteosarcoma model overexpressing CXCR4, the myeloid-derived suppressor cells in tumor inhibited T cell proliferation by up to 80% (K. Jiang, et al., 2019). *Dietrich et al.* found that patients with high PD-L1 expression and aberrant CXCL12 methylation demonstrated significantly shorter survival rates compared to those with low PD-L1 expression or normal CXCL12 methylation, supporting the link between CXCR4/CXCL12 and PD-1/PD-L1 pathways (Goltz, et al., 2016). To overcome these barriers, *Zeng et al.* combined AMD3100, a known CXCR4 antagonist (Plerixafor), and aPD-1 antibody alone or in combination in an immunocompetent syngeneic mouse model of ovarian cancer to prolong survival rates by enhancing T-cell function, and reprogramming the T regulatory cells into T-helper cells (Zeng, et al., 2019). In sorafenib resistant hepatocellular carcinoma, *Chen et al.* combined sorafenib, a broad tyrosine kinase inhibitor, PD-L1 antibodies and AMD-3100 to prevent immune cell suppression, enhance immune cell tumor infiltration, increase apoptosis/necrosis of tumor cells and overall delay hepatocellular carcinoma progression/metastasis (Y. Chen, et al., 2015). The K7M2 mouse osteosarcoma earlier mentioned was also treated with a CXCR4 antagonist with aPD-1 to increase survival of their subcutaneous osteosarcoma model in immunocompetent BALB/c mice. Combination of PD-L1 and K7M2 significantly upregulated the Ki67, intracellular granzyme B, IFN-γ, and TNF-α expression in CD8+ TILs relative to AMD3100 and aPD-1 monotherapy respectively. More recently, *Wu et al.* showed evidence of modulation of the tumor microenvironment and tumor vasculature in murine glioma models and increased survival *in vivo* in C57 BL/6 mice with anti-CXCR4 and aPD-1 combination compared to monotherapies (A. Wu, et al., 2018). All of these findings suggest suggesting that CXCR4 antagonism can potentially synergize with the immunotherapies (K. Jiang, et al., 2019).

2.3 Cancer cell vaccines

Interest in the use of DCs or other antigen-presenting cells as a possible immunomodulation therapy for solid tumors has grown immensely. For example, targeting dendritic cells using a B16F10-OVA-liposome in combination with a DC maturation signal such as IFN-gamma or GMCSF liposomes was shown to have potent antitumor activity and decreased lung metastasis *in vivo* (van Broekhoven, Parish, Demangel, Britton, & Altin, 2004). *Waeckerleman et al.* used PLGA microspheres as delivery tools for antigen loading of human monocyte-derived DC (hMoDC). They found that this process prolonged antigen presentation of the dendritic cells compared to soluble antigens without impacting the migration, cytokine secretion, survival and allostimulation of T cells (Waeckerle-Men & Groettrup, 2005). Similarly, GM-CSF derived-dendritic cells acted as both antigen donor and antigen presenting cells in murine fibrosarcoma (Ebrahimi-Nik, et al., 2018). Other research has shown the benefits of using a combination of photodynamic therapy (to increase antigen-presenting capacity) with dendritic cell vaccination in squamous cell carcinoma to induce an effective immune response (H. Zhang, et al., 2018). Another combinatorial therapy used an adenoviral vector to selectively activate dendritic cells (Chondronasiou, et al., 2018).

2.4 Limitations

In some cases, viruses can replicate in the patient, so development of replication-incompetent virus is crucial (Chiocca & Rabkin, 2014). Some virus or adjuvant treatments require that the therapeutic agent cross the blood-brain barrier, which is a challenge. Potential approaches that may overcome this problem include using smaller viruses (e.g., parvovirus) or injecting the agent directly into the central nervous system. Moreover, the long-term effect of in situ vaccinations as an immunotherapeutic or prophylactic treatment have not been extensively studied to date.

3. Nanoparticles

NPs can be either inorganic (i.e., gold, gadolinium, etc.) or organic based (i.e. carbon, hydrogen, nitrogen, phosphorus, sulfur) on the elements they incorporate. NPs vary widely in size and shape, but a broad spectrum of acceptable sizes is 1 to 100 nm. Typically, spherical NPs (~100–200nm) are translated for biological applications since they are easy to fabricate and load drugs, but non-spherical structures are also starting to emerge due to their ability to achieve superior accumulation in targeted tissues (Jurney, et al., 2017; Truong, Whittaker, Mak, & Davis, 2015). NPs have unique physical characteristic and capabilities, such as a high surface area to volume ratio which gives the ability to conjugate many different moieties like antibodies, antigens, and small molecules for cell labeling/targeting. NPs have been primarily investigated for targeted delivery of a payload (Fischer, Rasley, & Blanchette, 2014). Other unique features of NPs include optical, thermal, and/or magnetic properties. Within this scope, fine-tuning of NP synthesis has led to the development of agents that are endowed with specified, desired properties such as optimal light-scattering for use in sunscreen lotions to limit direct skin exposure to UVA and UVB rays. Depending on the desired use, NPs can be tailored (Hewakuruppu, et al., 2013), and increase the tumoral exposure by the enhanced permeability and retention (EPR) effect (Baetke, Lammers, & Kiessling, 2015). For example, EPR methodology was used to decrease doxorubicin cardiotoxicity as seen in the case of Doxil®, which is FDA-approved while also enhancing tumor delivery and therapeutic effects (Barenholz, 2012).

Whether organic or inorganic, NPs are typically modified with polyethylene glycol (PEG) groups to help evade detection by the immune system and circumvent a systemic response (Amoozgar & Yeo, 2012). In particular, the most significant interaction between NPs and phagocytic cells appears to be through pattern recognition receptors recognizing pathogen-associated molecular patterns (Ward & Rosenthal, 2014). In contrast, B cell activation within current forms of immunomodulation (outside of the dendritic cell cascade) with nanoparticles is not well known or understood to date.

Although initially NPs were primarily designed to evade the immune response, many recent studies have focused on their use as immunotherapeutics due to their tendency to expand the population of immune cells and alter the cytokine profile around the tumor microenvironment (Almeida, Chen, Foster, & Drezek, 2011). These studies utilized NPs primarily in combinatorial regimens to additively or synergistically enhance specific immune effects in comparison to individual modes of therapy. Some NPs can also work as adjuvants to push T-helper cell activation to either the Th1 or Th2 cell phenotype to promote

cell-mediated immunity or humoral immunity. For instance, fullerene cages achieved higher Th1/Th2 ratio in comparison to controls in murine models by decreasing the production of Th2 cytokines (IL-4, IL-5, and IL-6) and increasing the production of Th1 cytokines (IL-2, IFN, and TNF) (Y. H. Luo, Chang, & Lin, 2015).

3.1 Inorganic Nanoparticles (INPs)

Metal/metal oxides NPs, referred to here as INPs, have high optical, thermal, and magnetic properties that are amenable to contrast imaging and targeted therapy. As of current cancer therapies/motifs, the most prevalent INPs seem to include gold nanoparticles (GNPs), superparamagnetic iron oxide NPs (SPIONs), gadolinium NPs and quantum dots (QDs). GNPs can be used with photothermal therapies (Hwang, et al., 2014), as a radiosensitizer (Butterworth, McMahon, Currell, & Prise, 2012) (Jain, et al., 2014), or as a therapeutic/therapeutic carrier (Kumar, et al., 2012) (Ekin, Karatas, Culha, & Ozen, 2014). Additionally, a clinical trial in 2015 reported that GNPs are safe to use in combination with laser ablation in prostate cancers (Stern, et al., 2016).

Iron oxide nanoparticles (e.g., SPIONs) are very popular medicinally because of their unique properties (Kandasamy & Maity, 2015), such as a T2-MRI contrast agent (Yoffe, Leshuk, Everett, & Gu, 2013) (Kato, et al., 2015), their accumulation in tumors (Baetke, et al., 2015), and their highly magnetic properties (Tietze, et al., 2015). In the presence of an external alternating magnetic frequency, SPIONs can also generate local heating by molecular vibrations either via Neel mode that rotate its magnetic moment toward the magnetic field or by rotational friction that is provided by the resistance of the suspending medium (Dan, Bae, Pittman, & Yokel, 2015; DeNardo & DeNardo, 2008). If controlled well, mild heating with AMF do not impact the function of both the SPIONs and proteins/antibodies (Solar, et al., 2015; Thirunavukkarasu, et al., 2018). *Johnson et al.* showed that SPIONS have potential to replace “sentinel lymph node biopsy in the management of early breast cancer with *in vivo* pre-operative metastasis detection” by mainly accumulating in the lymph node and surrounding areas of metastasis in a clinical trial (Johnson, Pinder, & Douek, 2013). *Maier-Hauff et al.* showed the safety and efficacy of intratumoral injections of iron oxide NPs in combination with an alternating magnetic field in 66 patients (59 with recurrent glioblastoma); they described significantly longer overall survival after diagnosis of first tumor recurrence, the safety of therapy along with smaller radiation doses, and general safety with minimal adverse side effects (Maier-Hauff, et al., 2011). The exploitation of SPIONS with cancer biomarkers as targets, such as high overexpression of mucin 1 and folate receptors in most malignant adenocarcinomas (Aghanejad, Babamiri, Adibkia, Barar, & Omidi, 2018) or VEGF-A (vascular endothelial growth factor-A) overexpression in tumor angiogenesis also appears promising (Tsoukalas, et al., 2018). Other imageable NPs include those containing gadolinium (Y. Li, Song, Cao, Peng, & Yang, 2018), as it is an ideal f-block metal for maximizing MRI imaging capabilities. Gadolinium oxide NPs can be synthesized readily (T. J. Kim, Chae, Chang, & Lee, 2013). However, to date the research using these NPs has focused mainly on imaging capabilities and less on immunomodulation.

In this review, we focus on designs that have been synthesized and studied in either *in vivo* or *in vitro* models and on the data available in the literature. GNPs of all sizes and shapes

have been tested for their potential immunomodulatory effects (P. Singh, et al., 2018), but before delving into this topic, we must understand how different cells interact with these NPs. To understand the interaction and uptake of GNPs, *Liu et al.* examined accumulation of differently charged GNPs (positively or negatively charged) in phagocytic RAW 264.7 macrophage cells and nonphagocytic HepG2 cells. They reported that RAW264.7 cells accumulated similar amounts of positive and negative NPs, but HepG2 cells accumulated more positive than negative GNPs, suggesting that phagocytic immune cells clears the GNP regardless of the type of surface charge (X. Liu, Huang, Li, Jin, & Ji, 2013). In fact, GNPs are known to stay in liver macrophages for up to 6 months post-treatment (Sadauskas, et al., 2009). Additionally, in macrophages GNPs can show size-dependent inhibition of toll-like-receptor (TLR9) function (C. Y. Tsai, et al., 2012), which illustrates their role in inflammatory pathways. As an immunomodulator, GNPs can act as both a pro-inflammatory and an anti-inflammatory agent (Almeida, Figueroa, & Drezek, 2014). Naked GNPs tend to have a pro-inflammatory response and a cytotoxic effect in macrophages *in vitro* by increasing levels of TNF-α, IL-1, and IL-6 (Yen, Hsu, & Tsai, 2009), whereas modified GNPs, such as those coated in citrate, tend to be immunosuppressive by downregulating IL-1β, a proinflammatory cytokine, both *in vitro* and *in vivo* (Sumbayev, et al., 2013). Functionalization of GNPs with peptides molecules typically aids anti-tumor immunity. For example, GNP bound to TGF-B1 decreased IL-10 to alter the tumor microenvironment, significantly reducing the protective immunosuppressive cytokine profile, and increased levels of tumor-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes in murine bladder cancer models (Y. S. Tsai, et al., 2013). Additionally, certain peptide-functionalized GNPs effectively polarizes liver macrophages to either M2 or M1 (Bartneck, et al., 2012). Overall, data indicate that GNPs increase the immune cell numbers and cytokine profiles depending on the synthetic approach (e.g., coating, shape and size to give a specific, desired therapeutic response).

Iron oxide NPs are generally used for their ability to be imaged by MRI or their magnetic properties (Tietze, et al., 2015). Studies focused on altering the immune profile using iron oxide NPs have reported magnetic field-induced T cell activation with 4-fold greater naïve T cell proliferation compared to the no magnet control (Perica, et al., 2014) or magnet-enhancement in T cell activation with 2-fold more T cell receptor signaling with MHC-Ig and anti-CD28 antibodies bound to iron-dextran NPs in murine B16 melanoma models (Yigit, Moore, & Medarova, 2012). Another advantage of magnetic NPs, such as SPIONs, is their ability to initiate hyperthermia in the tumor while minimizing heat transfer to surrounding tissues (Hoopes, et al., 2018). *Huang et al.* reported a tumor temperature of up to 66 °C while maintaining muscle tissue at no more than 42 °C (H. S. Huang & Hainfeld, 2013). We and others found that combining iron oxide NPs with Alternating Magnetic Field (AMF) heating aids the immune stimulatory potential of radiation therapy (RT), resulting in an extended tumor control and systemic immune effects in canine and rodent models (Hoopes, et al., 2017). In particular, in murine melanoma models, heating B16 primary tumors at 43°C for 30 min with iron oxide/AMF combination activated dendritic cells (DCs) and propagated melanoma specific CD8(+) T cells in the draining lymph node, conferring systemic resistance against re-challenge with melanoma cells (Toraya-Brown, et al., 2014). More recently, the FDA-approved iron oxide nanoparticle compound, ferumoxytol was

found to delay mammary cancers, and lung cancer metastases in liver and lungs of murine cancers by inducing a pro-inflammatory M1 phenotype, protecting against cancer recurrence even in the absence of AMF (Zanganeh, et al., 2016). The proposed mechanism was by creating a cascade of reactive oxygen species (ROS) via the induction of Fenton reaction, followed by iron oxide-induced M1 macrophage polarization. This process augmented the production of TNFα and nitric oxide to promote cancer cell apoptosis. In summary, iron oxide NPs undoubtedly enhances anti-tumor immunity, however, more studies are needed to fully understand how to utilize them in combination with other immunomodulators to achieve superior outcomes against a variety of tumor types.

Quantum dots (QDs) are becoming more and more relevant with the increased manufacturing of them for consumer-based applications. Beyond their industrial use, QDs have shown some potential for imaging within live cells (Michalet, et al., 2005). Like the other NPs discussed, QDs can have immunomodulatory effects. Sub-lethal doses of CdSe/ZnS (cadmium selenide/zinc sulfide) QDs in human epidermal keratinocytes and human dermal fibroblasts have inflammatory effects by increasing multiple cytokines and chemokines such as TNF-α, IL-1β, IL-1, IL-10, IL-6, CASP1 and others associated with immune-activated apoptotic responses. *Romoser et al.* suggested that QDs control immunomodulation primarily through the NF-κβ pathway in human skin cells (Romoser, et al., 2011). Graphene QDs show a similar trend of NF-κβ inducing generation of ROS, autophagy, apoptosis, and inflammation by promoting TNF-α, increasing phosphorylation of p38 and p65, and increasing expression of caspase-3, caspase-9, IL-1β and IL-8 in THP-1 activated macrophages (Qin, et al., 2015). Data about the effects of QDs will increase drastically over the next decade as more cell studies testing newer QD formulations are conducted.

Other metal-based, inorganic NPs can have immunomodulatory effects as well. For example, silver NPs have been shown to alter the IL-1β profile (Yang, Kim, Kim, & Choi, 2012), or titanium dioxide NPs have been shown to increase the Th2 immune response (Larsen, Roursgaard, Jensen, & Nielsen, 2010). However, only the most popular NPs to date are included in this discussion. Other examples can be found in Table 1.

3.2 Organic Nanoparticles

Liposomes, and core-shell polymeric structures are commonly used organic NPs. Liposomes have an internal water-soluble cavity with a phospholipid bilayer membrane (W. D. Wu, et al., 2015). A meta-analysis of 10 randomized controlled trials showed that a water-soluble therapy, such as doxorubicin, could be encapsulated in a liposome to lower cardiotoxicity of normal routes of therapy (Xing, Yan, Yu, & Shen, 2015). Doxorubicin (DOX) can also be attached to the surface of micelles or dendrimers. Functionalization of the surface can be achieved in all cases with specified methodologies. Altering the lipid profile of liposomes lends the ability to burst them in response to hyperthermic conditions and aids in the concept of a targeted, deliverable payload (Kong & Dewhirst, 1999). Additionally, manipulation of the surface with proteins or ligands to target certain processes or cell types further enhances the specificity of targeted delivery for a desired effect.

This review focuses mainly on liposomes and poly(d,l-lactide-co-glycolide) nanoparticles (PLGA) polymeric nanoparticles for immunotherapy. Liposomes can be used as hollow transportation vessels for chemotherapies, but some formulations (e.g., cationic liposomes) can also have immunomodulatory effects via stimulation of the antigen presenting dendritic cells (DC) leading to the expression of co-stimulatory molecules, CD80 and CD86 (Vangasseri, et al., 2006). *Collins et al.* showed that ovalbumin (OVA)-encapsulated acid-resistant liposomes could generate class I MHC-restricted T cell responses *in vivo* by lysosomal processing and recycling of immunogenic peptides (D. S. Collins, Findlay, & Harding, 1992; Harding, Collins, Kanagawa, & Unanue, 1991). *Suzuki and colleagues* used unmethylated cytosine-phosphorothioateguanine oligodeoxynucleotide (CpG-ODNs)-encapsulated cationic liposomes as an adjuvant to actively target antigen presenting cells (e.g., dendritic cells) by binding to TLR9 and increasing serum IL-12 levels, increasing type I innate immunity (Suzuki, et al., 2004). TLR9, the target of CpG typically localizes in the endosomal/vacuolar/vesicular compartments but not to the cell surface. Data suggested that liposome encapsulation of unmodified CpG-ODN enhanced the dendritic cell uptake and IL-12 production. Additionally, this approach induced IFN-γ but not IL-4 production by natural killer cells by ligating TLR9 to CpG-liposome coencapsulated ovalbumin (OVA); features essential for Th1-dependent cytotoxicity against OVA-expressing tumor cells. If used in combination with a stimulatory RNA or DNA, liposomes can also be used for targeted epitope expression and activation of T-cells. RNA encoding tumor associated, or tumor specific epitopes, were encapsulated in liposomes to enhance the targeting of antigen-presenting cells (i.e., splenic macrophages, dendritic cells, Kupffer cells) for epitope encoded activation of the T cell (Sayour, Mendez-Gomez, & Mitchell, 2018). The key benefits of the RNA-liposome approach include immune activation following parenteral injections, simultaneous loading of multiple epitope generating mRNAs and the induction of type I interferon response. Liposomes and/or liposome-type NPs have also shown to deliver plasmid DNAs that normally would be very difficult to achieve (Moon, et al., 2011). For instance, cationic liposomes were shown to enhance the delivery of plasmid DNA to elicit antitumor immunity in murine model of cervical cancer (Sayour, et al., 2018). Unlike viral vectors that risk mutagenic integration with host cells, liposomes are a promising and safer gene drug delivery technology (Seow & Wood, 2009), and thus are starting to enter clinical trials for immunotherapy investigations (Madhusudan, et al., 2004; Wakabayashi, et al., 2008; D. Wang, Sun, Liu, Meng, & Lee, 2018; Yoo, et al., 2001). Studies are currently underway to further improve the transfection and expression efficiencies in a targeted manner in tumor cells while reducing side effects to healthy organs and induction of autoimmunity.

PLGA NPs have been thoroughly investigated and found to be very compatible with cells and tissues, as they have a very low toxicity profile and provide controlled release of encapsulated proteins. Many researchers have investigated different preparation techniques, drug encapsulations, and surface modifications (Sadat Tabatabaei Mirakabad, et al., 2014). PLGAs have been synthesized with a combination of Poloxamer 235/encapsulated docetaxel to treat the multidrug resistant human breast cancer cell line MCF-7/TXT, but they had a significantly higher toxicity profile than the control (Tang, et al., 2015). Several recent studies have assessed potential modalities of PLGA NPs in combination therapy and/or as a

targeted delivery transporter (Heit, Schmitz, Haas, Busch, & Wagner, 2007). As an immune therapy, PLGAs can incorporate other moieties, just as liposomes do, to increase potential application in a combination therapy (e.g., as a carrier for a vaccine or drug). Using the B16 cell line, *Zhang et al.* reported data that support effective induction of an antitumor cytotoxic T cell response when PLGA NPs containing murine melanoma antigenic peptides or TLR4 agonist were used in a prophylactic setting. Moreover, when administered with interferon gamma (IFN-λ), the antitumor activity was significantly augmented (Z. Zhang, et al., 2011). Furthermore, exogenous antigens seem to have a much higher MHC class I presentation *in vivo* when loaded into PLGA NPs. It was reported that "in primary mouse bone marrow-derived dendritic cells, the MHC class I presentation of PLGA-encapsulated OVA stimulated T cell interleukin-2 secretion at 1000-fold lower concentration than soluble antigen and 10-fold lower than antigen-coated latex beads"(H. Shen, et al., 2006; Waeckerle-Men, et al., 2006). *Chen et al.* showed that PLGA-NPs coencapsulated with indocyanine green (ICG), a photothermal agent, and imiquimod (R837), a TLR7 agonist was effective in inducing simultaneous photothermal ablation locally, and triggering release of TAAs for superior immunological responses with checkpoint therapies in malignant and orthotopic murine breast and colon cancer models (Q. Chen, et al., 2016). It may be noted that although PLGA-NPs loaded with antigen can enhance anti-tumor immunity, however, they also generate high population of T-regulatory cells, suggesting a need to combine them with checkpoint blockade. In summary, considering that PLGA material is FDA-approved, and achieves multifunctional outcomes as listed above, the improvement in particle design principles hold significant potential for immunotherapy applications.

Interest in the NP approach has even extended into synthesizing combination organic/inorganic NPs and synergizing their therapeutic effects with device applicators. Combinations could include incorporating metals into the film preps of organic NPs, adding polymers during metal NP synthesis, crosslinking metal-containing molecules to previously made organic NPs, or crosslinking proteins to previously synthesized inorganic NPs. Examples of combination NP approaches could include organic-coated silver NPs (V. K. Sharma, Siskova, Zboril, & Gardea-Torresdey, 2014), fluorescent sulfonate-based inorganic-organic NPs (Poss, Zittel, Meschkov, Schepers, & Feldmann, 2018), zinc-doped iron oxide core NPs with a biocompatible mesoporous silica shell with miRNA and DOX (Deng, et al., 2018), hydrophilic surface-functionalized superparamagnetic NPs to be used in magnetic fluid hyperthermia-based thermotherapy (Kandasamy, Sudame, Luthra, Saini, & Maity, 2018), and biocompatible chitosan-functionalized upconverting nanocomposites (Duong, et al., 2018). Many NPs combination have been extended for immunoadjuvant capabilities with increased efficiency. For instance, iron oxide-zinc oxide core-shell nanoparticles with ZnO-binding peptides were used for improved delivery of tumor antigens and the real-time imaging of dendritic cells trafficking in lymph nodes. *In vivo* studies in murine tumor models by magnetic resonance imaging suggested an effective uptake by the dendritic cells. Additionally, an antigen specific T-cell activation resulting in a superior tumor regressions and survival rates were noted (Cho, et al., 2011). Likewise, core-shell lipidic nanoparticles composed of Fe_3O_4 magnetic nanoclusters (MNCs) and anti-CD205 decorated shells were shown to facilitate efficient dendritic cell uptake and MHC I cross-presentation in 4T1 murine breast cancer model. The resultant immune activation efficiently propagated $CD8^+$ T

cells and tumor control of malignant tumors, while also providing efficient tracking of immunotherapy responses by MRI imaging (F. Li, et al., 2019). Like lipids, iron oxide along with perfluoropentane (PFP) and anti PD-1 (aPD-1)-coated with PLGA shells have been investigated for local checkpoint inhibitor delivery (N. Zhang, et al., 2019). Several other nanoparticles incorporating PLGA have been seen to improve immunotherapy outcomes in murine models not discussed here have also been reported in literature (Cruz, et al., 2014; Jin, Luo, Zhang, & Pang, 2018; Saengruengrit, et al., 2018).

Other combinatorial approach to aid NP immunotherapy includes synergizing its therapeutic effect with photothermal modality. For instance, PLGA NPs effectively delivered aPD-1 locally especially when combined with photothermal therapy and that this combination is effective in tumor control via increased CD8+ T cell infiltration in melanoma (N. Zhang, et al., 2019). Similarly, chitosan-coated-CpG hollow copper sulfide (CuS) NP achieved tumor size reduction in both primary and secondary untreated tumors in a mouse breast cancer model with photothermal therapy (L. Guo, et al., 2014). Therapeutically, following laser irradiation, the intratumorally injected NPs disintegrated and reassemble into chitosan-CpG nanocomplexes, allowing CpG tumor retention and uptake by dendritic cells and enhancing the cross-priming of the tumor antigen-specific T cells. In another study, administration of CpG oligodeoxynucleotide-coated Prussian blue nanoparticles (CpG-PBNPs) induced tumor cell death and antigen release with photothermal therapy in vitro and in murine model of neuroblastoma (Cano-Mejia, Bookstaver, Sweeney, Jewell, & Fernandes, 2019). Importantly, PBNPs alone enhanced the anti-CTLA-4 checkpoint inhibition effect to result in significantly higher survival (55% survival vs. 12.5% aCTLA-4 alone) and increased protection to re-challenge in the neuroblastoma murine model (Cano-Mejia, et al., 2017). Other types of NPs such as PEG-PEI-covered nanosized graphene oxide sheet encapsulating CpG oligodeoxynucleotide has also been found to have immunostimulatory properties with photothermal therapy (evaluated by TNF-α and IL-6) and enhanced survival *in vivo* in a colon cancer model (Tao, Ju, Ren, & Qu, 2014). In the future, the NP approach with the most usage among the previously listed options could be used in a combination treatment to effectively synergize therapies while limiting negative effects such as toxicity or high excretion rate. INPs can be incorporated in organic materials to increase the immunomodulatory effects and increase stealth delivery and local biological effects.

3.3 Immunotoxicity/Limitations

For the most part, NPs have been thoroughly investigated for their general acute and/or chronic toxicities. Metal or metal oxide NPs exposure by inhalation due to recent industrial and medicinal advancements remains a concern (Bakand, Hayes, & Dechsakulthorn, 2012). Previous studies have shown that oral dosages are significantly less toxic in comparison to the inhalation route of exposure. The general mode of toxicity can be necrotic and/or apoptotic separately and independently (Buerki-Thurnherr, et al., 2013). As a general rule when comparing metal and metal oxide NPs, the toxicity profile increases with a reduction in size; however, there still is a difference depending on the metal used (Sutunkova, et al., 2018). Table 1 shows the immunotoxicity profile of different NPs. Organic NP toxicity is generally less cumbersome because they are naturally occurring biocompatible compounds that tend to be less hazardous than metals. A few cases of NPs with a high level of toxicity

have been reported, but many adverse side effects can be decreased and minimized by incorporating some modification of surface chemistry (e.g., lipids or proteins) to make them “stealthier” and not as cytotoxic. Considering that the NP immunomodulation approach is a relatively new field, the continued learning of managing adverse toxic effects should be a key goal. We propose that combination immunotherapies (e.g., modified NP plus checkpoint inhibitor) rather than nanoparticle alone is the way to translate this approach for clinical use. Furthermore, development of targeted approaches such as the use of anti-CD8a F(ab’)2 fragment decorated NPs for CD8+ T cell attachment (Schmid, et al., 2017), and leveraging the inherent physicochemical properties of nanomaterials for modification of innate immunity (Y. Liu, Hardie, Zhang, & Rotello, 2017) can provide innovative ways to improve therapeutic outcomes in patients.

4. Checkpoint Inhibitors

Checkpoint inhibitors are proteins or monoclonal antibodies that function to downregulate the natural immune response mediated by CTLA-4 and PD-1 pathways to foreign cancer cells. The two predominantly discussed checkpoint inhibitors include anti CTLA-4 and anti PD-1/PD-L1. Some less studied and more recent checkpoint inhibitors are briefly discussed as well. Table 2 describes the drugs used for the clinically relevant PD-1 and CTLA-4 pathways, their pathway of inhibition, and which cancer cell lines are most affected to date.

4.1 Anti PD-1

PD-1 is a cell surface protein expressed on both activated T cells and professional B cells. This immune checkpoint receptor in the immunoglobulin superfamily and in the extended family of CD28/CTLA4 binds PD-L1, also known as CD274 or B7-H1, which is a transmembrane protein in tumor cells (P. Zheng & Zhou, 2015) (Afreen & Dermime, 2014). The normal function of this receptor interaction is downregulation of apoptosis of T regulatory cells and suppression of antigen-specific immune cells proliferating in the lymph nodes, therefore promoting an immunosuppressive environment. These help reduce autoimmunity complications and maintain homeostasis of immunity. However, certain cancers (e.g., metastatic melanoma, non-small cell lung cancer, and renal cell cancer) can disrupt PD-1/PD-L1 expression to evade immune detection (Hargadon, Johnson, & Williams, 2018). The recent interest in blocking the cascade reaction of immunosuppression from PD-1/PD-L1 by blocking either the protein, PD-L1, or the actual receptor, PD-1, to activate effector T-cell function and tumor cell killing has resulted in a plethora of recently synthesized antagonists (Table 2). The PD-1/PD-L1 axis has also been exploited in HIV-1, which perpetually downregulates the activation of T cells by binding PD-1; this shows the potential of using aPD-1 therapies as an anti-HIV therapy (Gay, et al., 2017). Interestingly, PD-L1 can also be expressed and upregulated in immune cells via IFN-γ–induced adaptive regulation in tumors through pre-existing immunity, and a high PD-L1 expression in tumor immune cells generally correlates with a more durable clinical responses to aPD-L1s (Fehrenbacher, et al., 2016; Kowanetz, et al., 2018; Yamazaki, et al., 2002). A meta-analysis of 18 studies involving 3647 patients in breast cancer, 4,549 cases of non-small lung cancer and 37 stage III malignant melanoma patients showed that the expression of PD-L1 in tumor

infiltrating immune cells correlated strongly with enhanced survival rates (Jacquelot, et al., 2017; Kowanetz, et al., 2018; Zhao, Li, Wu, Li, & Zhang, 2017).

Clinical trials are increasingly being reported for a variety of PD-1 checkpoint inhibitors. For example, aPD1 Nivolumab was tested versus docetaxel in advanced non-small-cell lung cancer during or after platinum-based therapy in over 500 patients to determine if the treatment significantly increased overall survival rates (Borghaei, et al., 2015). Data showed that the median overall survival was significantly higher for nivolumab group (12.2 months; 292 patients) versus the docetaxel group (9.4 months; 290 patients). *Lipson et al.* reported results from a pilot phase I clinical trial in 3 patients diagnosed with colorectal cancer, renal cell cancer, and melanoma who were treated with aPD 1 antibody BMS-936558; they found a complete response in colon and renal cell cancer, and a partial response in melanoma. Also, the re-induction therapy in melanoma was shown to be effective upon remission (Lipson, et al., 2013). In another study, *Lipson et al.* characterized the therapeutic efficacy of pembrolizumab (aPD-1) in a patient with metastatic PD-L1 (+) basal cell carcinoma. A partial response was noted up to 14 months after initiation of therapy (Lipson, et al., 2017). Likewise, a phase 3 double-blind clinical trial conducted to evaluate the PD-1 inhibitor, pembrolizumab, as an adjuvant therapy in high-risk stage III melanoma after complete resection showed significantly longer recurrence-free survival in comparison to placebo with a dosage of pembrolizumab every 3 weeks for up to a year (ClinicalTrials.gov number,) (Eggermont, et al., 2018). Yet another clinical trial showed evidence of aPD-1 activity in enzalutamide-resistant prostate cancer in two out of 10 patients (Graff, et al., 2016) (Fig. 2). Both of these patients partially responded to the treatment and the outcomes correlated with microsatellite instabilities in the genome of prostate cancer cells. Although promising, symptoms of myositis, hypothyroidism, and hypothyroidism were noted in the patients. More recent studies have shown that the toxicities can be addressed in combinatorial regimen, and several inhibitors are starting to enter large scale trials, or receive FDA approval for therapy of malignant therapy (J. M. Collins & Gulley, 2019; X. Shen & Zhao, 2018). Additionally, it has been established that checkpoint therapies are most effective when PD-L1 is expressed in immune cells (T-cell, macrophages) along with tumor cells, personalizing therapies and outcomes.

4.2 Anti CTLA-4

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a homologue to CD28 on T-cells and binds to CD80 (B7–1) or CD86 (B7–2) on APCs (e.g., dendritic cells) to regulate immune function. Unlike CD28 that stimulates immune response, CTLA-4 has a regulatory/inhibitory function, and demonstrates a higher affinity for APC receptors than CD28. Anti CTLA-4 drugs are in clinical use as an immunomodulatory therapeutic because certain cancer cell lines, such as non-small cell lung cancers (Clinical trial identifier: , , ,), small cell lung cancers (), gastric adenocarcinoma (), hepatocellular carcinoma (), and various others (), inhibit immune activation and cytotoxic killing through this pathway (Walker & Sansom, 2015).

Initial studies with ipilimumab demonstrated the early foundation for this idea (Blank & Enk, 2015). Later *in vivo* studies in which human CTLA-4 was “knocked-in” in mouse

models allowed successful screening of anti-human CTLA-4 therapies (e.g. hu-PBL-SCID mouse models of Epstein-Barr virus-associated lymphoma) (May, et al., 2005). Like anti-PD1, anti-CTL4s enhance survival responses in melanoma, renal cell carcinoma, squamous cell carcinoma, and non-small cell lung cancer patients when compared to conventional chemotherapies (Seidel, Otsuka, & Kabashima, 2018). Importantly, in malignant melanoma, PD-1 blockage is traditionally more effective than CTLA-4 inhibition, and when combined, a significantly longer progression-free survival than monotherapy alone is generally seen. This important finding paved the way for the first checkpoint to receive FDA approval as a combinatorial regimen (Larkin, et al., 2015). Currently, nivolumab (aPD-1) combined with ipilimumab (fully humanized aCTLA-4) is under investigation in over 100 clinical trials in various stages (Rotte, 2019). The proposed mechanisms of enhanced effects of aCTLA-4 and aPD-1 combination are through an enhancement of tumor induced lymphocyte (TIL) activity, and by a decrease in the frequency and function markers of T_{reg} cells (Chae, et al., 2018). A benefit of such an approach is also the reduced immune related adverse events in patients. For example, reduced-dose ipilimumab followed by standard-dose pembrolizumab (aPD1) in 153 melanoma patients was found to provide robust anti-tumor immune effects, and reduced toxicities (Long, et al., 2017). Thus, the current clinical data suggest that checkpoint combinatorial regimen are likely to result in enhanced outcomes against a variety of tumor types.

4.3 Toxicity/Limitations

The efficacy of aPD-L1 therapies have been shown to correlate with PD-L1 expression in patients especially in melanoma patients (PD-L1 positive: 43.6% vs. PD-L1 negative patients: 20.3%) (Daud, et al., 2016). Thus, newer innovations that increase the expression of PD-L1s in tumor are needed to optimize treatment outcomes. Another limitation of using checkpoint inhibitors is the adverse side effects that are commonly experienced. Long-term follow-up of previous clinical data has shown therapy-induced hypophysitis after ipilimumab that involved hormonal imbalances (Albarel, et al., 2015). A phase 2 study using pembrolizumab in advanced soft-tissue and bone sarcoma showed post-treatment adverse events including symptoms of adrenal insufficiency, pneumonitis, and nephritis. The primary endpoint was not completed according to the researchers due to the aforementioned side effects (ClinicalTrials.gov, number) (Tawbi, et al., 2017). Another phase 2 clinical trial that used nivolumab as a treatment for adult T cell leukemia-lymphoma showed an increase in disease progression in all three of the first three patients within a week rather than it being an effective therapeutic as a PD-1 therapy (ClinicalTrials.gov number,) (Ratner, Waldmann, Janakiram, & Brammer, 2018). Another pitfall is the lack of an overly expressed PD-1/PD-L1 or CTLA-4 axis for all cancer models. Additionally, despite profound clinical benefits, a large proportion (>50%) of patients still do not respond to the immunotherapy. This is attributed to a lack of a baseline T-cell infiltration, the dependence of immunotherapies on interferon gamma (IFN-γ) producing CD8+ T-cells, and selection of less-immunogenic tumor cells such as those with loss of function mutation of PTEN that blunt tumor-cell antigen cross presentation, and *c-Myc* signaling that influences expression of checkpoint molecules (Casey, et al., 2016; Horton, Williams, Cabanov, Spranger, & Gajewski, 2018; W. Peng, et al., 2016; Rizvi & Chan, 2016; Spranger, Bao, & Gajewski, 2015; Szczepaniak Sloane, et al., 2017). Furthermore, not all cancer cell lines that express checkpoints respond

favorably to inhibitors. In one study, only 45.3% of 72 cases of pulmonary neuroendocrine tumors showed positive expression of PD-L1, and positive expression was not associated with overall survival or progression-free survival (H. Wang, et al., 2018). Overall, the most useful direction for checkpoint inhibitor therapies seem to be in chemoand radio-resistant tumors, but mitigating the adverse side effects evident in the current approaches is an unmet gap; therefore, further research is needed to optimize these approaches.

5. Noninvasive Imaging of Immune Cells

A challenge in current immunotherapy and immunomodulation therapy is the uncertain nature of immune activation depending on the tumor type. In general, it is extremely difficult to assess immune responses longitudinally in solid tumors for multiple reasons. First, the tumor microenvironment is highly adept at generating an immunosuppressive response to avoid immune detection. Second, compared to immune activation, relatively longer time is typically required for tumor regression, so correlating immune modulation with therapeutic outcome is difficult. Thus, it is critical to develop new abilities to determine the immune modulations during and after immunotherapy, so that it can be linked to treatment planning and dosing. Towards this goal, an effective non-invasive imaging approach should image tumors without impacting the immune response, provide feedback to both patient and clinician in a longitudinal manner, and identify immune cell types rank them in order of their tumor infiltration rates to provide efficient tracking of immune therapy success.

5.1 T cell imaging

In terms of imaging a broad, systemic immune response, the most obvious target is the T cell. The tracking of T cells was reported using Cerenkov luminescence imaging (CLI) and radioluminescence imaging in murine tumors in both *in* vivo and *ex* vivo models with ^{32}P-ATP labeled T lymphocytes (Boschi, De Sanctis, Ugel, & Spinelli, 2018). CLI is an optical imaging technique that provides detection of beta emitter's radionuclides with endpoint energy of 261 keV in water and around 219 keV in biological tissues. For the detection of the T-cells in *vivo*, the research team generated orthotopic Panc02 tumors in C57BL/6 mice and adoptively injected the^{32}P-ATP labeled T lymphocytes by intravenous and intraperitoneal route. Bioluminescent images were acquired at different time points (from 30 minutes to 18 hours) from the pancreatic regions. Results suggested a high increase of light emission in the body compared to pre-injected time and the control group. Similarly, another group used T cells labeled with NIR-797-isothiocyanate as a near-infrared photoacoustic and fluorescent agent to noninvasively determine the location of T cells in diseased tissues at different time points following intravenous injection (S. Zheng, et al., 2018). More recently, a T cell-specific PET agent, [18F]F-AraG was evaluated in a rhabdomyosarcoma model. A high accumulation of the PET agent in CD8+ T cells in the murine tumors was observed. The PET agent could also differentiate the differences in signal between mice that responded to and those that did not respond to aPD-1 therapy (Levi, et al., 2019). In another study, genetically modified CD8+ autologous cytotoxic T lymphocytes (CTLs) co-expressing the chimeric antigen receptor (CAR) interleukin-13 (IL-13) and the herpes simplex virus type 1 thymidine kinase gene (HSV1-tk) were imaged using radiolabeled 9-[4-[18F]fluoro-3-(hydroxymethyl)butyl]guanine ([18F]FHBG) penciclovir (antiherpes drug in humans). Data

suggested an increase in PET signal after cytotoxic T lymphocytes (CTL) infusions in patients, suggesting that this approach can provide a means to image immune cells in real-time (Keu, et al., 2017). Alternative approaches that combine contrast-enhanced CT images, RNA-seq genomic data, and histopathological features of tumor biopsies to assess CD8 cell tumor infiltration are also being widely reported for characterization of immune cell phenotypes and likely success to checkpoint therapies (R. Sun, et al., 2018). Furthermore, a variety of approaches has also been reported for imaging of T-regulatory cells with via two-photon (or multiphoton) microscopy; detailed review of which was recently published (Hickey & Chow, 2017).

5.2 Macrophage Imaging

Macrophage phenotypes play an important role in determining effectiveness of immunotherapy. Within the tumor microenvironment, M2 macrophage phenotype can be the predominant macrophage phenotype (Hobson-Gutierrez & Carmona-Fontaine, 2018). Repolarization therapy that modifies macrophages from pro-tumoral M2 macrophage to anti-tumoral M1 phenotype has the capability to sensitize and/or effectively kill tumor cells.

Macrophages' membrane receptor targeting, or passive uptake can be utilized for their imaging. For example, M2-oriented macrophages were imaged using ^{18}F-labeled camelid single-domain antibody fragments that bind to the macrophage mannose receptor for PET imaging (Blykers, et al., 2015). Such selective phenotype-specific macrophages imaging can allow monitoring of the disappearance or the appearance of M2 macrophages to show the progression of therapy that is otherwise unobservable. In a 4T1 mouse breast cancer cell model, *Sun et al.* developed NIRF-dyed anti CD-206 antibodies to track the location of M2 macrophages in tumors (X. Sun, et al., 2015). While targeting a specific macrophage subpopulation such as M1 versus M2 has not yet been reported, their carbohydrate linker offers the potential to link affinity ligands to bind to specified or desired locations/cells (Nahrendorf, et al., 2008). Additionally, transgenic mouse model for *in vivo* imaging of luciferase-expressing macrophages has been reported to understand candidate immunotherapeutics. In one study, B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J mice expressing Cre recombinase under the control of the lysozyme M promoter (LysM) were crossed with Cre-lox Luc reporter mice (RLG), to produce LysM-LG mice, resulting in generation of mice with macrophages positive for luciferase expressions. Orthotopic induction of B16 melanoma and ID8 ovarian cancer cells in the skin and ovarian tissues of these mice allowed the tracking of macrophages in the tumor milieu. In general, a significant enhancement in the bioluminescence signal in the tumoral regions was noted longitudinally over time, with flow cytometry suggesting the macrophages to be mostly of the M2 origin, mimicking clinical conditions (Fig. 3) (He, et al., 2017).

Another means of noninvasively imaging macrophages can be to track the byproducts of macrophages or other phagocytic cells "at work." For example, a fluorescent probe to track hypochlorous acid (HClO) as a means of showing "apoptosis" or cell death via phagocyte activation in macrophages was developed (Zuo, et al., 2012). HClO is produced through a respiratory burst in which the cell takes up oxygen and forms superoxide anions. Superoxide reacts with hydrogen ions, or protons, to form hydrogen peroxide. Although HClO can also

be formed by neutrophils, this may still be a useful approach for tracking the metabolic profile of macrophages.

5.3 Limitations

An obvious drawback to this approach is the unintended change of function of immune cells while trying to monitor them. Imageable agents binding to T cells or macrophages can alter their function, resulting in flawed results. However, if the imaging process can be discretely controlled, it may dramatically improve our abilities to track the success or failure of immune therapies. For most approaches, intratumoral injection results in a highly localized response, but their application is mostly limited to easily accessible tissues such as skin, breast, and head and neck cancer, limiting their application for deep seated tumors (e.g., pancreas).

6. Conclusion & Perspectives

Many therapeutic approaches that attempt to employ immunomodulation as their main contributor are actively being investigated. NPs can be used as an immune stimulator or adjuvant, whereas checkpoint inhibitors can be used to reverse the immunosuppressive tumor microenvironment. In situ vaccinations can be used in combination with other approaches to further target the solid tumor microenvironment. Such processes can be aided by noninvasive imaging of the immune cells that can help monitor the short and long-term outcome of an immunomodulator therapeutic.

The type of therapy used to treat cancer must be based on an understanding of the tumor microenvironment, such as the presence or absence of T regulatory cells or expression of certain receptors/ligands that may potentially create an interruption in the normal immune response. Such an approach will lead to more personalized therapeutic regimens. The data presented above suggest that combination therapy would be the most efficient and effective approach to treating any solid tumor. It should be accompanied by an individual case-by-case analysis of the best route of therapy available by characterizing the genotypic and phenotypic features of the patient tumor. The topics presented herein may open the door to new possibilities and therapies in the fight against cancer in the near future.

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Abbreviations

NPs	nanoparticles
TAAs	tumor-associated antigens
MHC	Major Histocompatibility Complex
CTLA-4	anti cytotoxic T-lymphocyte Antigen 4

PD-1/PD-L1	programmed death-ligand 1
ISV	in situ vaccinations
PLGA	poly lactic-co-glycolic acid
Th	T-helper
IL	interleukin
IFN	interferon
TNF	tumor necrosis factor
INPs	inorganic nanoparticles
GNP	gold nanoparticle
SPION	superparamagnetic iron oxide nanoparticle
QD	quantum dot
MRI	magnetic resonance imaging
AMF	alternating magnetic field
DC	dendritic cell
FDA	Food & Drug Administration
ROS	reactive oxygen species
DOX	doxorubicin
OVA	ovalbumin
CpG-ODN	cytosine-phosphorothioate-guanine oligodeoxynucleotide
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
TLR	toll-like-receptor
OV	oncolytic virus
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSV	herpes simplex virus
APC	antigen presenting cells
CXCR	c-xc-chemokine receptor
Hsp	heat shock proteins
PET	positron-emission tomography

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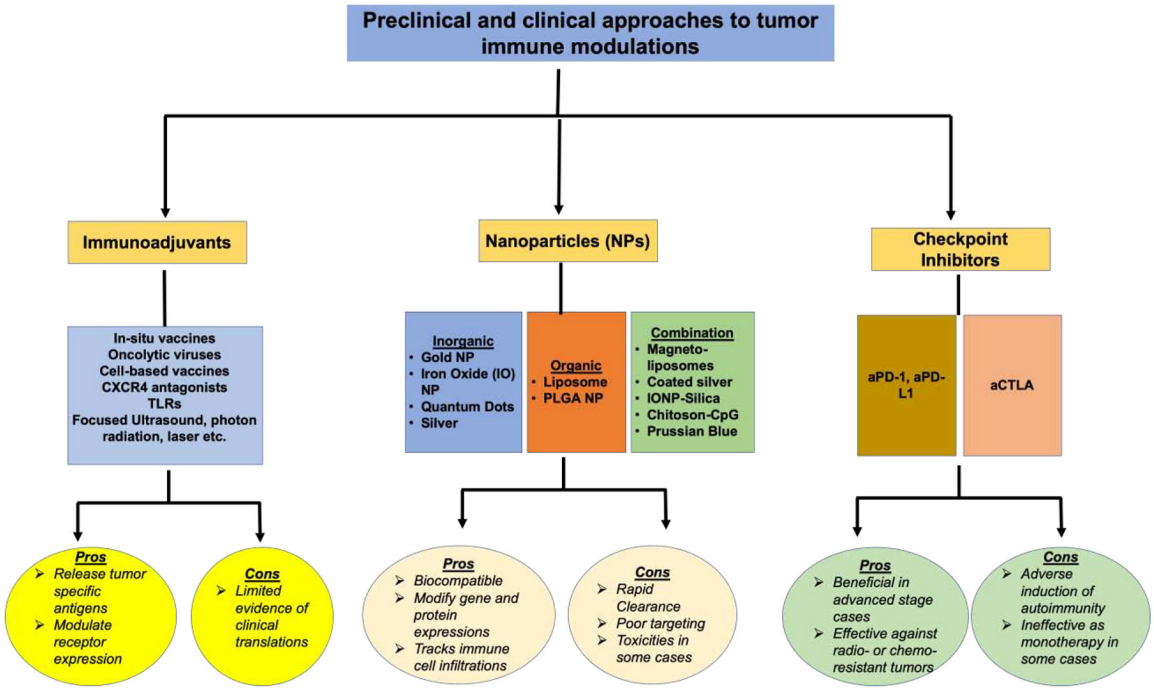

Fig. 1.

Pre-clinical or clinical agents in current use for modulation of tumor immune microenvironment.

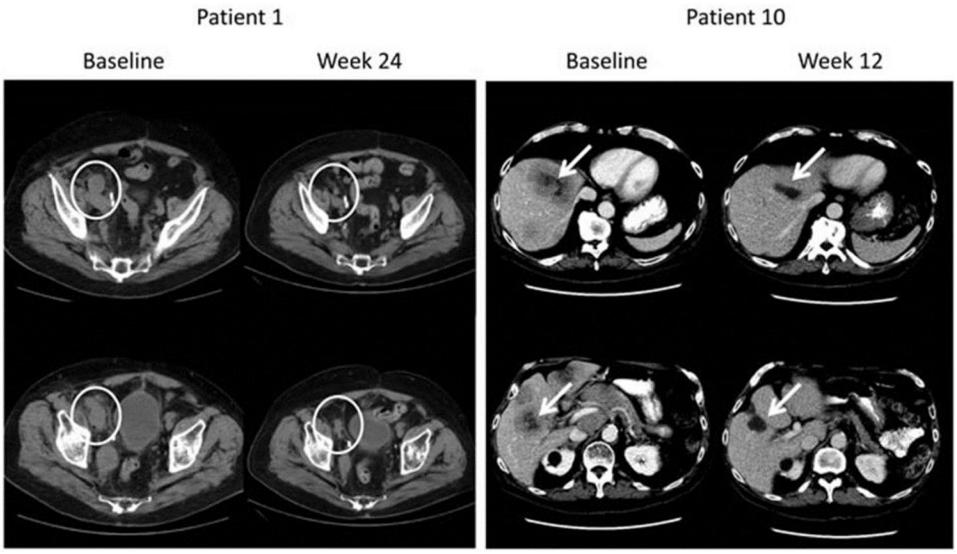

Fig. 2.

Anti-PD1 (pembrolizumab) therapy induces regression of castration resistant prostate tumors in patients. In patient 10, a significant reduction in the metastatic sites was noted 12-weeks post treatment with anti PD-1 and enzalutamide. Patient 1 also showed significant reduction in tumor size post treatment (Fig. adapted from Graff et al). Reproduced with permissions from *Oncotarget*, 2016; 7:52810–52817.

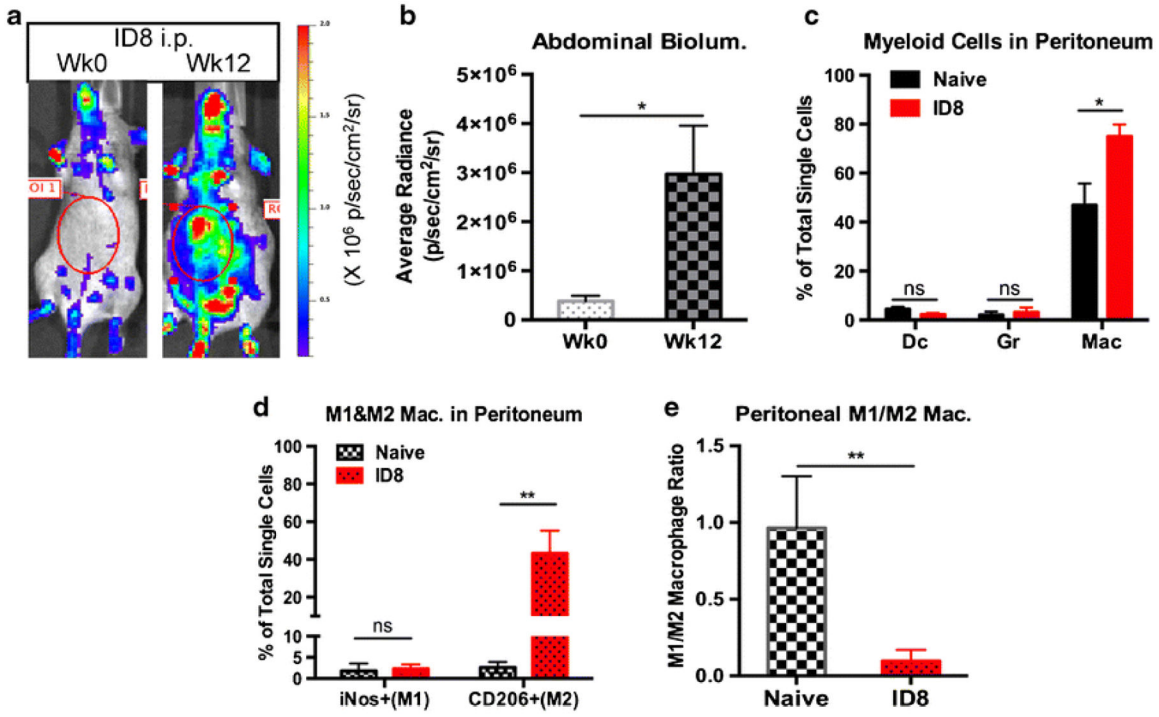

Fig. 3.

Tracking infiltration of tumor associated macrophages (TAMs) in murine tumors. A genetically engineered mouse model with bioluminescent TAM was developed. a-c) Upon implantation of ovarian cancer (ID8), a significant enhancement in the TAM infiltration in the peritoneal cavity was noted; d-f) The immunosuppressive ID8 tumors skewed the phenotype of macrophages towards pro-tumoral M2 macrophage. Reprinted with permission from Springer: Springer Nature [Molecular Imaging and Biology] [He et al.] (Imaging of Tumor-Associated Macrophages in a Transgenic Mouse Model of Orthotopic Ovarian Cancer, Huanhuan He, Alan C. Chiu, Masamitsu Kanada et al), [COPYRIGHT] (2017)

Table 1.

Nanoparticle immunomodulation information, altered immune profile/toxicity profile, and modified cell type

Type of Nanoparticle	Immune/Toxic Profile	Mode of Action/Cell type	Reference(s)
Copper oxide/ Platinum	DNA damage	Dendritic cells	(Karlsson, Cronholm, Gustafsson, & Moller, 2008) (Asharani, Lianwu, Gong, & Valiyaveettil, 2011)
Cobalt oxide	ROS production	Unknown (hypothesized Toll-like receptors)	(Chattopadhyay, et al., 2015)
Iron oxide (SPIONs)	Little to none found alone, NF-κβ activation with co-exposure with UVB radiation	Co-exposed with UVB rays alters glutathione and oxidative balance internally, creating cytokine release, cell death, and release of LDH	(Murray, et al., 2013)
Gold	Immunostimulatory-IL-1↑, IL-6↑ TNF-α↑, IFN-λ↑, MΦ−1 polarization, binds TGF-B1, IL-10↓ Immunosuppressive-IL-1β↓, MΦ−2 polarization, binds TLR9	Macrophages, dendritic cells, and neutrophils	(Asharani, et al., 2011)
Silver	High levels of DNA damage Immunosuppressive-IL-1β↓	Toll-like receptors, T lymphocytes, suppression of natural killer cells in spleen	(Asharani, et al., 2011) (Lappas, 2015)
Nickel	Less toxic than soluble nickel	Hypothesized Toll-like receptors	(Ispas, et al., 2009)
Zinc oxide	DNA damage and decreased cell viability	Toll-like receptors	(Y. H. Luo, et al., 2015)
Organic-coated silver	ROS production, DNA damage, inflammation	Damage to cell membrane, mainly observed in epithelial cells	(V. K. Sharma, et al., 2014)
Quantum dot	TNF-α↑, IL-1β↑, IL-10↑, IL-6↑, IL-1↑, IL-8↑, caspase-3↑, caspase-9↑	Skin cells, macrophages	(Almeida, et al., 2011) (Bilan, Fleury, Nabiev, & Sukhanova, 2015) (Michalet, et al., 2005) (Qin, et al., 2015) (Romoser, et al., 2011)
Titanium dioxide	ROS production, oxidative stress, mitochondrial damage	Toll-like receptors, T lymphocytes, inflammatory response resulting in T lymphocyte, B cell, and natural killer cell increases	(Czajka, et al., 2015) (Lappas, 2015) (Demir, Burgucu, Turna, Aksakal, & Kaya, 2013)
Calcium phosphate	Limited, negligible cytotoxicity	T lymphocytes and T cell maturation stage, negligible liver cell cytotoxicity	(Q. Wang, et al., 2015) (Petrarca, et al., 2015)
Zirconium dioxide	Little to none found, including no genotoxic effects	--------	(Demir, et al., 2013)
PLGA	Little to none reported, varies with other moieties involved or attached	Dendritic Cells and other antigen presenting cells through phagocytosis mainly	(D. Singh, Singh, Sahu, Srivastava, & Singh, 2016)
Palladium	Increase of stopping cells at checkpoints G0/G1 phase	Tested with human peripheral blood mononuclear cell	(Petrarca, et al., 2014)

Table 2.

Comparison of current checkpoint inhibitor drugs, mode of inhibition, and cancer treated with anti CTLA-4 and anti PD-1, anti PD-L1 targets

Drug/Antigen	Inhibition	Cancer Cell Line	Reference(s)
Pembrolizumab	PD-1	Hodgkin lymphoma, melanoma, non-small cell lung	(Long, et al., 2017), (Deeks, 2016)
Nivolumab	PD-1	Melanoma, non-small cell lung, kidney, colorectal	(Overman, et al., 2017)
Atezolizumab	PD-L1	Non-small cell lung and bladder	(W. Luo, Wang, Tian, & Li, 2018)
Durvalumab	PD-L1	Bladder and urinary tract	(Brower, 2016)
Avelumab	PD-1	Non-small cell lung, Merkel cell carcinoma	(Gulley, et al., 2017), (Kaufman, et al., 2016)
Ipilimumab	CTLA-4	Metastatic melanoma	(Williams, et al., 2017), (Long, et al., 2017)
Atezolizumab+vemurafenib, cobimetinib	PD-L1 +BRAF inhibitor and/or MEK inhibitor	$BRAFV^{600}$-mutated metastatic melanoma	(Sullivan, et al., 2019)
Pembrolizumab+Entinostat	PD-1 +class I HDAC inhibitor	Uveal malignant melanoma	(Jespersen, et al., 2019)
Pembrolizumab	PD-1	Resectable NSCLC stage II/IIIA	(Eichhorn, et al., 2019)
Nivolumab vs. Everolimus	PD-1 vs. mTOR inhibitor	Advanced renal cell carcinoma	(Tomita, et al., 2019)
Nivolumab vs. Ipilimumab	PD-1 vs. CTLA-4	Advanced esophageal squamous cell cancer	(Meindl-Beinker, et al., 2019)
Avelumab +Chemoradiatherapy	PD-L1	Advanced squamous cell carcinoma of the head and neck	(Y. Yu & Lee, 2019)
Nivolumab+Metformin	PD-1 +antihyperglycemic agent	Non-small-cell lung cancer or pancreatic cancer	(Kubo, et al., 2018)
Nivolumab+Ipilimumab	PD-1 +CTLA-4	Stage III or IV Unresecetable advanced melanoma	(Larkin, et al., 2015)
Durvalumab +Tremelimumab	PD-L1 +CTLA-4	Metastatic head and neck carcinoma, Non-small-cell lung cancer	(Bahig, et al., 2019) (Planchard, et al., 2016) (Antonia, et al., 2016)