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Engineered Multifunctional Nano- and Biological Materials for Cancer Immunotherapy

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Cancer immunotherapy is set to emerge as the future of cancer therapy. However, recent immunotherapy trials in different cancers have yielded sub-optimal results, with durable responses seen in only a small fraction of patients. Engineered multifunctional nanomaterials and biological materials are versatile platforms that can elicit strong immune responses and improve anti-cancer efficacy when applied to cancer immunotherapy. While there are traditional systems such as polymer- and lipid-based nanoparticles, there is a wide variety of other materials with inherent and additive properties that can allow for more potent activation of the immune system. By synthesizing and applying multifunctional strategies, it allows for a more extensive and more effective repertoire of tools to use in the wide variety of situations that cancer presents itself. Here, several types of nanoscale and biological material strategies and platforms that provide their inherent benefits for targeting and activating multiple aspects of the immune system are discussed. Overall, this review aims to provide a comprehensive understanding of recent advances in the field of multifunctional cancer immunotherapy and trends that pave the way for more diverse and tactical regression of tumors through soliciting responses by either the adaptive or innate immune system, and even both simultaneously.

1. Introduction

While the immune system plays a significant role in disease management, its dysregulation leads to the promotion of various diseases, including cancer.[1–3] The spatiotemporal modulation of the immune components can help target the specific cells to mitigate challenges that previous standards of care, like chemotherapy, are currently facing.[4–6] Through both arms of the immune system, innate and adaptive, immediate action can be taken against the intruding pathogens and dying cells while maintaining homeostasis. However, the mutagenic and aggressive nature of the cancer cells employs several mechanisms to pass the immunological barrier and create an immunosuppressive environment.[7–10] The excessive secretion of suppressive cytokines and growth factors such as IL-10 and TGF-β and upregulation of immune checkpoint receptor ligands significantly contributes to immune suppression.[11–13] Apart from this cytokine imbalance, infiltration of suppressive cells such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and alteration of macrophages toward protumorigenic phenotypes commonly known as tumor-associated macrophages (TAMs) are also found to be the key players driving the immunosuppression.[14–17] These protumorigenic cytokines and growth factors are abundantly present in the tumor microenvironment (TME) and facilitate tumor growth and immune evasion.[18]

Recently the focus has been shifted toward the in-depth understanding of intricate networks between the immune system and tumor microenvironment. These interconnections have revealed a new avenue that allows cellular and molecular level modulation of the immune system.[19,20] The chemical alteration of such a biological cascade that influences the immune system toward protumorigenic functions has shown great potential to educate and activate the immune system to perform its desired antitumoral functions. With these understandings, several cancer immunotherapy strategies such as the administration of therapeutic vaccines that stimulate dendritic cells (DCs), immune checkpoint blockade therapies that enhance the T cell function in TME have been studied.[21,22] Apart from those previously mentioned, cytokines, antibodies, immune modulators and immune adjuvants that target the T cells, B cells, and natural killer (NK) cells have also been developed in the last few decades. Among these attempts, the two therapies, namely chimeric antigen receptor (CAR-T cell therapy) and immune checkpoint blockade (ICB), have revolutionized cancer treatment through the lens of immunotherapeutic approaches and proved the potential of employing the body's defense system in combating cancer.[23–25]

Despite the fact that the impressive benefits of these therapies, only a small population of patients have benefitted,

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unfortunately leaving behind many more patients to suffer from a lack of efficacy.[26,27] For example, CAR-T cell therapies have been found to be very effective against hematological malignancies (i.e., multiple myeloma and B cell acute lymphoma). However, suboptimal clinical outcomes for solid tumors limit its broader applications. Moreover, heterogeneous antigen presentation, antigen-negative tumors, and treatment-related toxicities further limit their clinical success.[28] The inability to predict treatment efficacy, varying response rates, the host's pre-existing immunity, lack of specific biomarkers, and the presence of an immunosuppressive tumor environment are key hurdles limiting clinical success. Additionally, the problem of recurrence, acquired mutational resistance, intratumoral heterogeneity, and activation of alternative signaling pathways contribute to impeding the success of the immunotherapies.[29] The treatment-related side effects causing autoimmune diseases have also been a growing concern for the current approaches. Thus, to avoid these treatment-related toxicities and to increase the efficacy of the given treatment, target specific delivery strategies are essential. Nanomaterial- and biological-based delivery vectors have unique physical and chemical properties that allow for site-specific delivery and enable simultaneous delivery of multiples drugs from a single vector.[30–32]

Engineered nano- and biological materials can increase the efficacy of treatments through mechanisms like enhanced tumor penetration and cellular uptake via either enhanced permeability and retention effect or recently-discovered endocytosis mechanisms.[33] Nanomaterials with a long enough circulation time within the body have been demonstrated to achieve effective uptake into the tumors; however, toxicity must be minimized to avoid issues found in traditional therapies. One way that toxicity can be avoided is by functionalizing the surface of the nanomaterial or designing it so that it targets specific tumor-associated antigens (TAAs) or ligands on cancer cells make them the primary target, thus reducing off-target effects. In addition, the materials can be designed to enable a prolonged release of therapeutics, which in turn enhance a more durable immune response. This is done through the inherent properties of nanomaterials that allow for tunable degradation within the body. This goes hand-in-hand with creating responses to certain stimuli in a more targeted manner versus prematurely while in circulation, avoiding off-site toxicity.

Multiple groundbreaking nanotherapeutics have been reported and have demonstrated potential in clinical applications[34,35] as well as numerous FDA-approved nanomaterials, such as Abraxane,[36] Doxil,[37] and Marqibo[38] have seen clinical success. Regarding the immune system, nanomaterials have been shown to effectively deliver immunotherapy agents, as well as elicit immune responses in a wide variety of immune cells. By recruiting the immune system to eliminate tumor cells, it is possible to stimulate the once-hampered immune response to become active in a manner to act against the cancer cells, potentially reducing cytotoxic effects from traditional chemotherapy.

Unfortunately, several challenges have been identified when it comes to monofunctional nanoparticles and nanomaterial systems designed to inhibit or elicit specific pathways or responses, respectively. For example, multiple pathways can be responsible for how particular immune cell functions and single inhibition or activation of a pathway may not be robust enough to elicit an effective and long-term response. When this is the case, there needs to be a strategy to target tumors that have these mechanisms or ones that need more potent therapy without exceeding the toxic doses or increasing resistance to therapeutics.[39] When a more durable or more robust response is required, this is where multifunctional nanomaterials are effective, which can take advantage of the properties of tumors that were mentioned above. Herein, multifunctional materials will be defined as platforms and systems that have multiple components that, when combined within a system, cause combinatorial and/or systematic interactions, inhibition, activation, or redirected cell killing. Focusing specifically on cancer immunotherapy, multifunctional systems have shown to have a significant impact by targeting multiple cytokines, surface ligands, physical barriers, or a combination of these.[40] In comparison to the single-modality system, this can result in increased amounts of cancer cell death due to markedly higher amount of immune cell activation or re-education of the innate immune system, or even cause a sustained adaptive immune response in the form of vaccines, causing prolonged tumor regression and improved survival.[19] Additional factors like targeting-modalities, various drugs, material properties, and communication with the immune system can allow for a more diverse toolbox of therapeutics to aid patients not only in cancer, as we will discuss here, but other diseases as well (**Figure 1**).

Recent reviews[41–43] have extensively discussed the ability of nanomaterials to have an impact on cancer immunotherapy; however, these reviews do not focus specifically on the impact of multifunctional platforms that have shown to be effective in enhancing interactions with immune cells and improving cancer therapy response. Although cytokines and antigens for therapeutic delivery are biological materials and have been extensively reviewed,[44–46] this review will talk about the novelty of engineered biological materials such as multispecific and multifunctional antibodies. Here, we will discuss the benefits and upcoming trends in multifunctional nano- and biological materials and their impact on cancer immunotherapy, to summarize recent discoveries as well as showcase the direction of nanomaterial research that can create a more robust immunological reaction and increase patient quality of life (Figure 1).

2. Self-Assembled Multifunctional Nanomaterials for Combinatorial Immunotherapy

Self-assembly is the synthesis of highly stable and organized architectures from pre-existing disordered materials. This process is governed by attractive forces, including covalent, noncovalent interactions, hydrogen bonding, and supramolecular interactions, which impart unique properties to these now-ordered structures. In addition to their functional diversity, ease of structural modifications, excellent biocompatibility and multifunctional abilities give these nano biomaterials increased attraction in the biomedical field. These self-assembled nanomaterials provide an opportunity to deliver multiple therapeutic entities from a single nanocarrier system to accomplish combinatorial efficacy. The following section will cover the development of novel self-assembled multifunctional nano biomaterials and illustrates

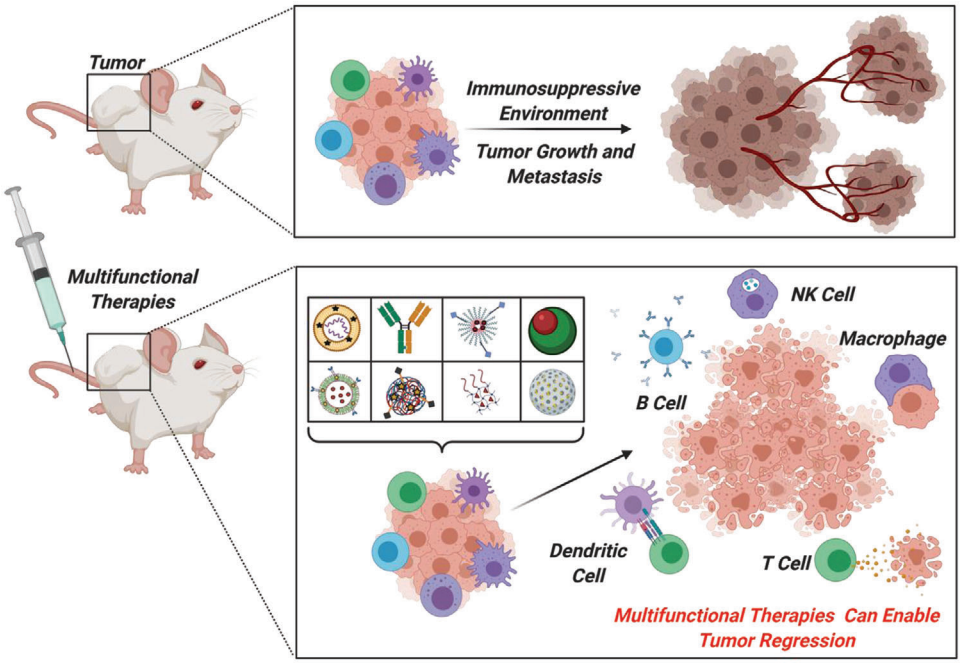

Figure 1. Multifunctional nano- and biological materials have the ability to inhibit tumor progression and metastasis through activating immune cells via a variety of mechanisms.

their therapeutic application specifically for combinatorial immunotherapy against cancer.

2.1. Liposomes

Liposomes are the lipid-based bilayer vesicles formed from the self-assembly of amphiphilic molecules. The simplicity in their preparation and unique features such as biocompatibility, biodegradability, low toxicity, and their ability to encapsulate both water-soluble and insoluble agents makes them an attractive nanocarrier for biomedical applications.[47] To date, several liposomal formulations have paved their way toward clinics, and some are undergoing clinical trials.[48] These liposomal carriers have also been extensively employed for cancer immunotherapy to harness the power of the immune system in controlling cancer progression. Liposome-based monotherapy platforms, though, have shown promising results, some of the advanced stage and hard to treat cancer develop resistance or adopts alternative pathways to diminish the therapeutic effects.[49] To circumvent these limitations, recently, multifunctional liposomes that enable the synchronized delivery of multiple therapeutics are being employed in cancer therapies. The general schematics for multifunctional liposomes and their subsequent intracellular actions are shown in **Figure 2**. The following section describes the synthetic design and application of multifunctional liposomal formulations that have been specifically applied for cancer immunotherapy applications.

Recent studies have shown that nanotechnology-based approaches can be harnessed to activate anti-tumor immune responses by modulating multiple immune pathways and by normalizing the physiology of the immunosuppressive tumor

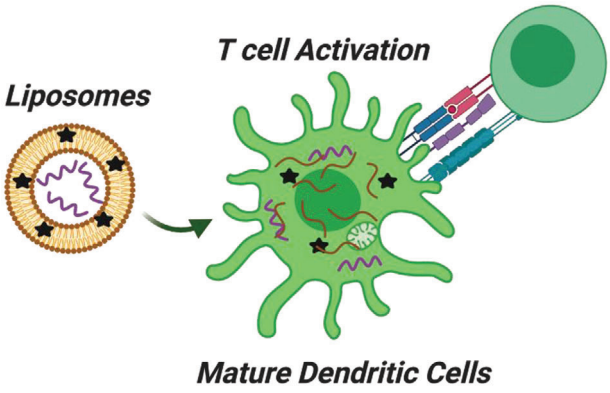

Figure 2. A generalized schematic depicting the interaction of multifunctional liposomes with immune cells to accomplish combinatorial response against cancer. Liposomes are phospholipid-based self-assembled structures and possess a hydrophilic core and hydrophobic layer. Liposomes provide unique opportunities to encapsulate water-soluble and water-insoluble drug molecules in the core and lipid bilayer, respectively. Here, a liposome was used for maturation of DCs for enhanced T cell activation.

environment. In this direction, Haung et al. have developed a liposomal platform that enables the combinatorial delivery of two chemically distinct oligodinucleotides (CpG) to activate the antitumor immune responses. These oligonucleotides being negatively charged and extremely sensitive towards intracellular enzymes, are difficult to deliver across the cell membrane. Once encapsulated in the liposomes, efficient intracellular trafficking of CpGs was achieved in the DCs and resulted in the activation of toll-like receptor (TLR) 9 signaling pathways to enhance the infiltration of the tumor antigen-specific T lymphocytes in the

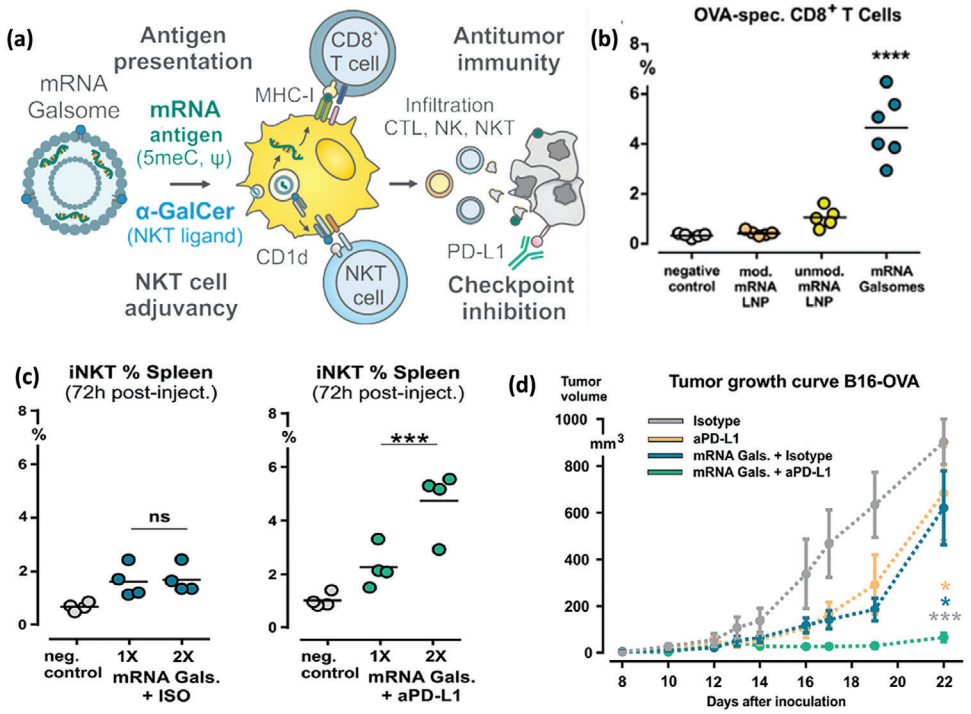

Figure 3. a) The intracellular delivery of m-Galsomes and subsequent activation of immune responses. b) m-Galsomes mediated antigen specific CD8$^+$ T cell response. c) m-Galsomes when combined with αPD-L1 antibody increases the number of splenic iNKT cells compared to the control, and d) graph shows a tumor progression for different treatment groups. Reproduced with permission.[51] Copyright 2019, American Chemical Society.

cancer milieu. The co-delivery of these CpGs at programmed ratios to DCs promoted the secretion of cytokines and IFNγ indicative of NF-kB and IRF 7 pathways activation to accomplish better anti-tumor immune response.[50] Certain cancers that do not respond to immunotherapy are considered to be immunologically "cold"; thus, simultaneous orchestration of different immune components can promote effective anti-tumor immune action. In recent studies, Verbeke et al. have developed a liposomal m-RNA nano-carrier "m-Galsomes" to promote the combinatorial delivery of antigens. The design schematics and its mechanism of action are shown in **Figure 3**a. These galsomes successfully delivered a nucleoside modified antigen-encoding m-RNA and glycolipid antigen α- galactosylceramide (α-Galcer) to activate the DCs and invariant natural killer (iNKT) cells (Figure 3b,c). The effectiveness of these mRNA galsomes was further enhanced by co-administration of checkpoint inhibitor anti-PD-L1 antibody in B-16 OVA melanoma, as evidenced by a synergistic reduction in tumor volume with complete tumor rejection in 40% of the animals[51] (Figure 3d).

Despite recent advancements in nanocarriers based delivery approaches; suboptimal treatment efficacies and slower response rates still remain a challenging task. While immunotherapy generates slower but long-lasting effects, the traditional chemotherapy induces immediate action, but the effects produced are short term. Thus, the combination of chemotherapy and immunotherapy can significantly increase overall therapeutic effects that are stronger and durable than those elicited by monotherapy. The dual responsive (matrix metalloproteinase and pH) liposomal carrier was designed by Chen and coworkers to integrate the chemo and immunotherapy approaches. Specifically, this strategy combines the PD-L1 inhibitor that acts as an ICB and a low dose of chemotherapeutic agent doxorubicin. The non-cytotoxic concentration of doxorubicin was found to sensitize the B16F10 melanoma cells to overexpress the mannose 6 phosphate receptors (M6PR) on tumor cells. This M6PR overexpressed tumor cells easily permeates the granzyme B released by cytotoxic T lymphocytes (CTLs) and thereby increases cytotoxic effects. This study could accomplish 78% tumor suppression in in vivo conditions, which were better than single therapies using a low concentration of DOX (40%) and inhibiting PD1-PD-L1 interaction alone (50%) in the B16F10 melanoma tumor model.[52]

Another way to improve immunochemotherapy efficacy is to activate the innate immune responses by utilizing cancer cell-specific antigen vaccines and combining them with a chemotherapeutic agent. The whole tumor lysates containing the repertoire of the cancer cell target antigens are recently being perceived to improve the CD8$^+$ T cell responses. These tumor cell lysates are particularly important since they can be used for patients regardless of their human leukocyte antigen (HLA) type. This concept was explored by Won et al. and thermosensitive liposomes (BG-TSL) were prepared for codelivery of chemotherapeutic agent doxorubicin along with tumor cell lysate. The introduction of the NIR (near-infrared) responsive component in the liposomes has enabled external stimuli to trigger degradation of the liposomes. The released tumor cell lysate promoted DC maturation and activation to induce antigen-specific immune responses. The DOX released also promoted cancer cell death. These DOX-BG-TSLs exhibited enhanced anti-tumor response in B16F10 tumor-bearing mice as compared to the control groups. These studies validated that the combination of tumor cell lysate to activate the

innate immune response with chemotherapy can enhance the anti-tumor effect.[53]

A combinatorial approach integrating immunotherapy with photodynamic therapy (PDT) is also an emerging trend. However, these PDT effects are often compromised by the immunosuppressive TME, such as; the up-regulation of indolamine 2,3 dioxygenase 1 (IDO 1) pathways in most of the cancers. The IDO pathway is known to suppress the effector function of T and NK cells and also promotes the activation of regulatory T cells and MDSCs. Thus, in an attempt to target this pathway in the tumor microenvironment, Liu et al. developed the redox activatable porphyrin-phospholipid conjugate-based liposomes (RAL) that can combine PDT and immunotherapy. These liposomes were designed to have the porphyrin-based molecule and IDO1 pathway inhibitor NLG-8189 together and enable the site-specific delivery of the cargoes in redox-rich TME. The enhanced tumor accumulation and TME specific (GSH responsive) activation of fluorescence signal made this system of particular significance to locate the response in primary as well as distant metastatic tumor lesions. This system facilitated the activation of immunogenic cell death through PDT. Further inhibition of the IDO1 pathway rendered the system to be immune supportive and enhanced the efficacy of PDT.[54]

Engineering the cell surface with nanomaterial-based systems is emerging as an effective way of targeting the TME. The Tregs are found in large numbers in the TME and are associated with poor prognosis in many cancers. Thus, isolation of these Tregs and modifying them with the nanocarriers system could open new opportunities for active targeting of the TME. This approach was explored by Ou et al. by employing Tregs for liposome delivery. Liposomes encapsulated with immunoadjuvant were conjugated on Tregs surface and TME site-specific delivery was accomplished using "antibody-receptor" chemistry. The CD25 antibody decorated liposomes loaded with interleukin-2 (IL-2), anti-PD-L1 antibody and drug imiquimod (IQ) have exhibited the pH-responsive release liposomes from Tregs in the TME. These liposomes promoted DC maturation and enhanced cytotoxic functions of the T cells in B16 melanoma tumor-bearing mice. The inhibition by immune checkpoint blockade using PD-L1 antibody further provoked a strong antitumor immune response to boost cancer immunotherapy strategy.[55]

Targeting the specific receptors on the cell surface can promote the cell-specific delivery of the antigens to activate the immune responses. One such receptor, CD44, has been extensively studied for targeting the cancer cells using hyaluronic acid as a ligand and exhibited good targeting ability to deliver the chemotherapeutic agents to cancer cells. These CD44 receptors are also present on the antigen-presenting cells, such as DCs; however, never been targeted to activate the immune response. Miyazaki et al. employed this hyaluronic acid-CD44 interaction for cancer immunotherapy using pH-responsive hyaluronic acid decorated liposomes. The liposomes were encapsulated with ovalbumin (OVA), which is widely used as a model antigen to activate an immune response. These liposomes showed better uptake in antigen-presenting cells such as DCs and macrophages than cancer cells. The ability of these liposomes to induce potent antitumor response was studied in the OVA-expressing murine T lymphoma model. With the liposomes, 25% of the tumor-bearing animals were found to become tumor-free at the end of the treatment. This work represents an elegant way to achieve specific delivery of antigen to antigen-presenting cells to activate the cellular immunity for cancer immunotherapy.[56]

Radiotherapy can make the cancer cells more susceptible to the immune attack and hence combining radiotherapy with different immunotherapy strategies could potentially yield synergistic anti-tumor effects. Thus, to combine immunotherapy with radiotherapy, Song et al. developed a simple yet efficient liposomal carrier to deliver H_2O_2 (H_2O_2@Liposomes) and catalase (CAT@Liposomes) to the TME. Upon sequential administration, these liposomes neutralized the hypoxic region in TME and orchestrated the T cell immune response. Further combination of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade with the cocktails of liposomes (H_2O_2@Liposomes + CAT@Liposomes) showed remarkable antitumor effects in mice bearing 4T1 tumor model as well as clinically relevant patient-derived xenograft tumor model.[57] In another study, Xu et al. explored a similar concept and synthesized a thermal responsive liposome (TRL). These liposomes can efficiently co-deliver the NIR sensitive indocyanine dye and immune-stimulatory molecule, polyinosinic: polycytidylic acid (poly I: C), to the TME. The indocyanine dye promoted the temperature-responsive apoptosis of the cancer cells upon NIR light irradiation. The poly I: C - activated the DCs and induced tumor antigen-specific immune response against the tumors. These bifunctional liposomes were found to be very effective in controlling the metastatic growth of cancers in both B16 melanoma and CT-26 carcinoma cells in vivo.[58] It was also known that the effective activation of TME specific antigen-presenting cells could trigger a potent immune response against the tumors. Thus, to achieve site-selective co-delivery of the clinically approved immune agonist, Atukorale et al. synthesized a lipid-based nanoparticle system. This system allowed a target-specific activation of antigen-presenting cells. The dual delivery of cyclic diguanylate monophosphate (cdGMP), a stimulator of interferon pathways, and TLR 4 agonist monophosphoryl lipid A (MPLA) from the nanoparticles was found to activate the production of type I interferons and strong proinflammatory cytokines. These nanoparticles were shown to activate the NK cells to recruit CTLs in the TME and exhibited significant tumor ablation in triple-negative breast cancer-bearing mice.[59]

Macrophages are key innate immune players that play a very crucial role in the innate and adaptive immune responses. These macrophages exist across a spectrum of phenotypes based on the stimuli they are exposed to. Recent studies have revealed that the macrophage polarization states are more complex and are not perfectly described by the simplified model of pro-inflammatory M1 phenotype and an immunosuppressive, anti-inflammatory M2 phenotype. However, this M1–M2 dichotomy is commonly used to describe macrophages in the tumor microenvironment. This phenotypic polarization due to cytokine imbalance in the tumor microenvironment drives them towards the pro-tumoral function and makes them TAMs. Thus, targeting these TAMs provides an opportunity to reverse the immunosuppressive tumor microenvironment. In recent studies, Kulkarni's group developed a lipid-based dual drug-loaded nanoparticles (DNTs) strategy to target the TAMs. The chemical components of the DNTs, their liposomal design and morphology are depicted in **Figure 4**a. These DNTs exhibited dual-inhibition of CSF1R and

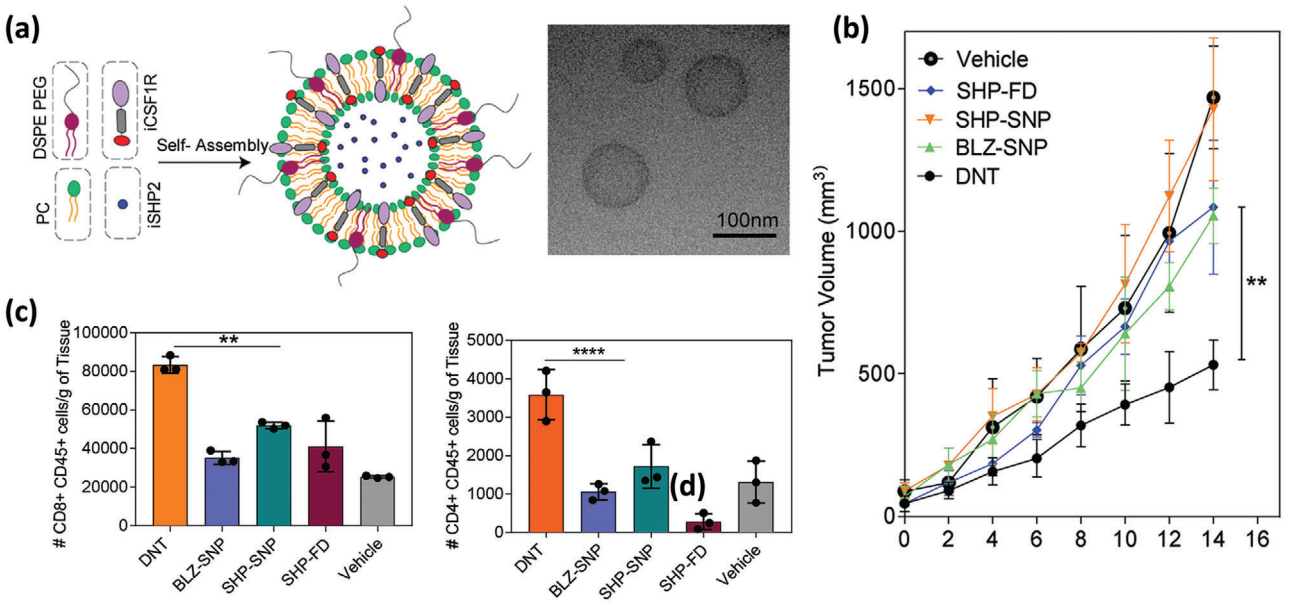

Figure 4. a) Schematic representation of lipid based DNTs, its constituents for targeting TAMs, and cryo-TEM morphology. b) Plot showing the tumor growth profiles for tumor bearing mice treated with DNTs. c) Plots showing the expression of different effector T cells in excised tumors. Reproduced with permission.[60] Copyright 2019, Wiley-VCH GmbH.

SHP2 pathways that are mainly responsible for immunosuppressive M2-like phenotype and to trigger "eat-me-not" signal from cancer cells. The DNTs treatment in highly aggressive breast cancer and melanoma tumors resulted in efficient repolarization of macrophages towards tumoricidal M1 phenotype and endowed superior phagocytic abilities. There was a significant reduction in tumor volume in mice treated with DNTs. (Figure 4b). The DNTs also showed enhanced infiltration of $CD8^+$ and $CD4^+$ T cells in excised tumors (Figure 4c). Overall the DNTs have shown remarkable antitumor effects in metastatic 4T1 tumor-bearing mice.[60] In another attempt, Brouillard et al. deployed lipid nanoparticles for targeting the multiple cellular pathways that govern the protumorigenic M2 polarization of the macrophages. The dual-inhibiting supramolecular nanoparticles (DSNs) having CSF1R inhibiting amphiphile and MAPK pathway inhibitor when administered in a highly aggressive 4T1 breast cancer model, showed excellent macrophage repolarization and significant tumor growth inhibition, which was significantly better than the free drug counterparts.[61] The recent work from Ramesh et al. also utilized these nanocarriers (DiLNs) made from phospholipids for dual delivery of PI3K and MAPK inhibitors to induce strong cytotoxic effects in D4M melanoma and TOV21G ovarian cancer cells. Further combination of these DiLNs with immune checkpoint blockade therapy using the anti-PD-L1 antibody has shown significant tumor reduction in mice bearing D4M tumors.[62]

Fusogenic liposomes are another potential carrier system that has been explored for therapeutic applications. These liposomes can directly fuse with the plasma membrane without causing any cytotoxicity and deliver the encapsulated entities into the cytoplasm. Highly pH-sensitive fusogenic liposomes were reported by Yuba et al. to deliver the antigenic OVA molecules to induce OVA-specific generation of the CTLs. These liposomes modified with hyperbranched poly (glycidol) were taken up by DCs to activate the CTLs. The nasal or subcutaneous administration of these liposomes in mice bearing E.G7-OVA tumors exhibited a significant reduction in tumor burden. The same group later employed these fusogenic liposomes to encapsulate (OVA)OVA-I (SIINFEKL) and OVA-II (PSISQAVHAAHAEINEAP) peptides that can bind to major histocompatibility complex I and II on DCs to achieve an effective response. The mice vaccinated with this OVA I peptide-loaded hyperbranched poly (glycidol) liposomes exhibited a complete rejection of E.G7- OVA cells. This approach represents a highly pH-sensitive strategy to deliver the antigenic molecules to the DCs efficiently. In another study, Aryal and co-workers have developed an NK cell membrane-associated targeting protein decorated fusogenic liposomes (NK-some) to specifically target the cancer cells over a normal cell. These NK cells, like NKsome, have been employed for delivering chemotherapeutic agent doxorubicin to the mice bearing MCF-7 breast tumors. The decorated NK cell-associated proteins have helped these liposomes locate the tumor cells and release the cytotoxic molecules to the cancer cells. The NKsome showed an excellent tumor homing potential and accomplished a significant antitumor effect against the human breast cancer model.

In addition to enhancing the efficacy of treatment, it is also imperative to read out the response of the given therapy through imaging (fluorescence or MRI) to differentiate between the responders and non-responders to decide the further course of treatment. To address this challenge, Grippin et al. have designed the multifunctional RNA-loaded magnetic liposomes that activate the immune system and function as a biomarker to track the treatment response. These liposomes activated the DCs more efficiently than the electroporation method, as confirmed by flow cytometry and fluorescent studies. Further coloading of

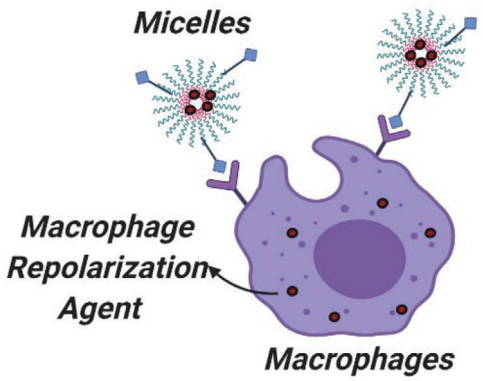

Figure 5. A generalized schematic showing the interaction of targeted micelles with macrophages to reverse the immune suppressive microenvironment and to active the antitumor immune responses against tumor cells. Micelles are specialized self-assembled structures that consist of a hydrophobic core and efficiently encapsulates hydrophobic molecules. Here, micelles decorated with surface marker ligands were used to target macrophages for repolarization within the tumor microenvironment.

carboxylated iron oxide nanoparticles to these liposomes have enabled the MRI imaging of the treatment response.[63]

The above-discussed strategies highlight some of the elegant liposomal systems that are engineered for multifunctional cancer therapy approaches. These liposomes have enabled the encapsulation of different immune modalities together in a single nanocarrier to maximize efficacy. The ability of the liposomes to incorporate the chemically and physically different moieties has made it possible to knot the immunotherapy with the traditional therapies to accomplish the combinatorial anticancer effects. Although liposomes have enabled the integration and delivery of various therapeutic moieties, premature leakage, short half-life, solubility issues, and physiological stability are some of the major limitations affecting clinical success. To circumvent these challenges, polymer-based amphiphilic systems capable of self-assembling into nanostructures are being utilized. These systems in aqueous medium generate various self-assembled structures such as micelles, polymersomes, nanoparticles, nanogel, reverse micelles etc. The following section will cover the recently developed smart multifunctional micellar systems that are being studied in cancer immunotherapy.

2.2. Micelles

Micelles are specialized self-assembled nanostructures made from amphiphilic molecules that can efficiently engulf lipophilic drugs to enhance their pharmacokinetics and pharmacodynamics. On account of their unique features such as size (10-200 nm), high physiological stability, biocompatibility, and, most importantly, the ability to accumulate in the tissues with compromised vasculature, micelles can accomplish site-specific delivery of the payloads.[64] The recent trend to engineer the multifunctional micelles capable of triggering multiple antitumor immune responses and their combination with traditional cancer therapies is evolving as a fascinating way to treat cancers.[65] The general schematics for micelles are shown in **Figure 5**. The following section will describe the application of multifunctional micelles in the immunotherapy-based combination approaches.

Immunotherapy, though, has gained significant attention in the past decade; the efficiency of certain immunomodulators is still compromised by their low aqueous solubility and lack of target-specific accumulation. On the other hand, chemotherapy though being clinically used, is associated with severe side effects. Moreover, the vulnerability of small molecular immunostimulators and chemotherapeutics towards the p-glycoprotein efflux mechanism also contributes to hampering the success of therapy. Thus, encapsulation of the small molecules in micellar nanocarriers can help to bypass these barriers. In the recent studies by Zhao's group, two different polymeric micelles were prepared to accomplish the target-specific cancer chemo-immunotherapy. The micelles were made from chondroitin sulfate with an affinity for mannose receptors on macrophages; thus, enabling the targeted delivery of TLR agonist R837 to TAMs. A different micellar system was designed from polycaprolactone-polyethylene block and employed to deliver chemotherapeutic agent doxorubicin hydrochloride to cancer cells. The R837 and DOX released from the respective micelles after intratumoral and intravenous injections were found to trigger many actions including, the maturation of macrophages to promote the secretion of cytokines (TNF-α, IL-6, and IL-1β), infiltration of $CD8^+$ T cells and DNA damage. These micelles acted synergistically in 4T1 tumor-bearing mice to achieve the highest tumor inhibition rate of 85% and the highest survival rate of 80% in in vivo studies.[66] Among the various immune adjuvants and modulators, ICB has shown promising results for cancer immunotherapy; however, diversity among the tumors and individual responses toward immunotherapy makes it challenging to accomplish the desired level of efficacy. The alternative strategy is to combine immunotherapy with traditional cancer drugs such as chemotherapeutic agents. Shuai's group has explored this strategy to make pH and matrix metalloproteinase dual responsive micelles for controlled delivery of anti-PD-L1 antibody and paclitaxel in solid tumors. The encapsulation of MnO_2 in place of paclitaxel in the micelles further provided the opportunity to track the response. This combination was shown to promote the immunogenic cell death and immune checkpoint blockade to synergistically induce a potent antitumor response in B16F10 tumor-bearing C57BL/6 mice and also enabled the MnO_2-mediated MRI imaging of the treatment.[67]

Although immunotherapies have offered several ways for tumor regression, site-specific delivery, stability against pathophysiological barriers, and low solubility of the immunotherapeutic agents limit their efficacy. To overcome this hurdle, Li's group developed multifunctional micelles that successfully delivered indoximod and doxorubicin drugs. The conventional chemotherapeutic agent doxorubicin, owing to its ability to induce immunogenic cell death (ICD) and thereby enhancing the action of DCs at low concentration, is gaining interest in cancer immunotherapy. On the other hand, indoximod triggered the infiltration of cytotoxic $CD8^+$ T cells and promoted the reversal of the suppressive immune microenvironment. This micellar system has shown significant tumor shrinkage in murine breast cancer models compared to their single drug-loaded counterparts.[68] In a different study, Wei et al. synthesized multifunctional micelles to enable the co-delivery of TLR7 agonist imiquimod and

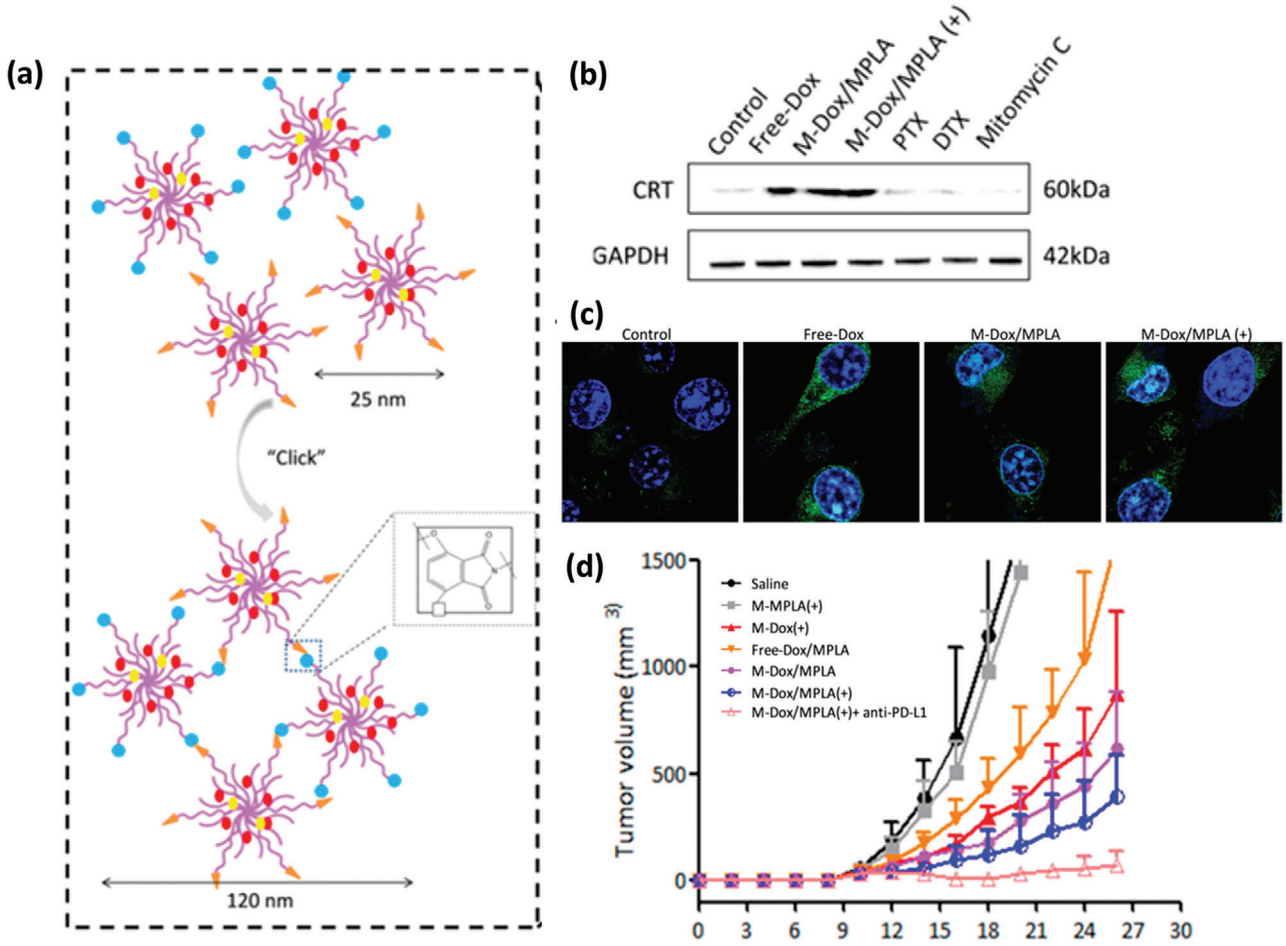

Figure 6. a) Schematics showing the Cu(I) click chemistry mediated aggregation of the micelles. b) Expression of calreticulin on the surface of immunogenic cell death detected by western blot. c) Immunofluorescence detection of calreticulin by confocal microscopy. d) Tumor growth curves for mice treated with different treatment groups. Reproduced with permissions.[71] Copyright 2018, American Chemical Society.

chemotherapeutic agent doxorubicin. Further, these micelles were also combined with the PD-L1 antibody and induced a robust antitumor action.[69]

Neoantigen vaccines are another immunotherapeutic strategy that can help personalized immunotherapy treatments and significantly inhibit tumor progression. A combination of neoadjuvant vaccines thus can impart significant efficacy if delivered through a single nanocarriers system. Ni et al. have developed a biadjuvant neoantigen nanovaccine that can deliver a peptide neoantigen (Adpgk) along with two adjuvants, TLR 7 agonist R848 and TLR 9 agonist CpGs. These micelles containing bi-adjuvant and neoantigen were further coupled with anti PD1 antibody to augment the infiltration of cytotoxic T cells and the activation of antigen-specific immune responses. These micelles have coupled three immune components (neoantigen vaccine Adgpk and two adjuvants R848 and CpGs) together in single nanocarriers and facilitated the activation of innate as well as adaptive immune responses in MC38 tumor-bearing mice. This methodology exhibited significant tumor progression up to 70% without any possibility of recurrence.[70] The amount of drug payload delivered through nanocarriers, their retention in the tumor tissues, and its substantial effect on the healthy cells plays a crucial role in the clinical translation of multifunctional nanocarriers. To increase the retention of nanocarriers carrying the combination of the drugs, He's group has proposed a strategy to aggregate the micelles in in vitro and in vivo conditions to secure the optimal size that can permeate and retain in the tumor tissue and increases the localized concentration of the drugs. The schematics representation of the micelle aggregation is shown in **Figure 6**a. These micelles have been shown to successfully deliver an immunoadjuvant monophosphoryl lipid A (MPLA) and doxorubicin. The released cargoes promoted ICD, DCs maturation, antigen presentation abilities and a strong T cell-mediated cytotoxicity. The calreticulin expression on the cell surface, confirming the immunogenic cell death, was assessed by western blot and immunofluorescence detection is shown (Figure 6b,c). Further combination of DOX-MPLA micelles with anti-PD-L1 monoclonal antibody accelerated the inhibition of metastatic melanoma B16F10 tumor progression (Figure 6d) and protected the mice from tumor recurrence by generating immune memory.[71]

Photodynamic therapies have shown promising results for some cancers but are rarely found to be efficient as monotherapy. The hypoxic environment in the metastatic tumors,

adaptive immune resistance, and the inability of laser penetration through tumor tissues limit the efficacy of the PDT. To overcome these limitations, Li's group has designed a strategy that integrates the PDT with an anti-PD-L1 antibody to achieve synergistic cancer therapy. The acid-cleavable pheophorbide conjugated micelleplexes were found to promote the PDT along with ICB. These micelles inhibited the immune surveillance escape of the tumor cells and also promoted the cytotoxicity through active T lymphocytes. This regimen exhibited a synergistic effect to inhibit the tumor growth in B16F10 melanoma tumor-bearing mice and demonstrated a way to integrate PDT with immunotherapy.[72] In a different study, Chen et al. employed a polyethylene glycol-IDO 1 inhibitor NLG 919 conjugate for codelivery of immunochemotherapy drugs to treat advanced cancer. The paclitaxel and sunitinib, a tyrosine kinase inhibitor, were delivered simultaneously to enhance the therapeutic efficacy. The production of chemokines responsible for the recruitment of MDSCs was drastically blocked by sunitinib and helped improve the overall efficiency of the combination therapy.[73] The recent studies from Rivas's group fabricated phospholipids based micellar nanocarrier to integrate the TLR 4 agonist Xcc lipooligosaccharide, OVA antigen, and iron oxide nanoparticles to facilitate the co-delivery of immune stimulators. The TLR agonist Xcc lipooligosaccharide induced the immune response by secreting costimulatory molecules on DCs and inflammatory cytokines (TNF-α and IL-12) and helps in creating an immunogenic environment. Combining these micelles with immune checkpoint blockade inhibition imparted a potent action against aggressive B16F10 melanoma tumors and induced long term protection against tumor challenge.[74]

In an attempt to achieve site-specific delivery of the immune adjuvants, Sun's group synthesized polymeric hybrid micelles (PHM) from a diblock copolymer. It was used for delivering tyrosinase-related protein 2 (Trp2) peptide and adjuvant CpG Oligodeoxynucleotide to the lymph nodes. The incorporation of a cationic polycaprolactone-polyethyleneimine block in the present design helped PHMs to localize in secondary lymphoid organs, thus enabling the target-specific delivery of these immunoadjuvants to antigen-presenting cells. This site-specific delivery was shown to activate the immature DCs, promote cytotoxic functions of T cell through receptor binding, and inhibit tumor growth in B16F10 metastatic melanoma tumor-bearing mice.[75] These recent studies demonstrated that with the help of nanotechnology and combination therapies, the efficacy of the therapeutic agents could increase drastically and the multifunctional nanocarriers may put forward a new avenue addressing long-standing challenges in cancer immunotherapies.

2.3. Polymeric Nanoparticles

Nanoparticles made of polymeric materials hold great mechanical stability and offer the production of significantly stable formulations of clinically relevant therapeutic molecules. The feasibility of structural engineering of the polymeric materials to incorporate the intracellular stimuli-responsive functional units has enabled the synthesis of tailormade nanoformulations. These formulations offer a way to fine-tune the release profiles of the encapsulated entities from burst to sustained release and

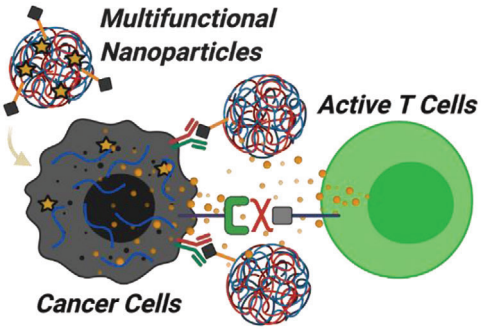

Figure 7. A generalized schematic showing the interaction of multipurpose polymeric nanoparticles with tumor cells and subsequent activation of cytotoxic T lymphocytes in the tumor microenvironment. Since nanoparticles are nanosized biomaterials they can act in the tumor cell immune cell synapse to accomplish the better therapeutic outcome.

help accomplish the on-demand site-specific delivery. The presence of multiple functional groups along the polymer backbone also provides the multiplexing ability, including the opportunity to increase the surface ligands density that enables the targeting ability to the nanoformulations.[76] The structural and chemical diversity amongst the polymer chains facilitates the efficient encapsulation of therapeutic molecules such as proteins, genes, cytokines, and enzymes through electrostatic force of attraction and helps to target specific intracellular compartments.[77] The ability of polymeric nanoparticles (**Figure 7**) to encapsulate the physically and chemically different therapeutic molecules in a single nanocarrier platform has led to the development of combinatorial cancer therapeutics. The following section will discuss some of the recently developed nanoparticles based combinatorial strategies employed in cancer immunotherapy.

ICB has shown great success in recent immunotherapy approaches. Recent studies have shown that cyclin-dependent kinase 5 (cdk5) protein has been involved in regulating the PD1-PD-L1 interaction and thus, knocking out this gene can significantly increase the function of cytotoxic T lymphocytes to evoke the antitumor immune response. Thus, cdk5 is emerging as a new target to prevent the immune surveillance escape of cancer cells. Zhang's group explored this idea and a pH-responsive nanoparticle delivery vector was synthesized from the PEI-PLGA polymeric system that can co-deliver CRISPR/Cas 9 plasmid (to knock out *cdk* 5 gene that attenuates the expression of PD-L1), an ICB agent and chemotherapeutic agent, paclitaxel. The schematic representation of the self-assembly and encapsulation of a gene is depicted in **Figure 8**a. This polymeric system has been shown to promote ICD (Figure 8b), DC maturation, and macrophage remolding towards the M1 phenotype. This combinatorial approach was found to be very effective in B16F10 melanoma bearing mice. The attenuation of PD-L1 expression on cancer cells by cdk5 has further shown a synergistic outcome through immunochemotherapy (Figure 8c-e).[78]

To integrate and deliver various therapeutic entities, Cruz's group has developed a PLGA-PEG nanoparticle platform that enables co-delivery of chemotherapeutic agent doxorubicin, poly I:C, and R848 immunoadjuvants, and MIP3α from a single platform. The encapsulation of NIR dye in the same nanoparticles has endowed this system with imaging abilities to monitor the

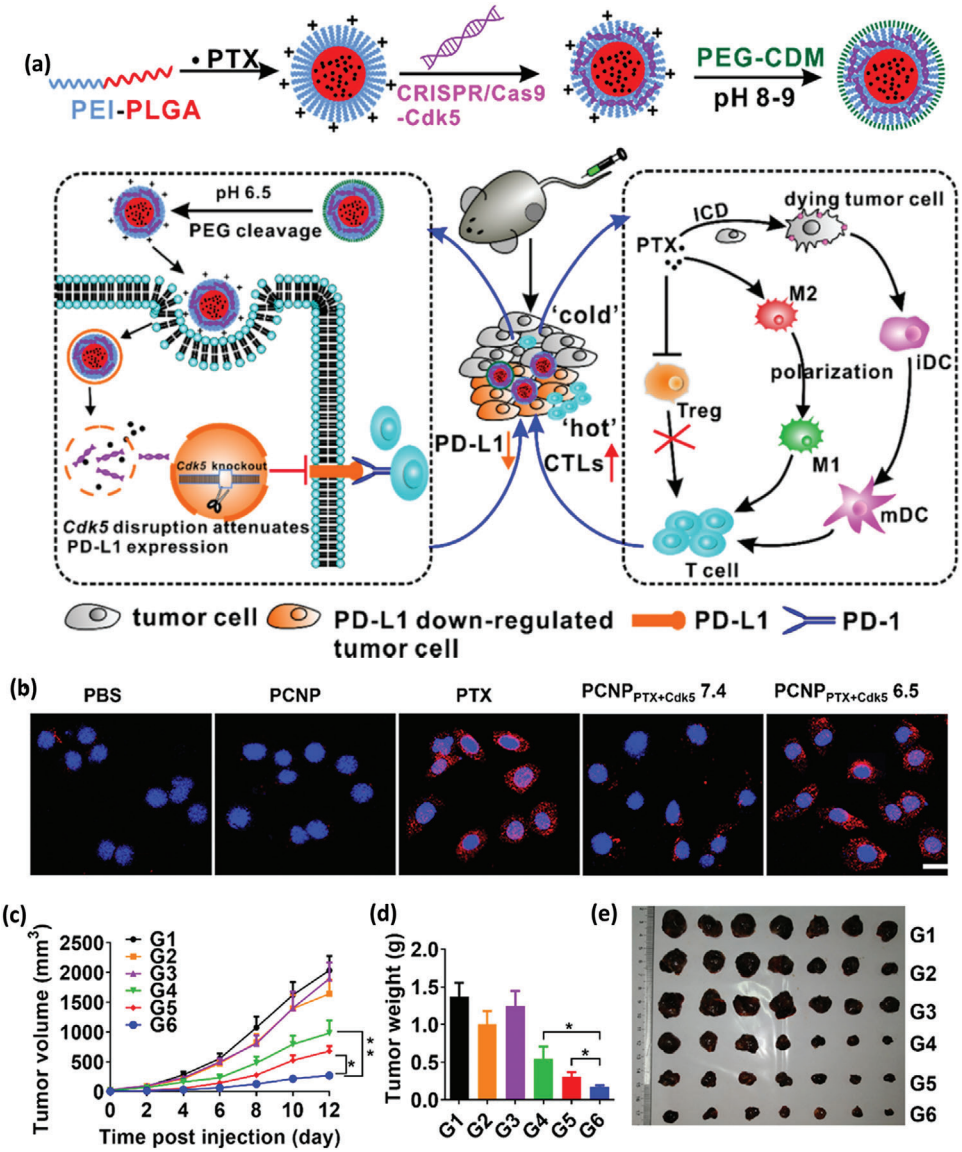

Figure 8. a) Schematics representation of pH responsive nanoparticles for PTX and CRISPR/Cas9-cdk5 delivery. b) Confocal microscopy images for different treatment groups showing the expression of calreticulin on B16F10 cells. c) Tumor growth curves for mice treated with different treatment groups. d) Tumor weight of the excised tumors and e) images for the excised tumors from mice treated with different treatment groups. Reproduced with permission.[78] Copyright 2020, American Chemical Society.

delivery. This platform has shown an excellent therapeutic efficacy on account of their ability to deliver multiple chemoimmunotherapy drugs in two resistant tumors, TC-1 lung carcinoma and MC-38 colon adenocarcinoma models.[79] In a recent study, doxorubicin, known to induce ICD as discussed earlier, was encapsulated in a PEG-PLGA polymeric nanoparticle and combined with an anti-programmed death 1 antibody by Salems's group. The nanoparticles with a low concentration of doxorubicin required for ICD were insufficient to produce enough toxicity and thus, a combination of PD-1 antibody was used to invigorate the therapeutic action. The mice bearing B16F10 melanoma tumors, when treated with DOX nanoparticles combined with an anti-PD-1 antibody, have shown complete eradication of tumors at the end of the treatment. This platform demonstrates an elegant way of combining ICB with agents that induce ICD to harvest an efficient therapeutic outcome.[80]

It is also important to activate cytotoxic immune cells in the TME and bridge them to cancer cells to extract the maximum outcome from the treatment. The nanoparticle design made by Li et al. has enabled the reduction of the synapse between cancer cells and immune cells to strengthen the immunotherapy approach for inhibiting cancer growth in hard-to-treat cancers. The nanoparticles system made from biodegradable PLGA polymer could encapsulate and deliver the IL-12 cytokine to target cells. The decoration of the surface of the nanoparticles with CD8 and GPC-3 antibodies further facilitated the specific binding of two target cells, $CD8^+$ and Hep-G 2 cancer cells, through antibody-antigen interactions. These target specific interactions have led

to the induction of the strong antitumor responses from CTLs.[81] The nanoparticles that can accomplish target-specific accumulation of the photosensitizer can increase the efficacy of PDT for advanced cancers. Thus, to combine PDT with immunotherapy, Dong and Li's group designed a system to deploy OVA as a nanocarrier to deliver photosensitizer Ce6 to the tumor sites. Further coating of these Ce6 encapsulated ova antigen nanocarriers with B16-OVA cancer cell membrane facilitated the tumor-specific accumulation of these nanoparticles. The enhanced antigen cross-presentation through OVA antigen, together with ICD, has completely eradicated tumors from B16-OVA tumor-bearing mice and showed a long-term antitumor immune memory effect to avoid tumor recurrence.[82]

To avoid the dose-related toxicities and to enhance the efficacy of a low dose of PDT, the reversal of the immunosuppressive environment is an essential factor to be considered. This idea was explored by Sun et al. and designed a TME specific-reactive oxygen species (ROS) responsive nanoparticles-based system which enabled the simultaneous delivery of Ce6 and a multikinase inhibitor sorafenib. These nanoparticles elicited strong ICD from PDT and also facilitated the effector function of tumor infiltrated T cells and reduced the immunosuppressive TME. This combinatorial platform enabled the complete ablation of local and distant metastatic tumors.[83] In another attempt, Kim et al. designed a polyglutamic acid-based nanoparticle platform for the photodynamic immunotherapy approach. The nanoparticles were used to co-encapsulate a photosensitizer Ce6 and a TLR-4 agonist MPLA and delivered to the target tumor site using the enhanced permeation and retention effect. The codelivery of these agents enabled the ROS-mediated calreticulin surface exposure, maturation of antigen-presenting DCs, and elicited ICD of the tumor cells. These dual-function nanoparticle systems induced a very significant tumor ablation in B16F10 tumor nearing mice and also protected the mice against tumor recurrence.[84]

The presence of an immunosuppressive microenvironment mainly influences the success of immunotherapy in advanced cancers. Thus, designing the strategy to reverse this microenvironment is highly desirable to overcome the hurdles that prevent the clinical translation of the immunotherapy approaches. Niaragh's group developed a hyaluronic-chitosan-based nanoparticles strategy to silence IL-6 cytokine and signal transducer and activator of transcription (STAT)3 signaling pathway to reverse the immunosuppressive microenvironment and to exert the anticancer effect. Small interfering RNAs (siRNAs) that can specifically target the genes for cytokine and STAT 3 pathway suppression were encapsulated in these nanoparticles and delivered to the CD44 receptor overexpressed cancer cells. The simultaneous delivery of these siRNAs has exhibited the excellent downregulation of IL-6 and STAT 3 in three different murine-derived cancer cell lines such as 4T1 breast cancer, CT-26 colon cancer, and B16F10 melanoma and elicited the synergistic anticancer effects.[85] The combination of small molecule drug icaritin, known to induce autophagy, and doxorubicin, can promote the synergistic ICD. Huang's group explored this strategy by employing PLGA-PEG polymer-based nanoparticles for co-delivery of these two drugs at an optimized ratio. The dual delivery of drugs has shown enhanced ICD and remolded the immunosuppressive TME in both mouse and human hepatocellular carcinoma models, resistant to chemo and immunotherapy. Besides, these combination nanoparticles, combined with lenvatinib, dramatically prolonged the survival time in mice at an advanced stage of HCC.[86]

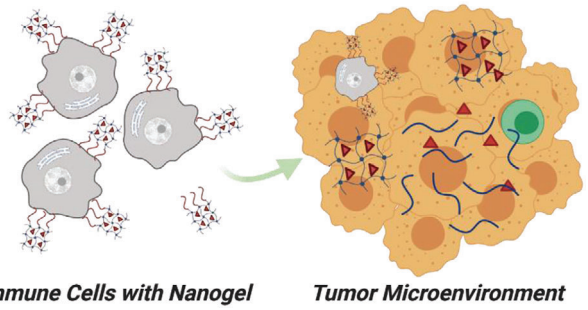

Figure 9. A schematic showing the decoration of a drug loaded nanogel on the cell surface of the cells to target the tumor microenvironment. This strategy enhances the accumulation of therapeutic molecules and T cells at the tumor microenvironment and thus helps to achieve better response.

The articles discussed above clearly reveals the importance of nanoparticles in extracting the maximum efficacy from combinatorial approaches in combating cancer progression. The detailed understanding of the mechanism adopted by these multifunctional nanoparticles and the key immunotherapy strategies discussed above have also proved that the modulation of the body's defense system against the cancer cells along with traditional therapies can yield synergistic antitumor responses.

2.4. Nanogels

The nanogels, mostly being prepared from crosslinked polymer networks, have high mechanical stability, higher encapsulation efficiencies and are also endowed with unique features such as excellent surface area, ease of functionalization, stimuli sensitivity, and swelling. The ability of these nanogels to encapsulate and shield biologically sensitive moieties such as nucleic acids, genes, RNAs, DNAs against physiological barriers also makes them potential candidates to be used in the biomedical field.[87] A general schematic of the nanogel and its immune cell-based delivery to TME is shown in **Figure 9**. The following section will shed light on important findings of the nanogels in combinatorial cancer immunotherapy.

Phagocytes exhibit a range of biological functions and provide the first line of defense against external pathogens, abnormal cells, or microbes. The neutrophil-mediated cell killing occurs via neutrophil lysosomes by the generation of singlet oxygen to destruct the foreign organisms. This idea of singlet oxygen generation to kill abnormal cells was used by Wu et al. and showed that the enzyme-mediated cascade reactions could be used for cancer therapy. The two biologically relevant enzymes, superoxide dismutase (SOD) and chloroperoxidase (CPO) that can trigger the generation of singlet oxygen, were encapsulated in supramolecular hybrid nanogel. The enzyme cascade reaction mediated generation of the singlet oxygen at the tumor site exhibited efficient inhibition of the tumor progression in a mouse bearing HepG2 tumors.[88] In another study, Kordalivand et al. developed a dextran-based nanogel system decorated with CTLs and $CD4^+$ T helper epitopes that enables the safe and

efficient delivery of long synthetic peptides. The nanogel exhibited superior peptide loading content up to 15% and their efficient uptake by DCs promoted their maturation and thereby inducing strong T cell-mediated antitumor immune responses.

Further co-encapsulation of poly I: C, a TLR3 agonist in this nanogel, augmented $CD8^+$ T cell-mediated immune response and helped in the efficient eradication of tumors from the mice.[89] To integrate the chemoimmunotherapy, Chen's group developed a nanogel delivery platform from FDA approved polyvinyl alcohol and vinyl ether acrylate and used for dual encapsulation of docetaxel, a chemotherapeutic agent and an indoleamine 2,3 dioxygenase 1 (IDO 1) inhibitor NLG 919. The folate modification of the nanogel facilitated the tumor-specific accumulation and pH-responsive destruction of nanogel helped in releasing the content at the tumor site. The simultaneous activation of immunogenic cell death, intratumoral accumulation of CTLs, NK cells, and reduced infiltration of MDSCs induced a significant tumor growth inhibition in mice bearing in metastatic 4T1 breast cancer model.[90]

Vaccines have long been used in the medical field and have shown excellent response against diseases by promoting antigen-specific immune response. The efficiency of the vaccines was improved by deploying nanocarriers for their delivery. Nanocarriers act as a host for vaccines and shield them from physiological barriers and degradation. Additionally, nanoparticles also enable the codelivery of antigen and adjuvants from a single system, which might produce a potent effect. Thus, nanocarrier-based vaccine delivery has also attracted much attention in the last decade to accomplish the desired level of antigen-specific immune responses. In recent studies, Miura et al. have developed a cholesterol-modified pullulan nanogel that can efficiently encapsulate OVA in its core and was employed for targeting lymph nodes (systemically) and antigen-presenting cells (cellularly). The delivery of OVA through nanogel was found to promote a robust cellular immunity by enhancing intratumoral infiltration of CTLs and in vivo results have also shown antigen cross-presentation with DCs in the presence of immune adjuvant CpG.[91] To invigorate the adoptive T cell transfer (ACT) therapy and reverse the immunosuppressive environment, Xie et al. designed a reduction responsive nanogel through chemical cross-linking of IL-12/Fc. This nanogel was further decorated with the polyethyleneglycol-*b*-polylysine polymer to suppress the non-specific protein binding and generate the cationic charge on the surface. This nanogel was then conjugated to the plasma membrane of the T cells through chemical coupling and electrostatic interaction and used for the ACT. The intravenous delivery of IL-12/Fc through T cell surface-modified nanogels to B16F10 melanoma bearing mice have shown to increase the density of tumor-reactive T cells in the TME as compared to the free IL-12/Fc.[92]

To target and manipulate TAMs towards an anti-tumoral phenotype, Nuhn et al. synthesized a nanobody conjugated nanogel and employed it for mannose receptor-specific targeting of TAMs. The intravenous injection of this nanobody functionalized nanogel showed an enhanced accumulation in pro-tumoral TAMs, as evident by the fluorescence microscopy imaging. This report provides a platform to efficiently target mannose receptor overexpressed TAMs and thus can help in remodeling the pro-tumoral TAMs to immune responsive ones.[93] The vaccines-based immunotherapy facilitates the presentation of tumor antigens to the immune system and augments the immune response to combat the cancer growth. Glycoprotein-100 (gp-100) is one such melanoma antigen that triggers the antigen-specific T cell responses against melanoma. The delivery of gp-100 is limited only by a parenteral route and thus faces an intestinal mucosal barrier before producing the antigen associated immune responses against cancer. To overcome the physical obstacles in gp-100 treatment, Shen et al. have developed a nanogel for safe and efficient oral delivery of the plasmid DNA gp-100 vaccine. This multifaceted nanogel was prepared by a blending-blending approach using transmembrane avidity TAT-1 protein and anionic alginate polysaccharide to bypass the gastrointestinal barriers. This nanogel showed enhanced activation of cytotoxic T cells and exhibited tumor growth inhibition in mice bearing B16F10 melanoma tumors.[94]

It is imperative to deliver the antigens to the draining lymph nodes where most of the immune cells reside, including antigen-presenting cells and their activation can produce effective antigen-specific immune responses against cancer. This challenge was addressed by Miura et al. by utilizing the structural properties of the polysaccharides and making a self-assembled nanogel from cholesterol-modified polysaccharides. The stealth nanogels were prepared from pullulan and dextrin-cholesterol conjugates separately and used for OVA encapsulation through hydrophobic interactions. This nanogel was found to deliver the OVA to lymph nodes safely and a superior activation of CTLs and antibody production was accomplished, as evident from flow cytometry and ELISA studies. These polysaccharide nanogels showed significant tumor growth inhibition in E.G7-ova tumors bearing mice.[95] In a recent study, a nanogel delivery vector to safely deliver chemo-immunotherapeutic agents was developed by Song et al. by using the natural biocompatible components. A pH-responsive nanogel was prepared from beta-cyclodextrin and chitosan and employed to encapsulate paclitaxel (PTX), a chemotherapeutic agent and IL-2, a cytokine. This nanogel promoted ICD of cancer cells along with IL-2 mediated activation of T cells and NK cells. This nanogel has also shown effective activation of DCs and CTLs in in vivo conditions in the B16F10 melanoma bearing mice model and resulted in significant tumor growth inhibition.[96]

The lack of presence of CTLs and the presence of immunosuppressive microenvironment are two key factors limiting the successful clinical utility of the immunotherapy regimens. To address this, Song et al. developed a syringe-injectable immunotherapeutic nanogel (iGel) using cationic clodronate liposomes and anionic multidomain vesicles. The schematic illustration of reversible iGel and its interconnection with dye loaded components are shown in **Figures 10**a,b. The higher encapsulation efficiencies for gemcitabine and imiquimod in this iGel system were observed compared to the conventional liposomes. The subcutaneous injection of this iGel in 4T1 and TC1 tumor-bearing mice stimulated the reprogramming of TME via ICD, enhanced recruitment of antigen-specific T cells, and induced suppression of MDSCs as evident by extensive immunological assays. The representative flow cytometry plots depicting the infiltration of different immune cells are shown in Figure 10c. Further combination of this iGel with ICB (PD 1 antibody) synergized the cancer immunotherapy and avoided tumor metastasis

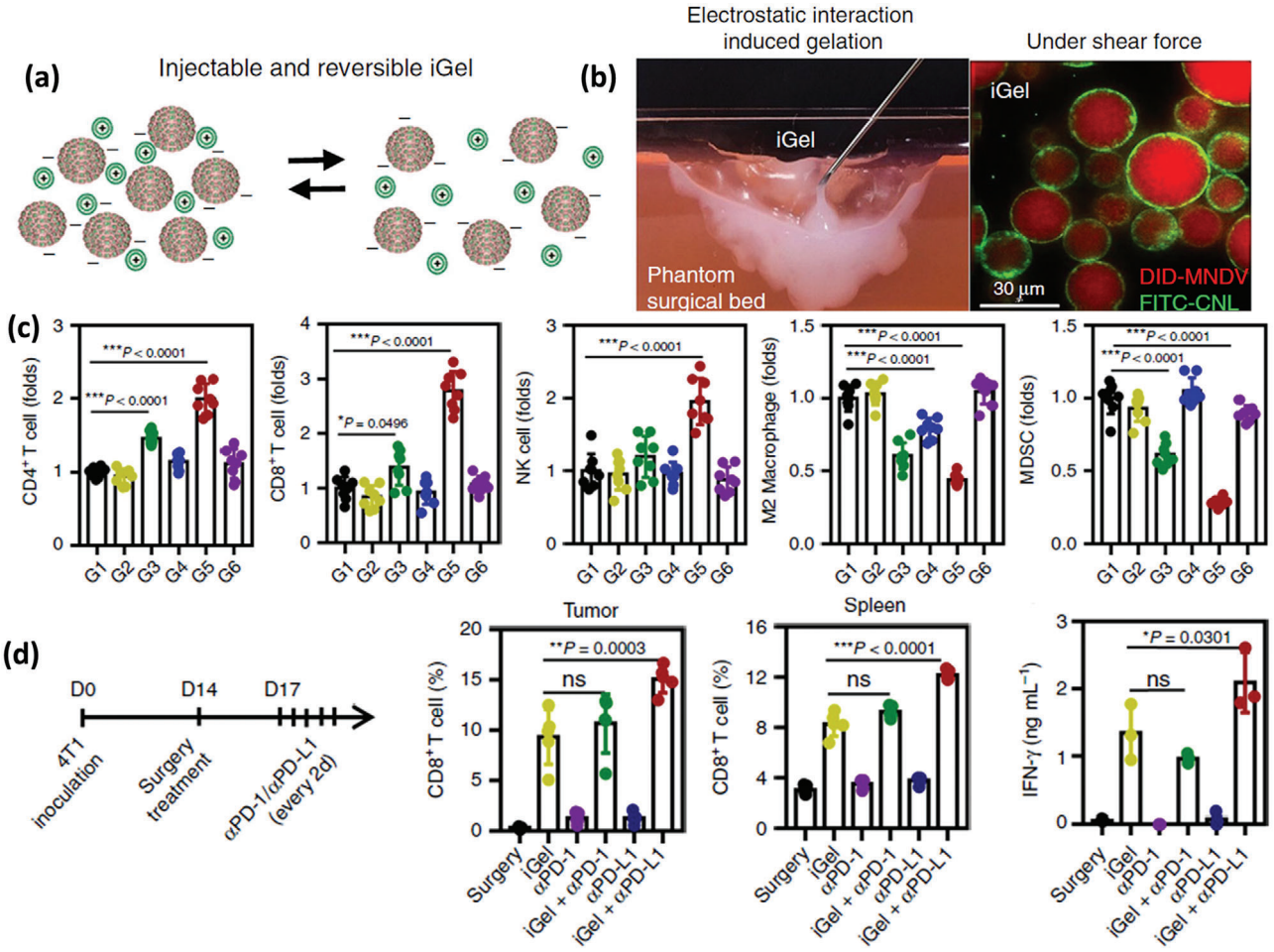

Figure 10. a) Schematics representation of reversible and injectable iGel that delivers gemcitabine and R837. b) Fluorescent images of iGel showing the interconnection between DID labeled MNDVs and FITC labelled CLVs. c) Quantification of the flow cytometry analysis demonstrating the infiltration of $CD4^+$, $CD8^+$ T cells, NK cells, M2 macrophages and MDSCs in recurring 4T1 tumors postsurgery at day 7. d) Schematics of treatment schedule of iGel with checkpoint inhibition, graph show the quantification of infiltration of $CD8^+$ T cells in 4T1 tumors and secretion of IFN-γ in combination therapy. Reproduced with permission.[97] Copyright 2019, Springer Nature.

and recurrence. The plot in Figure 10d showed the enhanced infiltration of $CD8^+$ T cells when mice were treated with iGel in combination with ICD.[97]

2.5. Polymersomes

Polymersomes are another class of self-assembled materials made from amphiphilic polymeric architectures that hold superior mechanical stability needed for stable encapsulation and thus stands as an excellent alternative for physiological application compared to any other self-assembled nanocarriers. Moreover, polymersomes possess a distinct hydrophilic core and hydrophobic layer that enables the encapsulation and delivery of drug molecules (hydrophilic and hydrophobic) to the site of interest (**Figure 11**). Additionally, polymersomes can be structurally engineered with various functional groups for effective conjugation of the drugs, targeting ligands, etc., for enhanced accumulation of the pharmacologically active molecules.[98] Thus, polymersomes provide a platform to explore the strength of different

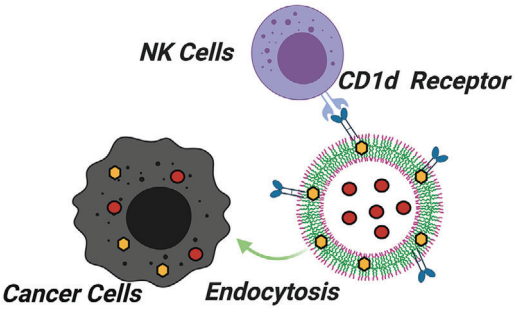

Figure 11. A generalized polymersome design targeting natural killer cells in the tumor microenvironment. These polymersomes are carrying other therapeutic entities to the tumor microenvironment, while also activating NK cell-based immune responses to accomplish a combinatorial antitumor effect.

therapies and their combinations to offset some of the limitations in cancer therapy. The following section will discuss some of the recent studies on polymersomes and their biomedical application.

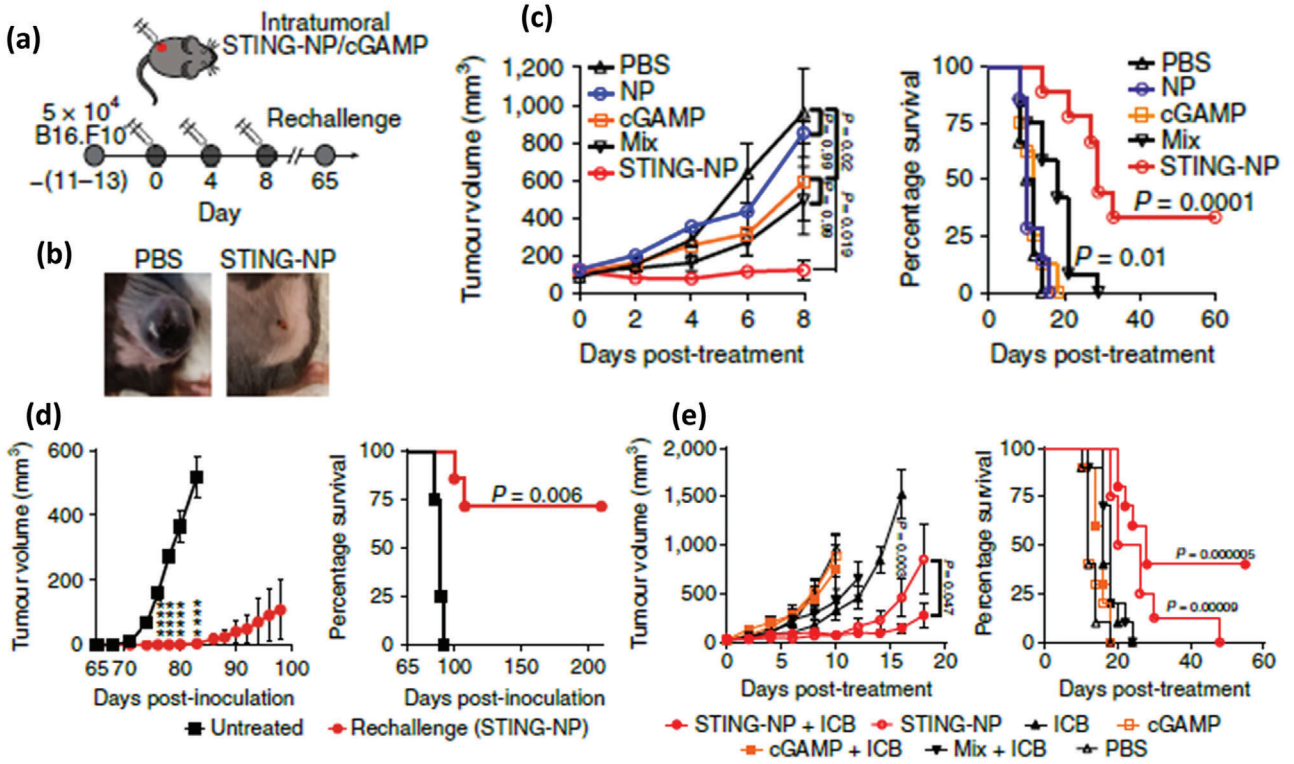

Figure 12. a) Schematic representation of intratumoral administration and tumor rechallenge in B16F10 tumor bearing mice. b) Pictures showing tumor sizes in PBS and STING-NP treated mice. c) Graphs depicting tumor volume and percent survival in mice administered with different treatment groups. d) Tumor volume and percent survival graphs for mice rechallenged with B16F10 cells on contralateral flank 65 d after inoculation without any further treatment. e) Graphs showing the tumor volume and percent survival curve for B16F10 tumor bearing mice administered with different treatment groups in combination with immune checkpoint blockade. Reproduced with permission.[101] Copyright 2019, Springer Nature.

In an attempt to make a chemoimmunotherapy system, the chimeric cross-linked polymersome (CCPS) delivery vector was designed by Yang et al. that found to successfully deliver the chemotherapeutic agent doxorubicin hydrochloride to induce ICD at a very low dose and a photosensitizer 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH) to induce photodynamic therapy. These CCPS systems were found to enhance the population of tumor-associated antigens and mature DCs in draining lymph nodes and $CD8^+$ T cells in tumor tissues and inhibits the growth of both primary and distant MC-38 tumors in vivo.[99] The same group also explored pH-responsive polymersomes or polymer vesicles (pRNVs) and attempted to integrate the PDT with an immunotherapy approach. The pRNVs were found to induce ICD through PDT along with inhibition of the IDO 1 pathway and promoted a significant abscopal antitumor effect in the B16F10 melanoma model in vivo at a very low dose.[100]

The site or target specific delivery of the immunotherapeutic molecules is crucial to augment the tumoricidal immune responses in cancer immunotherapy. Cyclic dinucleotides (CDN) are the class of immunotherapeutic molecules that acts as agonists of stimulator of interferon genes (STING) that mostly resides in the cytosol. However, CDNs efficacy was sacrificed by a lack of proper delivery vehicles that can deliver them to the cytosol. To overcome these challenges, Shae et al. have synthesized pH-responsive endosomolytic polymersome nanocarriers from diblock copolymer to accomplish cytosol specific delivery CDN ligand 2′3′ cyclic guanosine monophosphate–adenosine monophosphate (cGAMP). The schematic illustration of intratumoral STING-NP administration and the tumor images are shown in **Figure 12**a,b. These polymersomes were found to increase the efficacy of cGAMP by two to three folds compared to the free cGAMP administration and also improve the ICB therapy in murine melanoma models. The ability of these STING-NP to induce effective antitumor immune responses was studied by measuring tumor volumes and percent survival. The data are shown in Figure 12c. The effectiveness of the STING-NP was validated by tumor rechallenge in mice with B16 F10 cells (Figure 12d). Further combination of STING-NP with ICB have (Figure 12e) helped to enhance the survival of the animals undergoing the treatments. The translational potential of these cGAMP polymersomes was validated by studying their activity in resected human metastatic melanoma tissues.[101]

Chemokines and cytokines are the key regulators of immune responses, and recently, their modulation has been a potent immune target for cancer therapy. IL-12 is one such powerful immunoadjuvant that mediates the antitumor response by activating T and NK lymphocytes. The standard intravenous delivery of IL-12 protein has shown adverse effects; thus, loading and site-specific delivery of IL-12 using a nanocarriers platform are crucial in promoting the antitumor immune responses through this approach. To enhance the encapsulation efficiencies of such an important immunoadjuvant protein, Gao et al. synthesized cationic

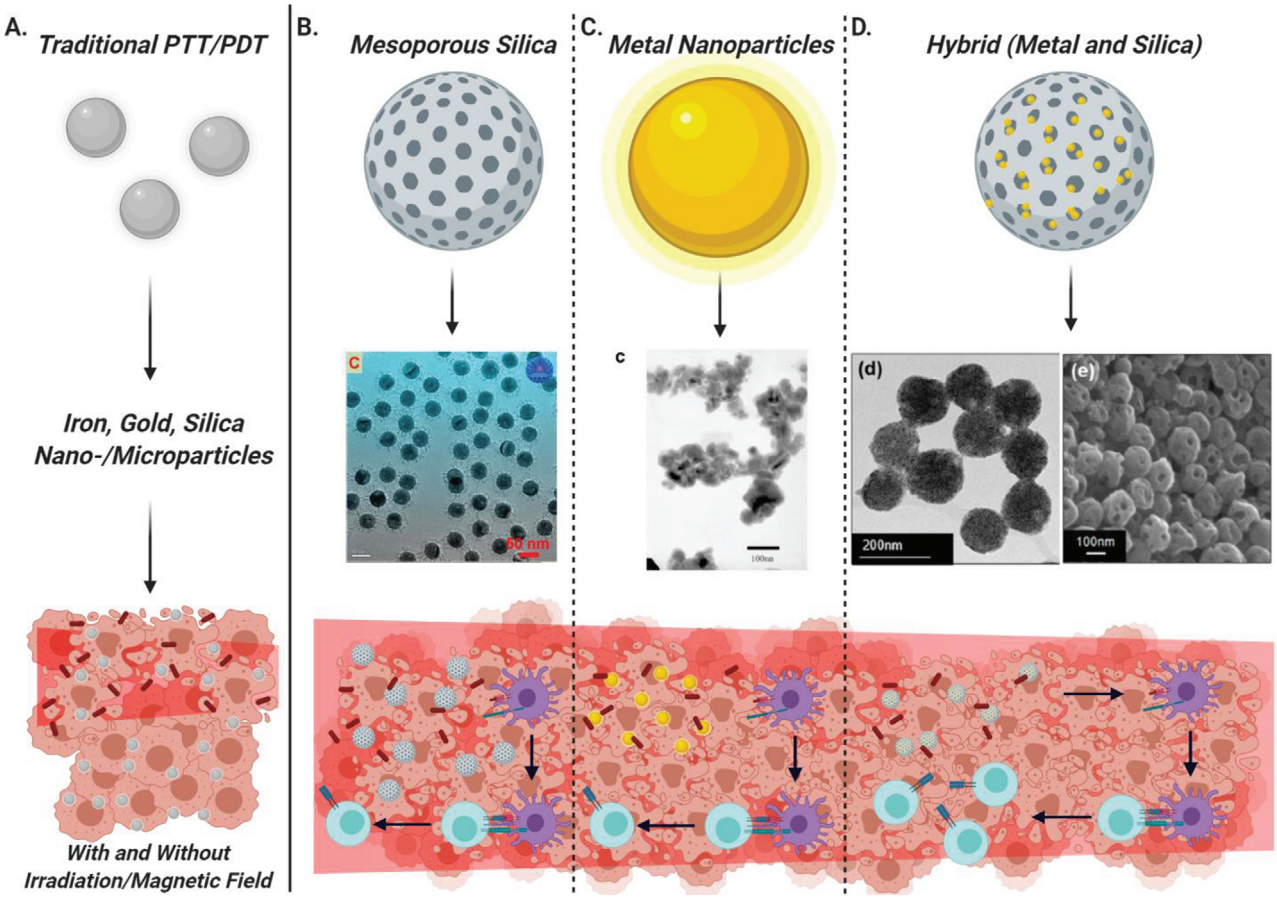

Figure 13. Schematic of traditional PDT versus PTT. a) While traditional systems increase cancer cell death and antigenicity when exposed to irradiation or magnetic fields, multifunctional systems allow for robust immune cell activation using a variety of b) silica-based, c) metallic-based, and d) hybrid-material-based systems. Micrographs depicted from left to right are micrographs. (Reproduced with permission.[126] Copyright 2018, Wiley-VCH GmbH). (Reproduced with permission.[115] Copyright 2018, Ivy Spring International Publisher). (Reproduced with permission.[125] Copyright 2019, American Chemical Society).

polyphosphazene vesicles that can encapsulate the plasmid IL-12 using physical and electrostatic interaction. The delivery of these plasmid IL-12 encapsulated vesicles in the intracellular milieu has stimulated tumor growth inhibition in BALB/c mice bearing CT-26 colon carcinoma.[102] The above-discussed ways of modulating the immune response to favor the antitumor efficacy and the integration of therapies mentioned hereof have validated the pivotal role of nanotechnology-based delivery vectors in combating the cancer progression.

3. Inorganic Nanomaterial Strategies to Elicit Immune Responses

As mentioned above, there are many traditional strategies for nanocarriers to deliver materials such as polymer- and lipid-based systems. One sector of nanomaterials that has been used for drug delivery and now moving into immunotherapy is inorganics, such as metal- and silica-based nanoparticles and nanoplatforms. Metallic and silica nanomaterials, in particular, have been of rising interest because of their ability to have tunable size, morphology, and composition.[103,104] Apart from these excellent features, metal-based quantum dots, owing to their NIR optical properties, are gaining a lot of interest as theranostic agents for cancer therapy. These quantum dots are usually packed in a self-assembled biomaterial and other therapeutic molecules and employed as imaging platforms in combinatorial therapies. The combination of these properties allows for careful tuning and optimization for preferential release of moieties or even targeting within the tumor microenvironment. Here we will discuss the strategies that take advantage of those properties in metallic-, silica-, and hybrid-based multifunctional nanomaterials (**Figure 13**). In the first section, we will discuss Janus nanomaterials, which are setting up an intriguing path for overcoming immunological barriers and eliciting an immune cell-specific response.

3.1. Janus Nanoparticles

Janus nanoparticles got their name from the Greek god, Janus, which is the god of beginnings and transitions who is depicted as having two faces looking opposite ways. This feature has been used in many nanoparticle strategies such as paint

coatings, sensors, and magnetic field imaging.[105–107] An area in which this has been underexplored is the field of biomedical engineering and immunotherapy specifically. Having completely different functionalities on either "face" of the particle can result in a variety of interactions that can be exploited in the field of immunotherapy depending on the interaction of separate targets.

Recently, this biorthogonal functionality has been taken advantage of by the Yu Lab at Indiana University, where they have developed Janus microparticles that act as artificial antigen-presenting cells, activating T cells because of their construction.[108] One article in 2014[108] reported a 3 µm silica-based microparticle platform that can undergo microcontact printing with a PDMS stamp that can imprint a protein onto the surface and then incubate those printed particles with another protein to give it a separate functionality. This system hopes to potentially act as an artificial antigen-presenting cells (APCs) to better activate T cells for cytotoxic functionality. Based on this microcontact process, they designed "bull's eye" and reverse "bull's eye" particles that had a small patch of T cell receptor (TCR) ligand anti-CD3 and the rest of the area covered with fibronectin, and vice versa, respectively. The spatial distribution of ligands was analyzed with Jurkat T cells to see what role this spatial pattern contributes to in T cell activation, in which Fluo-4 AM was used to detect intracellular levels of $Ca^{2+,}$ which is indicative of T cell activation. Upon stimulation of these cells with both native and reverse "bull's eye" particles, it was shown that the reverse pattern, because of the larger amount of anti-CD3 coverage, caused a greater level of T cell activation and caused colocalization of actin and PKC-theta with the Jurkat cells when the anti-CD3 was facing it, indicating intracellular signaling through TCR. On the other hand, the native "bull's eye" pattern only induced transient Ca^{2+} increase as well as dispersed actin and PKC-θ, which points this study in the direction that different surface compositions and patterns can have a huge influence on cytotoxic T cell activity and can be used to develop artificial APC-like systems further. Although this system is not on the nanoscale, the morphology and mechanistic approach to having completely separate surface areas of ligands can allow for manipulation and transient activation of T-cells in vitro.

In a different study, in 2017,[109] the Yu Lab developed nanoscale constructs that have clustered ligands that could have the potential to activate T cells. Instead of investigating clustering effects, a spatial arrangement was varied to understand this mechanism of T cell activation. These 500 nm silica Janus nanoparticles were made in relatively the same manner as their previous work;[108] however, the microcontact printed anti-CD28 antibody as a costimulatory ligand was used. Using the same Fluo-4 AM analysis as before, they were able to see peak T-cell Ca^{2+} concentrations within a few to ten minutes, followed by a decay in fluorescence, which aligned with previous experiments. To see if these results were contributed to the localization of anti-CD3 and costimulation by surrounding anti-CD28, one particle with the same surface density of each antibody similar to the Janus just scattered on the surface (U#) and another one with the same density relative to one another (Ud) were used. Both the Janus and Ud particles were able to have higher increased sustained T cell activation than the U# ones, but when the anti-CD28 was removed from the particles and relied only on the anti-CD3 present, they activated T cells to relatively the same extent. The data presented in this paper shows that clustering of ligands on the surface of nanoparticles enhances T cell activity because of the increased local surface density of ligand stimuli and, in general, shows that the spatial arrangements of activating and costimulatory molecules can have a significant impact on how T cells respond. This gives further evidence that Janus materials have the potential to impact T cell activation in immunotherapeutic applications.

Similar to the micron-sized particles first discussed, the Yu lab investigated the impact of locomotion and orientation of these magnetic microparticles to see the effect on the initiation of T cell activation.[110] Monodisperse silica microparticles were synthesized to have magnetic capabilities by applying nickel and aluminum to one hemisphere and keeping the other as just bare silica. This was done through sequentially using thermal evaporation deposition and resulted in 3 µm silica microparticles with 50 nm of nickel on one hemisphere covered by 30 nm of aluminum to halt nickel oxidation. With the other side as plain silica, it was functionalized using streptavidin-biotin-linked anti-CD3 antibody and passivated with bovine serum albumin (BSA) to avoid protein adsorption. The final product was a half-magnetic, half-T cell targeting microparticle, and this was manipulated through a rotating permanent magnet adjacent to the imaging apparatus in the *x*, *y*, or *z* directions. The rotation of these particles was successfully remote-controlled by rotating a permanent magnet and testing on T cell activation was conducted. Fluo-4 AM analysis shows that when the anti-CD3 portion of the microparticle was facing the T cell, a rapid increase in fluorescence intensity was observed and persisted before the gradual decline and disabled the particles from being controlled by the magnet. The metal side was shown to have no impact on T cell activation and was attributed to only rotating into contact with the anti-CD3 side for activation to occur. The studies done by the Yu group show that Janus materials have the potential to be used in further studies for remote T cell activation based on orientation and clustering effects on TCR-signaling ligand interactions and costimulation.

While multifunctional and multi-"faced" particles can offer morphological advantages to immunotherapeutic applications, traditionally synthesized metal- and silica-based nanoparticles offer inherent advantages such as ease of synthesis of the base materials and highly tunable and predictable properties that make them simple to work with.[103,104] Unfortunately, it has been shown that certain metallic and silica nanoplatforms negatively impact the immune system and activate unwanted mechanisms such as inflammasome activation[111,112] or alter immune cell function.[113,114] Despite these disadvantages, these nanoparticles can be used for tuned drug release, PTT, and PDT that has the ability to ablate tumor cells, causing them to release TAA and increase DCs' antigen presentation capabilities and cytotoxic T cell activity. It is possible to further stimulate immune cells by using multifunctional systems that allow for increasing the antigenicity of the TME through PTT, PDT, or through drug release mechanisms in tandem (Figure 13). Knowing this, it is worth mentioning the recent advances in synergistic vaccines and nanoplatforms that take advantage of the thermal properties of metals, the high loading possibilities with mesoporous silica nanoparticles (MSNs), and some novel materials that shine a light on other areas of potential immunotherapy research.

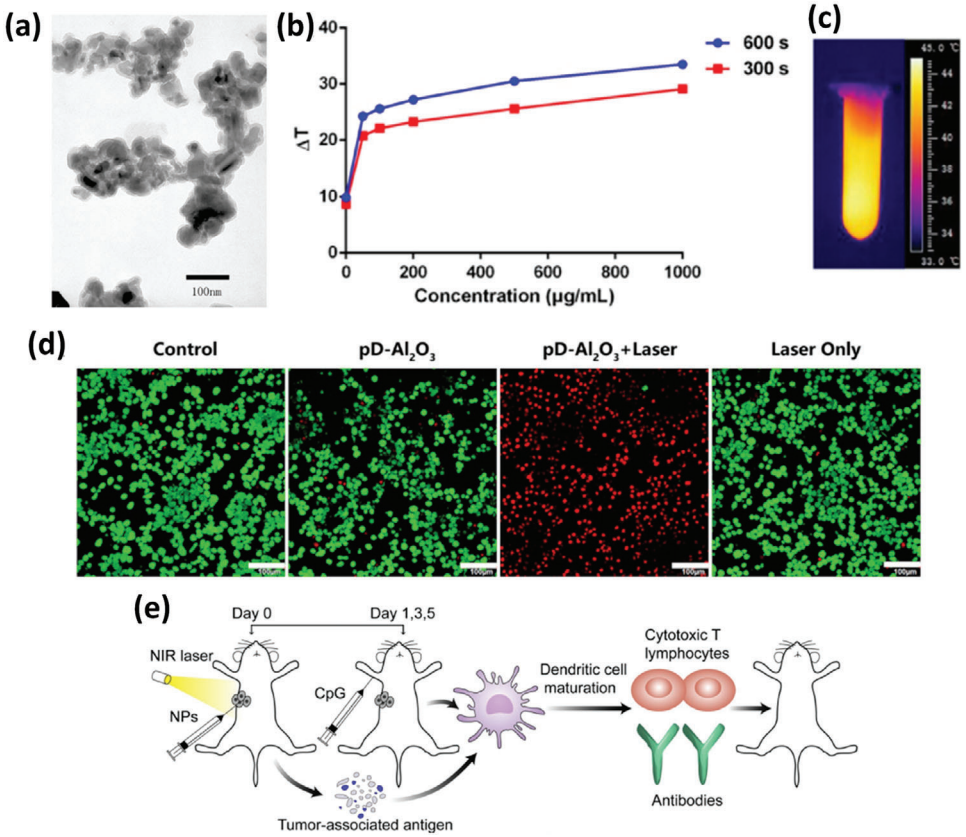

Figure 14. a) TEM micrograph of the pD-Al_2O_3 nanoparticles. b) Temperature changes of pD-Al_2O_3 NPs at various concentrations after 808 nm irradiation for 300 or 600 s. Irradiation causes increasing temperatures at varying concentrations and time scales. c) Infrared thermal images of pD-Al_2O_3 nanoparticles a 1000 μg mL^{-1} after 808 nm irradiation for 300 s. d) B16F10 cells show significant cell death when incubated with pD-Al_2O_3 nanoparticles and irradiated 5 min with a near-infrared laser operating at 808 nm and 1.18 W cm^{-2}. (green = live (fluorescein diacetate), red = dead (propidium iodide). Scale bar = 100 μm. e) Schematic illustration of PTT based on pD-Al_2O_3 nanoparticles followed by immunotherapy in the presence of CpG. Adapted with permission.[115] Copyright 2018, Ivy Spring International Publisher.

3.2. Metal-Based Nanoparticles and Nanomaterials

PTT is becoming of rising potential in the field of immunotherapy because of the possibilities to increase concentrations of free-TAA in the body through heat-induced apoptosis of cancer cells. These free-TAAs can be used to maturate DCs so that coordination and activation of T cells specific for those antigens can occur, causing tumor cell apoptosis and further tumor regression. PTT is used for biomaterials that can efficiently convert light energy, like near-infrared (NIR) light, into heat to create localized heating of nanoplatforms to induce cancer cell death. One study[115] used the photothermal conversion efficiency of polydopamine (PDA), one of the primary components of melanin, in conjunction with Al_2O_3 nanoparticles as an adjuvant to both cause cancer cell death and took advantage of the inherent adjuvant properties that aluminum has to offer. The nanoparticles consisted of PDA-coated Al_2O_3 cores, which self-polymerizes to the surface of the core through dopamine oxidation (**Figure 14**a). This system was able to be heated to over 50 °C within 5 min when exposed to an 808 nm wavelength laser, which will induce cell death (Figure 14b,c). In vitro analysis exhibited that B16F10 melanoma cells can sufficiently uptake these nanoparticles within 1 hour and without the presence of NIR irradiation, causes negligible impacts on cell viability, but reduces to 20% after 24hour incubation followed by irradiation for 5 min (Figure 14d). Bone marrow-derived DCs (BMDCs) were incubated with the pD-Al_2O_3 nanoparticles with or without CpG (to enhance immune activation) and those alone were able to elicit increased expressions of CD40 and CD80 on DCs showing significant maturation and activation in comparison to the untreated cells. In addition to biomarkers, pD-Al_2O_3 was able to increase TNFα and IFNγ cytokine secretion and this was increased even more significantly with the addition of CpG. These in vitro results correlated to in vivo such that this pD-Al_2O_3 + CpG PTT therapy (Figure 14e) was able to cause localized heating of the TME to cause significant to complete regression of tumors (without and with CpG, respectively), significantly increase the maturation of $CD40^+CD80^+$ DCs and secretions of IFNγ and TNFα, as well as increased lymphocyte proliferation rates and $CD4^+/CD8^+$ T cells in the TME. This study exhibits that the use of adjuvant cores in combination with PTT can create versatile, multifunctional nanovaccines.

Aside from using adjuvants to increase antigen presentation, one study shows that palladium (Pd) can be used as a PTT nanoparticle material while including traditional chemotherapeutic agents like DOX. These two materials were incorporated

in one study, in which DOX was released through the degradation of triglycerol monostearate (TGM) in response to high MMP2 levels in cancer cells.[116] The synthesized system, Pd-DOX@TGM, has the capability to heat above 50 °C for efficient cancer cell death, shows favorable release kinetics and only shows release in the presence of MMP2 while maintaining cell viability in 3T3 cells. CT26 multicellular spheroids, when exposed to Pd-DOX@TGM+MMP2, show increased spheroid penetration and dysregulation. When this system is exposed to an 808 nm NIR laser, it further exhibited chemo- and PTT therapeutic potential by killing cancer cells and inhibiting tumor spheroid growth. Also proving these systems capabilities, in vivo CT26 tumor studies reveal increased retention of Pd and DOX into the tumor site and corresponded with significant tumor regression in combination with laser. Immunogenic cell death (ICD) as a result of DOX + PTT treatment in the system show increase IFNγ and TNFα, as well as DOX synergistically boosting the ability to improve the immunogenicity of dead tumor cells. When anti-PD-L1 was added to boost efficacy against distant metastases, CT26 tumor-xenografted mice showed significantly reduced numbers of lung metastases and increased survival rates compared to traditionally used DOX and anti-PD-L1. Combination anti-PD-L1 and Pd-DOX@TGM NPs exhibited increased levels of $CD8^+$ T cells in the tumor in conjunction with decreased $FOXP3^+$ T cells. This is a prime example where immune checkpoint inhibitors can be useful in combination with PTT to increase immunotherapeutic efficiency, aside from the already synergistic impact of having a tumor-sensitive system that can cause PTT efficacy.

Not only can metal-based nanoparticles be used for PTT, but metallic nanosheets made out of single to few-layered transition metal dichalcogenides (TMDCs) have also drawn some attention. These are part of a novel class of 2D materials that have the potential to provide drug loading capabilities while allowing for generating heat upon irradiation in the NIR spectrum. One study[117] synthesized MoS_2 nanosheets that had the capability to not only load CpG to increase immunogenicity but also be tagged with SH-PEG. The resulting MoS_2-CpG-PEG nanosheets gave anywhere from single to few layers in thickness that is stable in physiological conditions at room temperature in PBS or culture medium for 2 weeks. Immunostimulatory analysis of the platform was performed on RAW264.7 immortalized macrophages and it was shown that in comparison to controls, the MoS_2-CpG-PEG system was able to significantly increase TNFα secretion more so than CpG alone or MoS_2 system because of the ability to increase internalization of CpG, which cannot cross the cell membrane on its own. Macrophages exposed to the system were irradiated as well in order to see cell viability and under the exposure conditions used for all experiments, there was mild hyperthermia present but nothing seemingly enough to kill cells. Not only was uptake driving macrophage secretion of TNFα, immortalized DCs (DC2.4) were able to have markedly higher CD80 and CD86 expression than controls. This was only accentuated when the MoS_2-CpG-PEG was irradiated with the laser, proving its potential to increase maturation of DCs in vitro, all while showing a lack of inherent toxicity of the platform to a variety of cancer cell lines, showing the apoptotic effects of cancer cells would be solely due to the effects of PTT. Finally, coculture, transwell experiments were conducted with macrophages (upper chamber) and 4T1 cells (lower chambers) in the presence of controls and MoS_2-CpG-PEF to see if the system with or without irradiation would cause. Without exposure to irradiation, the platform was able to inhibit the viability of some tumor cells, but upon radiation caused significant inhibition of proliferation, which was likely caused by a release of antitumor cytokines. This study pushes the narrative that diverse types of nanomaterials are required to find alternative strategies in order to induce more potent adaptive immune responses. Finally, not all metallic nanoparticles need to be used as PTT vessels but instead can be their own multifunctional nanomedicines.

One study proves that ZnO nanoparticles can be used, in conjunction with DOX, as a multitargeting platform that can impact macrophages and cancer cells, as well as cancer stem cells.[118] Since the polarity of macrophages has been proven to have an impact on cancer progression and cancer stem cells are known to instill drug resistance and self-renewal in tumors, it was implored that ZnO nanoparticles that have DOX are cargo can be used since ZnO NPs can be dissolved at low pH which is advantageous for release of drugs into the TME or intracellularly. This system was tested in both DOX-sensitive (MDA-MB-231, HeLa) and DOX-resistant (NCI/ADR-RES, MES-SA/Dx5) cell lines. It was shown that both cell lines had sufficient uptake through NP-mediated endocytosis and were higher than free DOX showing that the NP system was able to achieve internalization. ZnO/DOX nanoparticles were able to increase intracellular ROS and trigger caspase 3/7 mediated apoptosis, indicating there are potentially multiple signaling pathways that can be impacted by this platform. In addition to impacting cancer cells, RAW264.7 macrophages exhibited high internalization with ZnO/DOX and decreased cytotoxicity compared to free DOX showing that the nanosystem is able to be safe as we as an effective nanovehicle for cytotoxic cargo delivery. In addition to increasing TNFα and IL6 production at dose-dependent concentrations, CD80, CD86, and MHCII expression increased significantly compared to the two components alone. This culmination of data suggests that the ZnO/DOX system can synergistically be used as an immunoadjuvant system to increase the production of proinflammatory cytokines and M1-macrophage activity.

Quantum dots (QDs), with their unique optical properties in the NIR region and owing to their ability to produce the reactive oxygen species, have been used as theranostic agents in biomedical fields. However, their extremely small size, rapid renal clearance, and lack of functionalization limit their clinical utility. In a recent study, Li et al. have used black phosphorous quantum dots (BPQDs) in combination with PEG and ROS responsive polypropylene sulfide (PPS) to synthesize BPQD vesicles through a self-assembly approach. These formed BPQD vesicles were used to encapsulate CpG and were employed for PDT applications. These BPQD vesicles promoted ICD and created the tumoricidal TME to suppress metastatic 4T1 tumor growth in vivo conditions.[119] In another study, Gao et al. synthesized a sheddable bifunctional prodrug vesicles that can efficiently deliver a photosensitizer to elicit PDT and reduction responsive prodrug IDO-1 inhibitor NLG 919 to overcome adaptive immune resistance. The specific accumulation of the prodrug vesicles at the tumor site and MMP2-mediated cleavage of PEG has enabled PDT in addition to imaging to restore the immune-supportive microenvironment. This combination immunotherapy with prodrug vesicles has shown significant antitumor efficacy in

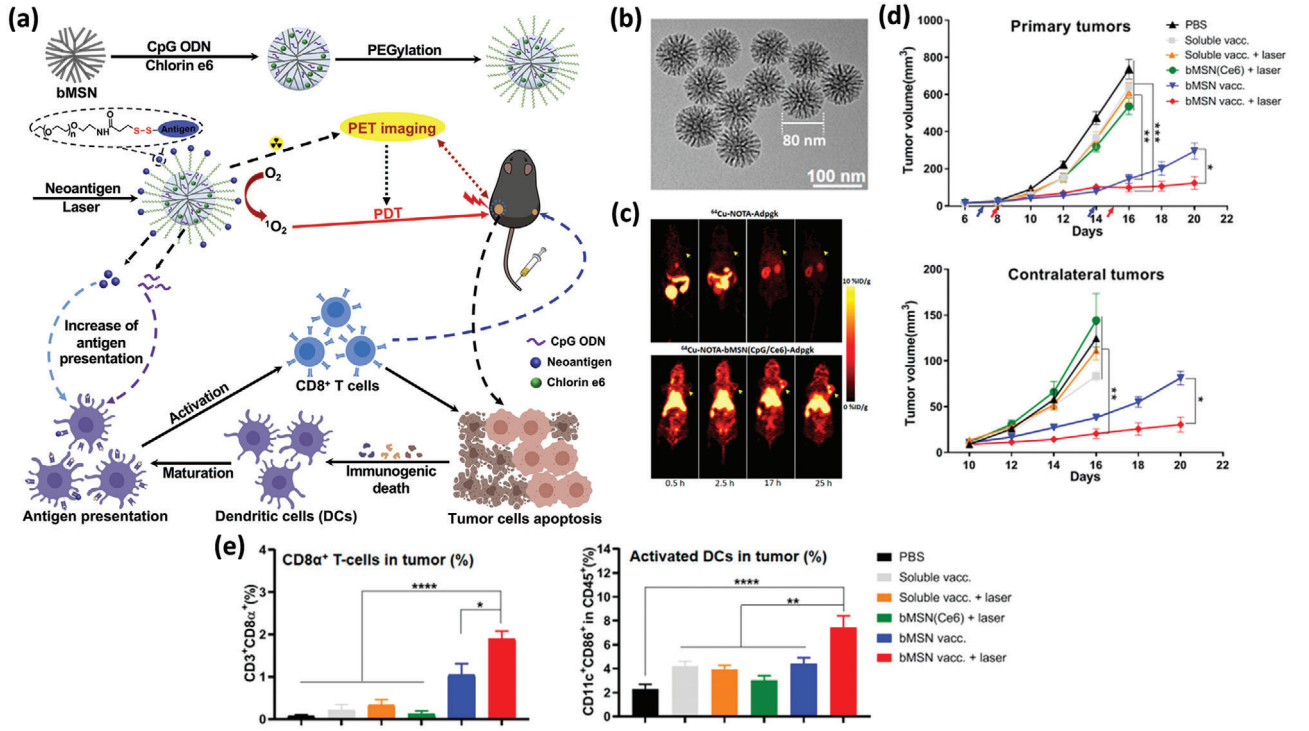

Figure 15. a) Overall schematic of how the described bMSN vaccines can be used in combination with PDT and PET imaging to cause tumor regression and robust DC and $CD8^+$ T cell activation, causing higher levels of antigen presentation and tumor cell apoptosis. b) TEM micrographs of bMSNs. c) Serial PET images of MC-38 tumor-bearing mice at various time points post-injection of 64Cu-NOTA-Adpgk or 64Cu-NOTA-bMSN(CpG/Ce6)-Adpgk. Tumors are indicated by yellow arrowheads. d) Tumor growth reduction is observed when bMSN vaccines + laser are used against primary and contralateral tumors in comparison to control groups. e) Significant increases in activated DCs and $CD8\alpha^+$ T cells in the tumor microenvironment caused by bMSN vaccines + laser. Adapted with permission.[121] Copyright 2019, American Chemical Society.

CT-26 colorectal and 4T1 breast tumor models.[120] Although quantum dots are increasingly employed in biomedical research, it is imperative to consider their potential toxicities within the body. These toxic effects depend on various factors, including their size, charge, concentration, oxidative state, photolytic activity and, most importantly, the metal base used in their preparation. Overall, QDs are extremely useful nanomaterials for biomedical research; however, their various physicochemical parameters prior to biomedical application should be tuned in order to avoid toxic side effects.

3.3. Silica-Based Strategies for Effective Immunotherapy

While PTT has the promise of inducing ICD, photodynamic therapy works along the same line, although it requires photosensitizing agents and molecular oxygen to induce death. This is where silica-based strategies come in, where mesoporous silica nanoparticles (MSNs) have potentially favorable toxicity profiles, high loading efficacies, and act as efficient PDT vehicles for immunotherapy. A study performed by Xu et al.[121] shows how PET-guided PDT can be useful with biodegradable MSNs (bMSNs) with large pore sizes that are loaded with Ce6 photosensitizer and CpG-ODN (**Figure 15**a). This system was also tagged, when appropriate, with neoantigen peptides (MC38 Adpgk) that were readily cleaved in the acidic environment of the tumor. TEM imaging confirmed average diameters of 78 nm and exhibited highly porous structures (Figure 15b) with relatively large pore sizes ranging from 5 to 10 nm, larger than the 2–4 nm shown in conventional MSNs.[122] These bMSNs were able to simultaneously load CpG, Adpgk, and Ce6 (known as bMSN vaccine), all while still having the same chemical activity of free Ce6. This system shows favorable cell viability and only induces killing upon irradiation showing the targeted therapeutic capabilities of this system. Potential toxicity of the system was shown to be possibly mitigated through PET-tracking of ^{64}Cu-NOTA-Adpgk exhibiting increased concentrations of the bMSN system, in comparison to just Adpgk, in the tumor microenvironment within 25 h postinjection (Figure 15c). Not only was this system able to increase levels of CD40/80/86 on DCs, but it also elicited strong production of TNFα and IL12p70 proinflammatory cytokines. In vivo studies using a contralateral two-tumor model with MC38 colon carcinoma, consisting of one tumor inoculated on day 0 with another contralaterally on day 8 were conducted to see tumor growth progression during and prior to tumor establishment. Mice treated with the bMSN vaccine were shown to have significantly reduced tumor volumes, as well as increased levels of Adpgk-specific $CD8\alpha$ T cells both with and without tumor irradiation in both primary and contralateral tumors. IFNγ levels analyzed through ELISPOT show that the bMSN vaccine with laser irradiation produced significantly enhanced Adpgk $CD8\alpha$ T cell responses compared to the single component controls and

increased activation of intratumor $CD11c^+CD86^+$ DCs. Finally, this was tested in a highly aggressive B16F10 melanoma model with relevant neoantigens and show very similar results to the MC38 trail (Figure 15d,e), proving this is a novel platform that can make use of synergistic PDT therapy with immunotherapy to elicit a robust response across multiple tumor types.

Without the influence of photosensitizers, one study suggests that MSNs can be used for traditional nanomedicine with conjugated poly(2-vinylpyridine) (PVP) to create a pH-sensitive prerequisite for potential therapeutic applications.[123] The particles were synthesized through a previously reported MSN-NH_2 process,[124] which resulted in porous structures that can be polymerized with Boc-protected PVP. The unique aspect of this MSN-PVP system is its ability to have open and closed porous structures based on the solution it is incubated in, wherein water, the porous surface is "closed off" due to the interactions of PVP and water. The 550 nm structures were able to show high uptake into macrophages and DCs in a concentration-dependent manner and colocalized into the endosomal/lysosomal pathway, but not into adaptive immune cells like T and B cells. Annexin V/PI staining indicates that when incubated with primary immune cells, they are nontoxic over a wide range of concentrations and can these particles alone do not increase the concentrations of IL6, IL12p70, or IL1beta. Low levels of CD80 on primary DCs and monocytes were found, showing that this bare system will only create low immunological responses. Furthermore, this system was loaded with R848, a TLR7 agonist, and was able to be absorbed at lower pH, but was locked into the MSN at pH 7. Since the local environment in the endo/lysosomes is acidic, it should be released in that environment and was shown to induce activation of DCs with upregulation of CD80 and secretion of IL6, in addition to upregulation of CD69 on T and B lymphocytes showing that indirect stimulation of these cells through the MSN-PVP-R848 system can increase adaptive immune responses.

3.4. Hybrid Inorganic Systems

While both silica- and metal-based nanosystems have their distinct advantages, the combination of the two can act as ways to increase the loading of therapeutic agents. In contrast, they still have the capabilities to take advantage of PTT. One study[125] fabricated extra-large pore MSNs (XL-MSNs) that were decorated with nanoscale gold nanoparticles (AuNPs) (now Au@XL-MSN) in combination with high CpG (Au@XL-MSN-CpG) loading to deliver effective PTT. TEM and EDS analysis confirmed the conjugation of AuNPs onto the XL-MSN system, and thiolated CpG was able to stably bind to the Au and allowing for relatively high loading into the system. To increase colloidal stability and cell viability, XL-MSNs were further modified with thiolated PEG (Au@XL-MSN-CpG/PEG) and correlated to higher tolerance by 4T1 cancer cells in comparison to non-PEGylated systems. Even with PEGylation and further modification, AuNPs were stable able to undergo irradiation and heating potential at approximately 800 nm. BMDCs were incubated with the Au@XL-MSN-CpG/PEG that both CD86 and MHCII expressions were significantly increased even when compared to the effects of just CpG-ODN, followed by higher levels of pro-inflammatory TNFα and IL12, showing this system has enormous potential to act as a way to activate DCs. B16F10 tumors in vivo were treated with Au@XL-MSN-CpG/PEG and saw that this alone cause effective tumor regression, but was furthered through the use of 808 nm laser irradiation, which points to high heat destroying tumors can cause TAA release and downstream DC activation and adaptive immune responses.

Instead of using a traditional metal nanoparticle in conjunction with MSNs, one group[126] synthesized upconversion nanoparticles (UCNPs), converting NIR irradiation to visible light, which has high tissue penetration depth and coated them with mesoporous silica to increase the loading efficiency of photosensitizers to achieve better PDT. A separate recent review has discussed that when UCNPs are silica-coated, these pores allow for high-dose loading of drug or moiety payloads.[127] These large pored mesoporous-silica-coated Beta-$NaYF_4$:20%Yb,2%Er upconversion nanoparticles (UCMSs) were less than 100 nm and had large pores up to 30 nm. Encapsulation with the photosensitizer MC540 (UCMSs-MC540) exhibited relatively high loading at more than 12%. Coating with CT26 tumor fragments (TF) and model antigen, OVA, was still possible even with the loading of MC540 (UCMSs-MC540-TF or -OVA), and these combinations showed high and sustained release of antigens, which is beneficial since prolonged exposure to TAA can improve immune responses. The ability to generate ROS from the UCMSs-MC540 was confirmed to occur at 980 nm laser illumination. It was shown only to inhibit cell viability when irradiation occurred; otherwise, the system possessed low cytotoxicity and good biocompatibility. To test this system's immunoadjuvant potential, CT26 tumor-bearing mice were treated with UCMSs-MC540 with and without -OVA and/or laser irradiation, and splenocytes were extracted to look at T cell activity and cytokine profiles. This system ended up not only significantly increase IFNγ, TNFα, and IL4/12 secretion with the UCMS-MC540 group, it was improved significantly more with OVA loading and laser irradiation, leading to higher levels of $CD8^+$ and $CD4^+$ T cells, proving that combined protein and PDT treatment synergism can have huge potential for immunopotentiation. Finally, this was tested as a therapeutic vaccine in the same mouse model and the groups that provided the best survival and tumor regression results were the UCMSs-MC540-TF both with and without laser irradiation, showing this treatment can pave the way as another multifunctional delivery system for cancer vaccination. A study conducted by Yan et al. takes advantage of a similar system, lanthanide (Ln)-doped UCNPs.[128] This system takes advantage of previously discussed PDA and a Ln upconversion shell layer of $NaGdF_4$:Yb/Er, which has been shown to cause downshifting luminescence, increasing immunotherapeutic efficiency with NIR-based photothermal and photodynamic systems.[127] The PDA core is synthesized through self-polymerization in an alkaline solution and has very potent photothermal properties via strong absorption in the NIR spectrum. These particles were then coated with Ln-doped carbonate hydroxide through urea-mediated coprecipitation (PDA@$Ln(OH)CO_3$). Treatment with low concentrations of NaF/NH_4F solution yielded the now PDA@UCNPs that were capable of efficient PTT conversion and upconversion emission under 980 nm laser irradiation. To add a PDT element, Ce6 was introduced, which was loaded through mixing with multiarm PEG and the PDA@UCNPs (PDA@UCNP-PEG/Ce6). This system was shown to exhibit no apparent cytotoxicity when

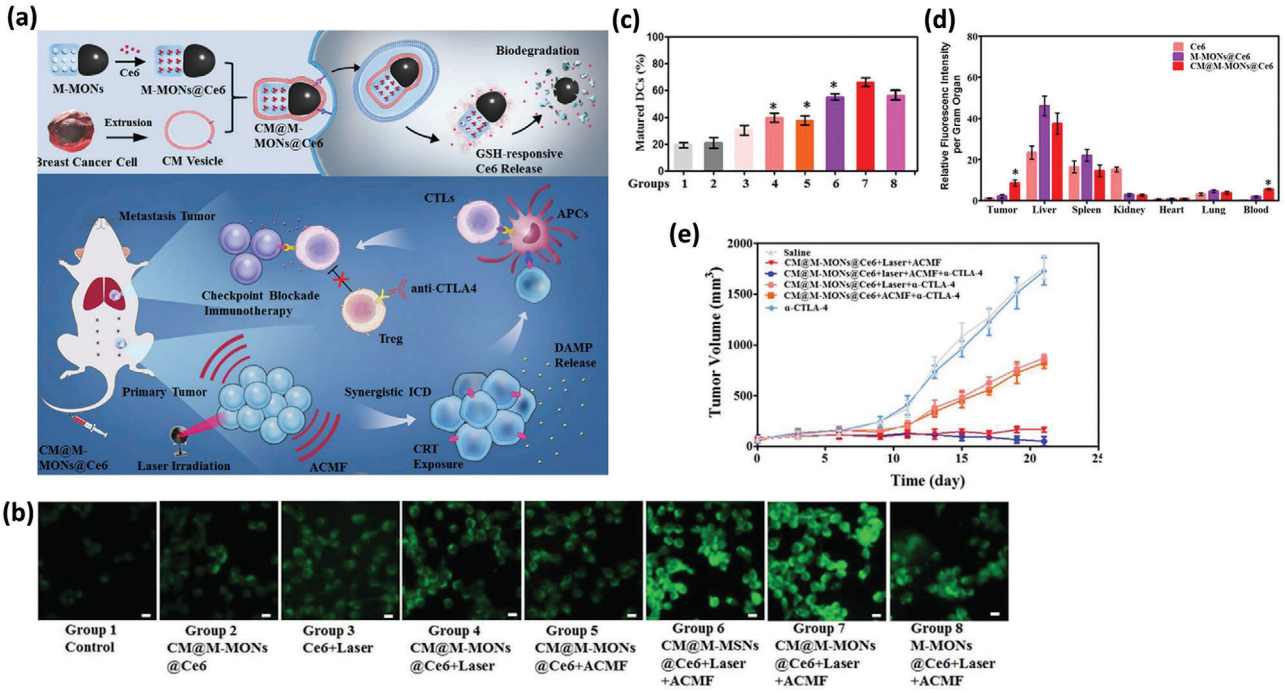

Figure 16. a) General schematic for CM@M-MONs@Ce6 mechanism. b) Intracellular reactive oxygen species, showing the highest release in Group 7. c) Increased levels of maturated DCs were found through M-MON-based systems exposed to laser+ACMF, with the highest being Group 7 (Group nomenclature is the same for b and c). d) Through the use of cancer cell membrane coating, increased accumulation in the tumor was observed. e) 4T1 membrane-coated CM@M-MONs@Ce6+ACMF+Laser exhibit efficacy in reducing tumor volume, which is furthered through the addition and incorporation of anti-CTLA4 immune checkpoint inhibition. Reproduced with permission.[30] Copyright 2019, Wiley-VCH GmbH.

exposed to 4T1 or RAW164.9 cells, in addition to a lack of generation ROS in cells in the absence of laser irradiation. This synergistic PDA@UCNP-PEG/Ce6 system with laser irradiation was shown to cause more tumor inhibition than the system without Ce6, additionally confirmed through TUNEL staining. Not only is tumor regression apparent, but increased DC maturation and CTLs along with decreased M2 macrophages show that this system can concurrently dampen immunosuppression and trigger a robust antitumor T cell response. Finally, this system was combined with ICB therapy using anti-PD-1, causing this treatment group to have a 77.8% survival rate (solid tumors), eradication of tumor invasion and metastasis (i.v. injections of cancer cells), and increased IFNγ^+ CTLs in the spleen and lymph nodes and spleen macrophages. This system shown can trigger a robust antitumor response with the maturation of DCs and activation of CD8$^+$ T cells, which is further through the addition of ICB using anti-PD-1.

Combining the themes that were just discussed, Janus, silica, and metal systems, one study[129] fabricated M-MONs@Ce6 nanobullets which are able to induce both PTT via magnetic hyperthermia and PDT on orthogonal sides of the system to elicit ICD (**Figure 16**a). These nanobullets were comprised of Fe_3O_4 spherical NPs attached to a disulfide-bridged mesoporous silica framework (M-MONs). The structures were determined to be 250 nm by 100 nm, and the silica portion was able to be degraded in simulated GSH solutions, which is found in higher levels in cancer cells, which can decrease oxidative stress in cells to reduce PDT efficacy. When exposed to an alternating current magnetic field (ACMF) for 20 min, they demonstrated stable magnetic-thermal performance. M-MON@Ce6, under light exposure, showed slightly less singlet oxygen (SO) production than free Ce6, but this could be resulting from the lack of Ce6 immediately available in the solution because of packing into the nanobullet. However, there was a reduction in relative intracellular GSH when incubated with M-MONs@Ce6, which could be due to the fact that disulfide bridges within the framework could consume GSH, resulting in more effecting PDT. To make the nanobullets exceptional at immune evasion, they were cloaked with cell-biomimetic vesicles (CMs) from MCF-7 breast cancer cells and coated with them to form CM@M-MON@Ce6, creating a thin layer surrounding the nanobullet and increasing stability in solution. This immune-evasion coating resulted in increased uptake and release of Ce6 into tumor cells and less in macrophages, which are postured to be due to the amounts of CD47 still present in the CM material on the nanosystem. When stimulated with light in MCF-7 cells, there was an increase in PDT-dependent killing compared to free Ce6 because of the nanobullets potential to reduce intracellular GSH. Alternatively, when stimulated with ACMF, there was a dose-dependent effect by magnetic hyperthermia, but there was no difference in cell viability when unstimulated. When combined ACMF and irradiation, ROS generation substantially increased compared to controls and induced the highest apoptotic rates in MCF-7 cells (Figure 16b). When exploring the impact on DCs, PDT and magnetic hyperthermia treatment with CM@M-MONs@Ce6 with ACMF and laser irradiation caused a significant amount of DC maturation (Figure 16c). Before in vivo efficacy studies, it was shown that there was an increase in tumor accumulation through this

cancer membrane coating (Figure 16d). In orthotopic MCF-7 nude mouse tumor models, ACMF was applied before laser irradiation because of the ACMF-induced hyperthermia caused by blood vessel damage resulting in tumor oxygenation. Tumor growth was slowed down when exposed to CM@M-MONs@Ce6 with either laser ACMF, but the combination of ACMF+laser exhibited the slowest tumor growth and the best tumor inhibition rates (Figure 16e). The results were repeated using 4T1-BALB/c models where instead of MCF-7 CM, it was 4T1 CM that was used to cloak the nanobullets. In addition to the different coating, anti-CTLA4 was combined to study the sensitizing of these tumors to ICB therapy. Correlating to previous results in the MCF-7 model, CM@M-MONs@Ce6+ACMF+laser show significant tumor growth delay and reduction in tumor weight, with the addition of anti-CTLA4 causing further decreases in tumor volume (Figure 16e). Metastatic nodules in the lungs of BALB/c mice were only slightly reduced, owing to the fact that local inhibition would occur from PDT plus magnetic hyperthermia. Proinflammatory IL6, TNFα and IFNγ were found in significantly higher quantities along with increased CTL recruitment to the primary tumor and ratio of $CD8^+$ to $CD4^+$ T cells, with a slight decrease in Tregs in the TME.

Silica and metal-based strategies will all have their benefits and caveats. It was shown here that the use of metal nanoparticles can be done in order to achieve a sort of adjuvancy in addition to, separately from PTT and PDT. Causing the shedding of TAA allows for a more robust immune response and is increased through the use of traditional chemotherapeutic drugs that can increase immunotherapeutic potential. Silica-based MSN systems increase the loading efficacy of several systems and can be used in addition to photosensitizing agents or metal to double down on the potential to cause cancer cell death. With all these in mind, it is still worth noting that traditional toxicities with these systems should not be overlooked, and careful design of these systems should be taken. Off-target immune cell activation is possible and can cause severe negative side effects, hampering the potential impact of these systems in the TME. There is hope that with these hybrid systems, combining both metal and silica, even potentially Janus type mechanism, can elicit the best of both worlds, creating advantageous systems that can allow for lower systemic toxicity and increase tumor regression possibilities.

4. Multispecific/Functional Antibodies: Powerful Bridges for Enhancement of Immune Cell Activation

Antibodies are biological materials that are synthesized in our bodies to overcome potential pathogens and are synthesized by our B-cells. They can find a home here with their high specificities, ease of conjugation to other materials, and can even be synthesized in an adaptive immune response or produced by bacteria.[130] Monoclonal antibodies have seen success in clinics through the use of blocking surface antigens on cancer cells, which reduce their capabilities to spread and induce clearance through the immune system targeting the Fc regions of the antibodies. While effective, this strategy has severe limitations, such as nonspecificity and targeting to healthy tissues, cytokine release syndrome, autoimmunity, and inadequate pharmacokinetic properties.[131] Recently, advances in the areas of bispecific- and multifunctional antibodies have shown promise by specifically targeting immune cells like macrophages, NK cells, and T cells and bringing them in close proximity to cancer cells. This allows for the immune-mediated attack to happen specifically to the cancer cells and decreasing the likelihood of attacking healthy tissues. Bispecific antibodies work through redirecting effector cells (mostly immune cells) towards target cells (cancer cells) in order to precisely attack those of interest (**Figure 17**, **Table 1**), an aspect that monospecific antibodies lack. With over 20 possible engineered formats, the possibilities to elicit specific interactions between immune and target cells are vast.[132] Not only are the interaction-capabilities extremely attractive, but it has been shown that these platforms have the ability to increase biological activity and have favorable pharmacokinetic properties.

One successful example is blinatumomab, the first FDA-approved bispecific antibody that targets CD19 surface markers on B cells in acute lymphoblastic leukemia to be killed through T-cell mediated cytotoxicity. The structure of this bispecific antibody contains the variable regions of anti-CD3 and anti-CD19 antibodies and conjugated via a G_4S linker. Although successful, there is still a growing need to be able to use this technology and evolve it to be practical in a variety of both solid tumors and hematologic cancers, each having their own challenges. In order to discuss the broad field of multispecific antibodies, the following sections will be broken into which effector cells are being used to elicit powerful antitumor immune responses. Since various immune cells have multiple surface targets, we will proceed with understanding why those targets were used and their

Table 1. Summary of multispecific and multifunctional antibodies, including their immune cell and cancer cell targets.

Cell type	Name	Immune cell target	Cancer cell target	Refs.
T cells	JN67571244	CD3	CD33	[133]
	MCLA-117	CD3	CLEC12A	[134]
	REGN4018	CD3	MUC16	[135]
	ERY974	CD3	GPC3	[136]
	PRS343	4-1BB	HER2	[137]
	LiTEs	CD3	EGFR	[138]
	COBRA	CD3	EGFR	[139]
	p95HER2-TCB	CD3	p95HER2	[140]
	ROR1xCD3	CD3	ROR1	[141]
NK cells	MesobsFabs	CD16	MSLN	[142]
	BiSS	CD16a	CEA	[143]
	BiSdAb	CD16	VEGFR2	[144]
	NKCE	CD16 and NKp46	CD19, CD20, or EGFR	[145]
	aTriFlex	CD16a	BCMA and CD200	[146]
	1615EpCAM	CD16 (IL-15 Linker)	EpCAM	[147]
	1 615 133	CD16 (IL-15 Linker)	CD133	[148]
Macrophages	RTX-CD47	CD47	CD20	[149]
	CD47/TAA biAb	CD47	MSLN or CD19	[150]
	Pan-SIRPαV-Fc	CD47	EGFR	[151]
	CD89-CD20	CD89	CD20	[152]
	A2V	Ang-2	VEGF	[153]

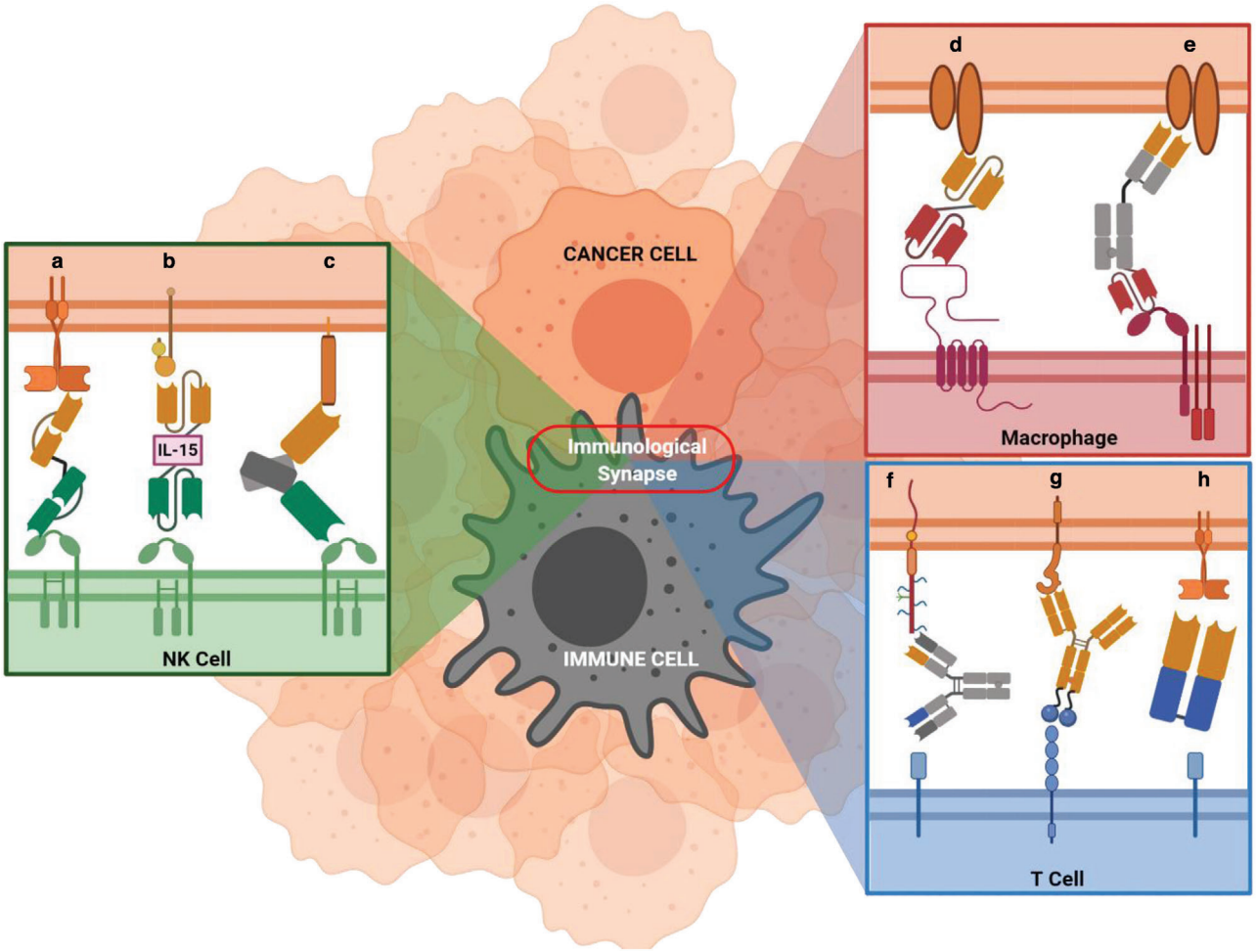

Figure 17. Bispecific and multispecific antibodies have the ability to increase immune cell activity through close-proximity interactions with a–c) NK cells, d,e) macrophages and f–h) T cells. All portions a–h show the different form factors that these antibodies can take on which allow for a variety of methods and strategies to be utilized in targeting receptors or ligands on both immune cells and cancer cells. The references for each of the following multispecific antibody forms are as follows: a[144], b[147], c [142], d[149], e[152], f [135], g[137], and h [139].

final impact on cancer cell-mediated targeting and killing via flexible, robust, and innovative multispecific antibody systems.

4.1. T Cells

4.1.1. Silenced/Mutated Fc-Based Bispecific Antibodies

One of the main side effects of monoclonal antibody (mAb) or bispecific antibody (BsAb) therapies that make them wary of being used is cytokine storm, which can wreak havoc on the body because of a lack of targeting. This occurs because of functional Fc regions on mAbs or BsAbs that can cause FcγR interactions, nonspecifically activating NK cells in circulation before reaching the tumor. To avoid this, the silencing of the Fc region of the antibodies can be done by using either Fab fragments, heterodimerization, or mutations that cause silence. One study made use of this through the synthesis of an anti-CD3 x anti-CD33 duobody (JN67571244) antibody format.[133] With CD33 being restricted to hematopoietic cell lineages and expressed in blasts and leukemic cells in 85–90% of patients, this targeted BsAb was used to target the C2-domain of CD33 to induce T cell-mediated killing. At a range of concentrations, JN67571244 was able to elicit T-cell mediated toxicity in vitro against primary acute myeloid leukemia (AML) cells and induce potent CD25 expression/activation, more so than the nullxCD3 control system. In addition to activation and cytotoxicity, levels of IFNγ, TNFα, IL2, and IL8 were increased in T-cell-CD33$^+$ cocultures. In vivo, NSG mice given KG-1 tumors saw approximately 90% tumor growth inhibition using 0.5–1 mg kg^{-1} treatments of JN67571244 with 6 and 7 complete responses by day 55, respectively. Using a MOLM-13-Luc to disseminate cancer cells, 0.05 mg kg^{-1} of antibody dose exhibited maximal tumor growth inhibition by 100% and increased lifespan by 72%. This was translated to cynomolgus monkeys, showing dose-dependent increases in IFNγ, IL-2,-6, and -10 two hours postdose, with IL-10 not returning to normal basal levels. This had led to sustained CD33$^+$ granulocyte and monocyte reduction and was well tolerated in these models from 0.01 to 30 mg kg^{-1}. Unfortunately, there are associated toxicities with CD33-targeted treatments, such as targeting towards non-cancerous

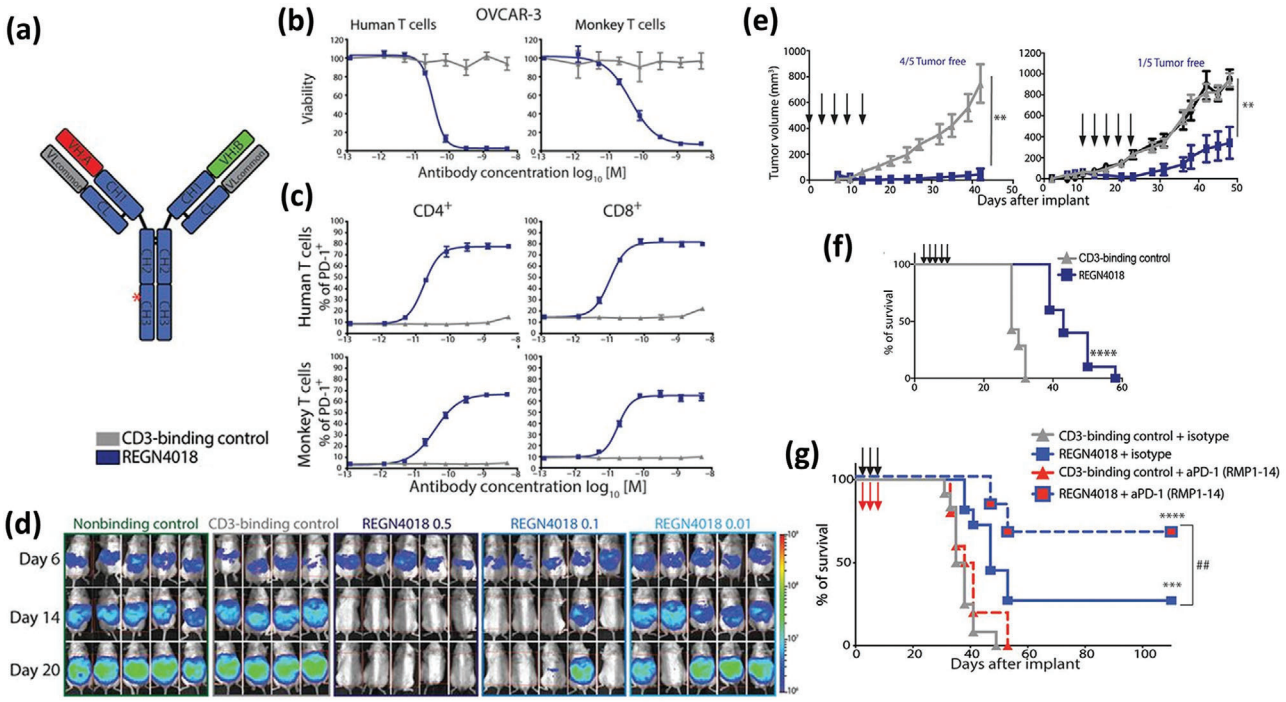

Figure 18. a) Format of the REGN4018 anti-CD3/MUC16 bispecific antibody (differing specificities in red and green, red asterisk is for an Fc mutation for purification). b) REGN4018 induces increased cytotoxic capabilities and c) CD4/8 expression in both human and monkey T cells. d) Bioluminescence imaging showing the impact of varying doses of REGN4018 in comparison to CD3- and nonbinding controls. e) Tumor volume reduction occurs due to increase T cell cytotoxicity in the tumor microenvironment when given treatments at the same time of tumor inoculation (left) and 10 d postinoculation (right). f) Effects of REGN4018 in ascites model, exhibiting significantly increased survival. g) Combination anti-PD1/REGN4018 therapy further increases immunotherapeutic efficacy leading to increased survival. Reproduced with permission.[135] Copyright 2019, American Association for the Advancement of Science (AAAS).

cells of both myeloid and lymphoid origin is a possibility.[154] To address this, van Loo et al. used the C-type lectin domain family 12 member A (CLEC12A, CD371), a myeloid differentiation antigen expressed on leukemic cells in 90–95% of patients with de novo or relapsed AML.[134] This is found on leukemic stem cells (LSCs) but not on hematopoietic stem cells (HSCs) like CD33. Using a full-length IgG1-format human BsAb with a silenced Fc region, MCLA-117 was used to bind specifically to CLEC12A$^+$ HL60 cells and CD3$^+$ Jurkat E6.1 cells to confirm specificity. With the engineered-silenced Fc region, pharmacokinetic analysis in C57BL/6J mice showed a $t_{1/2}$ of 9.4 d, indicating that the lack of Fc-binding capabilities will discourage premature clearance and increase surveillance in the body. Normal peripheral blood was used to show binding to CD3$^+$ CD4$^+$ and CD3$^+$CD8$^+$ T cells and CD3$^+$CD56$^+$ NK-T cells. In cocultures containing healthy donor resting T-cells and HL60 cells, the MCLA-117 BsAb was able to upregulate CD69 and CD25 expression on CD4 and CD8 T cells as well as show more effective and specific lysis of HL60 cells. Incubation of MCLA-117 with cocultured monocytes and autologous T cells efficiently induced CLEC12A-specific proliferation of CD4 and CD8 T cells even after 5 d, as well as induced the release of IL1beta, TNFa, IFNg, and IL-6 and -12.

In addition to hematologic malignancies, silenced or mutated Fc regions have also shown positive impacts in solid tumors by allowing for increased circulation time in the body.[155] Using the BsAb, REGN4018,[135] full length-MUC16 (CA-125) was identified as a target for ovarian cancers because of the high expression that lacks in healthy epithelia and has a short form, soluble CA-125 (sCA-125) as a potential targeting obstacle to overcome. REGN4018 consists of a typical IgG format; however, the V_h of each arm consisted of either anti-MUC16 or anti-CD3 targeting capabilities (**Figure 18**a), which show binding in both human and cynomolgus T-cells in PBMCs. Using CFPAC-1 and OVCAR3 cells to represent MUC16^{low} and MUC16^{high}, respectively, binding occurred in both but not against the ID8-VEGF parental negative control cells. In the presence of human OVCAR3 and monkey MUC16-expressing ID8-VEGF cells, NFAT-luc reporter T cells lines for human and cynomolgus both induce T-cell mediated killing (Figure 18b), followed by increased PD1 expression on CD4 and CD8 T cells in response to CD3 stimulation (Figure 18c), and human T-cells were shown to increase IFNγ, IL-10 and -12. Even using the antigen sink replication due to sCA-125, binding of REGN4018 to OVCAR3 cells is not impacted but the use of the control anti-MUC16 antibody did inhibit binding to the target antigen. Using OVCAR-3 xenografted mice and REGN4018, bioluminescence imaging on mice with human T cells exhibits significantly decreased tumor burdens at varying levels in comparison to CD3- and nonbinding controls (Figure 18d). Further, in a syngeneic ID8-VEGF/huMUC16delta model that treatments coincided with tumor inoculation or 10 d postinoculation still resulted in significantly increased tumor regression (Figure 18e). Even in an ascites model, REGN4018 exhibited markedly longer survival in comparison to control treated mice (Figure 18f). Not only was REGN4018 effective on

its own but showed increased immunotherapeutic efficacy and survival when coadministered with anti-PD1 antibody leading to four out of nine mice reaching complete tumor clearance (Figure 18g). Assessing the safety profiles in cynomolgus monkeys gave way to acceptable tolerance with 5 weeks of treatment and 12 weeks of treatment-free assessment., results in REGN4018 $t_{1/2}$ of approximately 10 d, no macroscopic changes in organs, and no depletion of circulating T cells even with postdosage changes in cytokine levels.

A final example of using Fc-silenced BsAb formats was done using ERY974, which targets CD3 and glypican-3 (GPC3), which is a highly tumor-specific antigen, unlike other methods that use EPCAM-targeting, and is involved in a multitude of pathways in cancer like Wnt and fibroblast growth factor pathways that control cell growth and apoptosis.[136] Various types of affinities were designed for each arm of ERY974 and assessed with T-cell dependent cellular cytotoxicity (TDCC). Using $GPC3^-$ SK-HEP-1 cells and $GPC3^+$ SK-HEP-1/hGPC3 transfected cells, PBMCs were cocultured and incubated with ERY974, eliciting 25% TDCC against the $GPC3^+$ cells compared to 5% with the $GPC3^-$ cells. There were marked increases in CD25 and CD69 activation markers in $CD3^+$ cells. In vitro testing with patient donor T cells with ERY974 also induced clear TDCC in both CD4 and CD8 T cells, in addition to the proliferation of both subsets. This system was successful in NOD-SCID mice using $GPC3^+$ xenografted tumors and human T cells and in immunocompetent CD3 transgenic mice that were xenografted with highly immunogenic Hepal-6/hGPC transfected tumors. These tumors showed marked regression ($P = 0.0068$), with most mice coming to complete remission, which was proven to be from the BsAb through increased degrees of T-cell infiltration, as shown through histopathology and gene expression. Translating this study to cynomolgus monkeys, a half-life of ERY974 was observed to be 5.1 d with main cytokine increases from IL-6 and increased in a dose-dependent manner. No apparent cytotoxic effects were evident using single-dose treatments, and cytokine release were manageable through premedication with corticosteroids without impacting antitumor efficacy.

Silenced-Fc-based systems allow for more focal targeting of T-cells without high-interference from NK-cells and other Fc-engaging cells. In comparison to therapies with functional Fc regions, these systems stand a better chance at reducing systemic toxicities while having a high affinity for target cells, whether in blood or solid tumors.

4.1.2. Bispecificity via Variable Domain or Protein Linking

There are also other strategies for T-cell BsAbs, such as linking proteins to the antibodies themselves or conjugating variable domains together. Conjugation of antibodies through anticalin proteins can be used to add additional binding sites to antibodies. Hinner et al. designed an anti-HER2×4-1BB/CD137 bispecific antibody–anticalin fusion (PRS343) that allows for binding to T-cells through the 4-1BB anticalin that is fused to a variant of trastuzumab that can target HER2.[137] These antibodies are produced through phage display and have been shown to bind to 4-1BB without the competition of binding 4-1BBL, and also have an Fc region that is silenced to reduce NK-cell participation through antibody-dependent cellular cytotoxicity (ADCC). Using NFkB-Luc2/4-1BB Jurkat T cells cocultured with $HER2^+$ NCI-N87 tumor cells and PRS343 leads to a 20-fold increase of NFkB luciferase reporter activity, showing an EC_{50} of 50 pmol L^{-1} that can effectively activate 4-1BB pathway signaling only in the presence of HER2 overexpressing cells. In comparison to cells with basal levels of HER2, PRS343, when used with T cells in the presence of HER2-overexpressing cells, caused increases of IL2, which correlated to increased T cell activation. When this was done in excess trastuzumab, T cell activation and IL-2 levels were abolished, highlighting the dependence of this action on the simultaneous binding of HER2 and 4-1BB. In vitro cytokine release assays, when done side-by-side with OKT3 anti-CD3 antibody as a control, do not induce a significant increase in cytokines over the background when incubated with PBMCs. Pharmacokinetics in CD1 mice and cynomolgus monkeys using 10 mg kg^{-1} injections or 60 min 3 mg kg^{-1} infusions, respectively, show half-lives of greater than 14 d in mice and about 4 d in cynomolgus monkeys. Using immunocompromised mice xenograft with $HER2^+$ SK-OV-3 tumors cells and engrafted with human PBMCs, dose-dependent antitumor efficacy was observed from 0.2 to 5 mg kg^{-1}, maximizing at 10 mg kg^{-1}. Peripheral blood samples show no sign of increased T cell activity, but tumors exhibited marked increases of $CD8^+CD45^+$ cells. This platform can overcome the issue of targeting and locally killing tumor cells with HER2 overexpression, and by using two high affinity and specificity targeting proteins, it is able to overcome toxicities associated with HER2 cancer and immunotherapies.

Another strategy for using linked bispecific antibody portions was done through in vivo generation of bispecific tandem V_{HH}-scFv proteins that consisted of fused anti-human EGFR Egal V_{HH} and anti-human OKT3 CD3 scFvs separated through a G_4S linker (EGFRxCD3 LiTEs).[138] These were produced by transfected HEK 293 cells with binding specifically to EGFR-expressing HeLa cells and CD3-expressing human Jurkat T cells, as evident through flow cytometric analysis. When incubated with coculture of HeLa cells and human peripheral T cells, dose-dependent expression of CD69 activation was found, but not when conducted with $EGFR^-$ 3T3 cells. Using transwell cultures of the transfected HEK 293 cells on the bottom of the well with HeLa and hPBMCs with an effector cell-to-target cell (E:T) ratio of 5:1, LiTEs secreted by the HEK 293 cells were able to redirect T cells to kill EGFR-expressing tumor cells with approximately nine times higher killing. While boasting high efficacy and killing potential, this platform was successful by incorporating a CD3 portion that only requires monovalent binding, which allows for disengagement to T-cells. This, in addition, to be able to produce these BsAbs in vivo allows for many of these multiple-cancer killing T-cell engagers to have very potent antitumor immunotherapeutic potential.

There are many stimuli that can cause the release of drugs or degradation of polymers when delivering to the TME, but this can also be done with protease-activated bispecific T-cell engagers (BiTEs) for treating solid tumors. A hemi-COBRA and full-length COBRA structure have been synthesized that allows for an inactive version of the bispecific to only become active in the presence of specific proteolytic stimuli.[139] MMP2/9 is highly expressed in solid tumors and is regulated in healthy tissues, allowing for the active prodrug to target these areas. Hemi-COBRAs were

designed with two domains of scFvs for CD3ϵ and EGFR. Briefly, an anti-EGFR single domain antibody (sdAb) was tethered to an active anti-CD3ϵ variable domain and attached to an MMP9/15 linker. Across this linker is an inactive anti-CD3ϵ variable domain and connected to an anti-human serum albumin sdAb to increase serum half-life. Using cocultures of EGFR-expressing HT29 and CD3-expressing Jurkat cells, it was shown that binding to CD3-expressing Jurkats only occurred in the presence of human MMP9 to create the active bispecific. In addition to CD3 binding, TDCC occurred was shown using human primary T cells in the presence of MMP9 and showed 500-fold more potency than that of the uncleaved or noncleavable hemi-COBRA in both LoVo and HT29 cell lines. Moving onto the full-length COBRA, cleavage with MMP9 would cause two active EGFR and two active CD3 binding sites that would not be expressed otherwise. The cleaved, active dimer COBRA had affinities for CD3 and EGFR of 58×10^{-12} M and 54×10^{-12} M, respectively, in comparison to the hemi-COBRAS of 330×10^{-12} M and 3×10^{-9} M, which translated to increasingly potent TDCC because of the active dimer on the surface of target cells. In NSG mice being administered every three days and seven doses, control treatments did not show tumor growth inhibition, whereas the COBRA eliminated the tumor after 21 d. This strategy shows how targeting to TME-specific stimuli can cause an increasingly potent targeting BsAb and will further reduce systemic toxicity concerns associated with similar platforms.

4.1.3. Targeting via Surface Marker Fragments/Epitopes

Another opportunity to target TAAs can be taken advantage of by targeting epitopes or even fragments of the surface markers that solid tumors can portray. One study has shown that targeting a C-terminus fragment of HER2 p95HER2, which is found in 40% of HER2$^+$ cells, can be an alternative since HER2-expression can still be found on some healthy cells types.[140] This p95HER2-TCB consists of a two-armed IgG1 that binds monovalently to CD3epsilon and bivalently to p95HER2 while having a silenced Fc-region. CD8 and CD25 expression were found only in coculture conditions, in addition to increased levels of IL-2, granzyme B, and IFNγ. Immunocompromised mice with MCF7 xenografts that express p95HER2 were engrafted with peripheral blood mononuclear cells (PBMCs) from different healthy donors exhibit varying degrees of efficacy; however, they still had potent antitumor effects. Anticytokeratin staining shows a decrease in tumor cells, which indicates that the tumor volume overestimates the tumor burden in the p95HER2-TCB treated mice. Since this method shows positive results in p95HER2 expressing cancers, it was conducted in breast cancer tumors, which were shown to have varying levels of p95HER2 expression. Using patient-matched PBMCs with the same patient-derived xenograft (PDX), there was increased TCR activation corresponding to higher p95HER2-ness as found evident with an NFAT reporter Jurkat cell line. By targeting a portion of the TAA, HER2, it is possible to increase specificity to target cancer cells while decreasing the potential to impact healthy cells and cause systemic toxicity.

In addition to fragments, epitopes of surface markers have the potential as potent targets. A ROR1xCD3 BsAb was investigated to target a ROR1 epitope, where ROR1 is uniformly expressed on the surface of malignant B cells in chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), but not on healthy B cells.[141] This antibody format contained a glycosylated and heterodimeric scFv-Fc format designed to decrease Fc interactions and extend circulation while targeting the kringle (Kr) proximal domain of ROR1 and human CD3. Through a panel of ROR1-specific binding domains, anti-human CD3 functional was combined and validation of binding shows specificity to K562/ROR1 and MCL JeKo-1 cells that ectopically and endogenously, respectively, express ROR1 and Jurkat T cells that express CD3, but not to parental K562 cells. Primary T cells isolated from healthy donors saw approximately 50% crosslinking with the ROR1xCD3 BsAb between T cells and K562/ROR1 cells compared to negative control antibodies that saw around 10% crosslinking. At E:T 10:1 at a range of concentrations, K562/ROR1 cells were killed in the presence of ROR1xCD3 BsAbs, but not K562 cells, which show that this is ROR1 dependent T cell killing mechanism with an EC_{50} value of 22 ng mL^{-1}. The transfected K562/ROR1 cells saw much higher induced activation of T cells in the presence of ROR1xCD3 than in negative controls, and upregulation of CD69 was found while incubating with 1 µg mL^{-1} over 16 h. In vivo studies using systemic human MCL JeKo-1 cells transfected with firefly luciferase (JeKo-1/ffluc) were xenografted into NSG mice and engrafted with human T cells. Bioluminescence observations show significant tumor regression and eradication starting on day 14 after one dose of T cells and 2 of ROR1xCD3 BsAbs. Pharmacokinetic results show that the half-life of this construct in mice approximately 6.5 d. Both of these studies show the potential for targeting not only main tumor-associated antigens but also portions that can increase specificity, which can decrease systemic effects and discourage cytokine storm from occurring. This is another example of how targeting specific portions of a TAA can allow for alternative strategies to induce T-cell mediated killing of cancer cells.

4.2. NK Cells

NK cells are part of the innate immune system that has the potential to kill target cells upon activation of FcγIII receptors, as well as families of receptors like NCRs[156] and the NKG2 C-lectin family.[157] Receptors and microenvironmental stress factors and cytokines can cause potent activation of NK cells while not relying on priming to a cytotoxic state first,[158] which is why they are an interesting alternative for multispecific antibody targeting to T cells. Here we will discuss the impact that bispecific and trispecific antibodies can have on multiple NK cell strategies such as interacting or inhibiting circulating cytokines, binding to TAA, and even interacting with other immune cells to increase immune cell activation and coordination.

4.2.1. TAA and Cytokine Receptor Targeting

Bispecific antibodies have mostly been used in binding to target cells where the killing capacity of T or NK cells can be used directly on cancer cells of interest. A recent study by Del Bano et al.[142] discusses the fabrication and impact of targeting triple-negative breast cancer (TNBC) through mesothelin (MSLN), a

membrane glycoprotein found overexpressed in aggressive tumors. They designed a bispecific antibody targeting MSLN and FcgRIII, or CD16, one of the most potent activating receptors expressed on NK cells. These MesobsFabs used a previously described[159] single domain-based antibody Fab format using anti-MSLN and anti-CD16 nanobodies. Using fluorescently labeled unstimulated human NK cells and HCC1806 spheroids, significant recruitment of NK cells was shown into the core of the spheroid from the perimeter. To understand the impact of ADCC, both monolayers and spheroids of HCC1806 and MDA-MB-231 cells were co-cultured with the same NK cells in the presence of MesobsFabs. Both cell monolayers saw cytotoxic effects in co-culture, with MDA-MB-231 showing lesser because of the lower expression of MSLN. When tested in 3D tumor spheroids, it was demonstrated that IFNγ expression was significantly higher in these models than in 2D monolayers, and TNFα was significantly higher in both cell lines, both 2D and 3D, however mostly in 10:1 E:T ratios. PBMC-humanized NSG mice were given orthotopic MDA-MB-231 and HCC1806 tumors, and both models sustained very significant tumor regression where HCC1806 tumors saw tumor drop off past day 19 and MDA-MB-231 saw decreasing growth past day 44.

Another single domain antibody was designed[143] to target carcinoembryonic antigen (CEA) derived from natural camel heavy-chain only antibodies (HCAbs) that do not consist of any light chains and do not have a CH1 domain. CEA is expressed in GI, pancreatic, lung, and breast cancers and not in healthy tissues,[160] making this a widely applicable target for cancer immunotherapy. Here a BiSS (bispecific antibody with a single domain, single-domain antibodies) antibody was constructed by targeting CD16 and CEA through expression and produced in *Escherichia coli*. BiSS was found to be specific to CEA-positive HT29 and LS174T cancer cells and caused significant cytotoxicity in NK cell cocultures even at 10^{-2} and $10^{-3} \times 10^{-9}$ M, respectively. In vivo studies with LS174T NOD-SCID mouse models were performed with transplanted LS174T with or without isolated human PBMCs, in which rapid tumor growth was observed; however, only the group treated with BiSS was able to cause significant tumor growth inhibition over 38 days. Another study focused on CEA-targeting[161] created a CD16a VHH or nanobody. This CD16a VHH was linked to an anti-CEA VHH through a $(G_5S)_3$ linker (anti-CEA-CD16a) and still exhibited high specificity towards their target ligands while only killing LS174T cells in the presence of NK cells. In vivo, antitumor activity in NOD/SCID mice transplanted with LS174T cells and isolated human PBMCs was observed, exhibiting minimal tumor growth only in conjunction with PBMCs and anti-CEA-CD16a treatment. Using TAA to target NK-directed cytotoxicity has been shown to impact tumor growth inhibition, which shows promise as an alternative to T-cell mediated bispecific antibodies.

In addition to TAA, cytokine receptor inhibition has exhibited the capabilities to inhibit tumor progression. By inhibiting cytokine receptor activity, the chances of activation of downstream signaling can be decreased, which causes increased tumor-specific activity while providing a useful targeting modality in the TME. One study synthesized bispecific single domain antibodies (BiSdAbs) through expression in *E. coli* that consisted of an anti-VEGFR2 and an anti-CD16a domain to promote an angiogenic blockade.[144] Using HUVECs as VEGFR2 positive cells, both the VEGFR2 single domain antibodies (SdAb) and BiSdAb had cytotoxic potential on these cells through a CCK-8 assay, as well as inhibited the ability for cell migration and capillary tube formation to occur, showing potential antiangiogenic properties. When examining the impact of NK cells on VEGFR2$^+$ BEL-7402 cells, human NK cell cocultures were incubated with either parental SdAbs or BiSdAbs, and relatively higher cytotoxicity was exhibited, pointing in the direction that NK cells in combination with VEGFR2 inhibition can cause a decrease in proliferation in conjunction with anti-angiogenic properties.

4.2.2. Trispecific Antibodies for Increased Targeting and Immune Cell Activation

Not only are bifunctional antibodies important for eliciting immune responses, but trispecific have also found a home because of their ability to improve one-to-one interactions with cancer cells as well as create more functional targeting capabilities. One study[145] works with a three-pronged approach where the three functionalities include TAA targeting of different cancer types with NK cell targeting of CD16 through an Fc fragment and NKp46, which is found in high expression in tumors and has been reported to be linked to the downregulation of other activating receptors like NKp44 and NKG2D in multiple cancers. Triggering of NKp46 mAb-mediated crosslinking has been shown to cause cytokine release in NK-cells in addition to cytotoxic activity. This group reports the synthesis of bi- and trifunctional NK-cell engager (NKCE) that consists of mAb fragments that target NKp46 in conjunction with a TAA and Fc fragment to induce ADCC.

One group[146] fabricated a trispecific antibody that uses a diabody motif of CD16a to have two orthogonal interactions with CD268 (BCMA) and CD200, which are multiple myeloma-expressed target antigens. This design was based on previous studies with AFM13.[162] This aTriFlex format was used to elucidate NK cell activity in challenging to target solid tumors. The C16a diabody core showed similar specificity and was comparable to its scFv parental components but showed weak binding in single positive BCMA$^+$ and CD200$^+$ cells, increasing the importance for dual targeting in a single cell. Dual-targeting experiments in BCMA$^+$/CD200$^+$ cells show increased NK cell-mediated cytotoxicity, CD69 expression, and mean higher IFNγ secretion. This diabody-focused construction shows promise for targeting in double-positive targeting cells in scenarios where the single-antigen binding is potentially not strong enough to elicit an immunological reaction. Finally, the Vallera lab has since inserted cytokines into the physical construction of multispecific antibodies to enhance ADCC and activation of NK cells[147] (**Figure 19**). Previously, bispecific NK-cell-engagers (BiKE) were designed[163] and were shown to be effective in eliciting antibody-dependent cell-mediated cytotoxicity. Through the insertion of an IL-15 linker between two scFvs for EpCAM and CD16, trispecific NK-cell engagers (TriKE) was shown to combine ADCC capabilities and mediation of NK cell expansion. Further, there was no compromise in binding to EpCAM$^+$ cells (Figure 19a), and when targeting HT-29 EpCAM$^+$ cells, there was a much higher cytotoxic activity of TriKE with NK cell cocultures at different levels of enriched NK cells and effector: target ratios (Figure 19b). CD107a

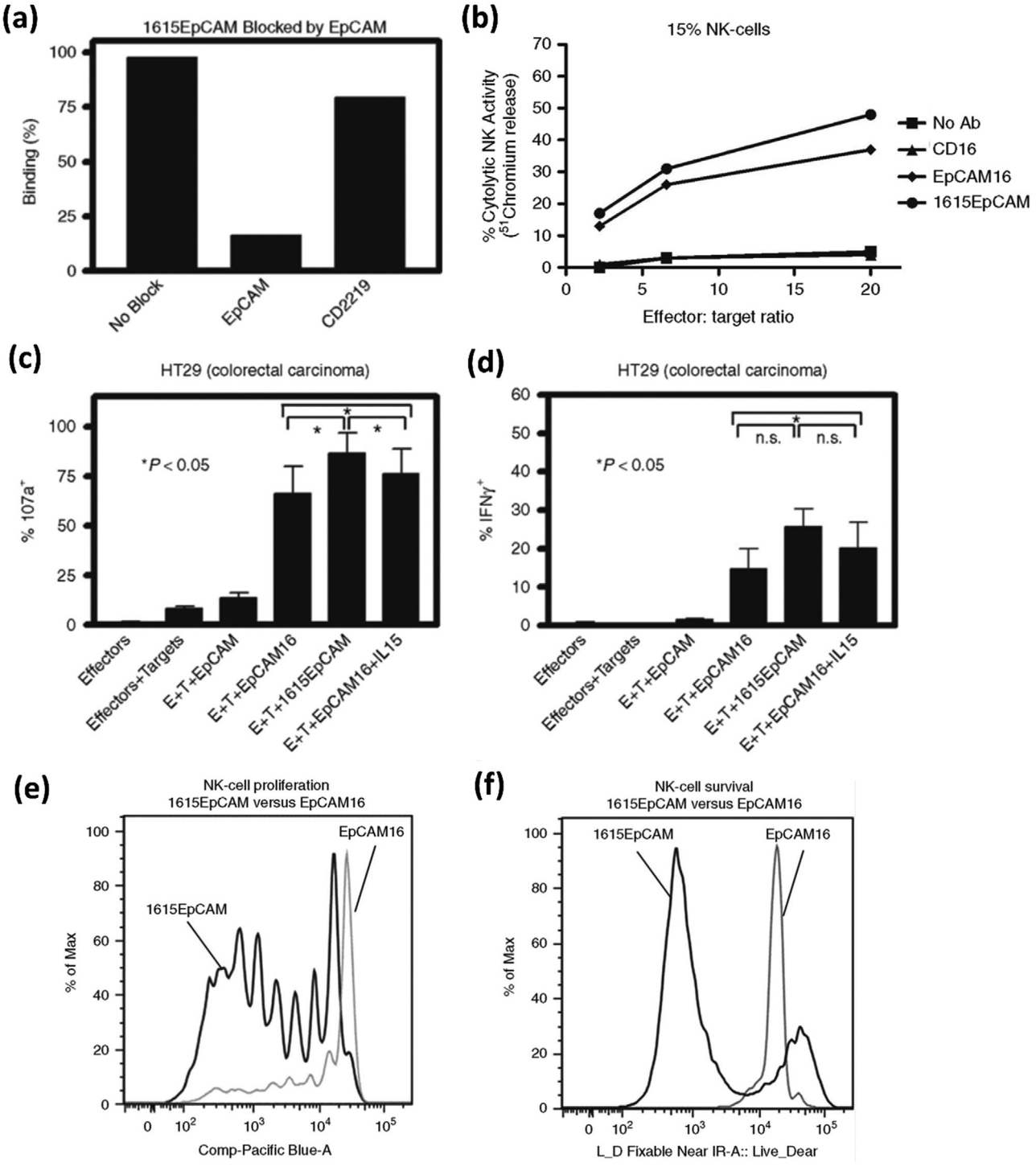

Figure 19. a) 1615EpCAM trifunctional antibodies have the capabilities to work only in the presence of EpCAM, shown that blocking with EpCAM causes a decrease in binding percentage. b) Cocultures of NK-cells with cancer cells exhibit increased amounts of cytotoxic activity in the presence of TriKEs. c) Increased NK cell activation is exhibited through higher levels of CD107a and d) IFNγ. e) TriKEs increased the NK cell proliferation as well as f) survival in comparison to EpCAM16 BiKE controls. Reprduced with permission.[147] Copyright 2016, Elsevier.

and IFNγ expression was analyzed in HT-29-NK cocultures and it was shown that TriKE was able to increase both of those levels significantly higher than even BiKE+IL15 (Figure 19c,d), indicating that IL-15 integration into the TriKE platform potently increases NK activity. Across multiple EpCAM$^+$ cancers, TriKE outperformed various controls, including BiKE) in terms of NK-cell degranulation signified by CD107a, as well as significantly higher levels of IFNγ, without inducing toxic IL-6 secretion. Since NK cells undergo more robust proliferation, human PBMCs were isolated, and TriKE could induce proliferation and survival (Figure 19e,f) to the same extent as those incubated with IL-15. These results exemplify TriKE's potential to not only activate anticancer NK-cell activity but induce robust proliferation. This work was continued in another study[148] but focused on CD133$^+$ cancer stem cells instead of EpCAM, which are found in multiple tumor types and can cause an issue with the management of cancer treatment. The new TriKE in this study, similar to the previous one proving the advantageous use of IL-15 as a linking entity, shows markedly increased IFNγ and CD107a expression, high cytotoxic activity when human PBMCs containing NK cells were cocultured in high effector to target ratios with CD133$^+$ Caco-2 cells, and expansive capabilities of NK cells.

4.3. Macrophages

While T cells are effective targets of immune activation because of their ability for robust activation and inflammatory properties, several studies have shown that forcible activation of T cells through BsAbs can trigger cytolytic cascades inducing cytokine release syndrome and create unwanted inflammatory side effects.[164] Because of possible systemic toxicities, exploring other immune cell options is becoming a growing priority of the multispecific antibody field. One cell of importance is the macrophage, which in the case of solid tumors, can comprise nearly 50% of tumor mass.[165] Their phagocytic capabilities can be mediated through two specific pathways, CD47-SIRPα (inhibition of phagocytosis signaling pathway)[166] and CD89 (for ADCC mediated phagocytosis),[167] and other targeting capabilities, which will be discussed in more detail, giving an introduction to the capabilities that macrophages have as being potent multi-specific antibody targets.

4.3.1. CD47-Mediated Targeting of Macrophages

Macrophages are known to be one of the most phagocytic cells in the immune system, making them very potent effector cells for causing tumor regression. Studies with single- and multitargeting[60–62,168] systems have been shown to increase the phagocytic capabilities of macrophages and convert them to an antitumor M1 phenotype. Several studies have recently demonstrated that through inhibition of CD47 stimulation by SIRPα, macrophages are more likely to induce potent and robust phagocytosis of cancer cells.[169–171] One study was able to overcome the CD47-SIRPα interactions by synthesizing a bispecific antibody derivative RTX-CD47.[149] RTX-CD47 was synthesized to have monovalent binding specificity through genetically fusing a CD47-blocking scFv to a CD20-targeting scFv derived from rituximab, a chimeric monoclonal antibody which can destroy CD20-presenting B-cells on their surfaces. This construct lacks an Fc domain, which has been shown in other studies to induce premature uptake into cells that rely on Fc receptor (FcR) signaling.[172] CD20 is a surface receptor on malignant B cells found in B-cell lymphomas, and inhibition of CD47 on that same cell will cause an "eat me" signal to induce phagocytosis. This novel system had expressed high binding specificity to malignant B cell lines with positive CD20 expression while ignoring those with negative expressions. Also, treatment with this BsAb showed induced macrophage phagocytosis of two-thirds of tested malignant B cell lines showing efficacy in a wide variety of disease models. Most importantly, this system could induce phagocytosis in a CD20-restricted manner without the reliance on Fc-mediated signaling, only inhibiting CD47/SIRPα interactions when in conjunction with CD20 binding.

Another promising system similar to the previous one is being used to target either B cell lymphoma and leukemias or mesothelin-expressing solid tumors using an anti-CD47/CD19 or anti-CD47/mesothelin (MSLN) IgG1 antibodies.[150] The mechanism of action is similar to the RTX-CD47 system in that both arms of the BsAb must bind to the surface of double-positive cells to induce a phagocytic response (**Figure 20**a). The difference between these two systems is that these anti-CD47/CD19 or MSLN systems have an Fc portion, allowing for potent macrophage-mediated antibody-dependent cellular phagocytosis. Since binding to only CD19$^+$ or MSLN$^+$ cells is strong, it shows a high affinity for those relative cells in comparison to the lower binding affinity of the anti-CD47 arm. In light of this, it was also demonstrated that significant phagocytosis would not occur if there were no coengagement of TAA CD19 and CD47 (Figure 20b). In vivo experiments showed significantly reduced tumor volumes compared to single antibody treatments, indicating the synergistic impact of binding to two specific sites on tumor cells, further reducing interactions with normal healthy cells (Figure 20c).

This same company, Glenmark Pharmaceuticals, went on to look at increased specificity to mouse models due to low cross-reactivity of anti-human BsAbs with mouse CD47, as well as how these forms of antibodies can have potent effects on the adaptive immune system.[173] They show that the affinity of the anti-CD47 arm is important to induce binding, where depending on the structure, it can impact its binding efficacy. Results show that CD47/CD19 binding can affect adaptive tumor immunity in such that enhanced phagocytic capabilities and antigen cross-presentation can impact in vitro efficacy. Transfecting A20-hCD19 tumors cells with hemagglutinin A (HA) and coculturing them with HA CD8$^+$ transgenic T cells and BMDMs were used to elucidate the impact that this treatment can have on the adaptive immune response. Treatment with this more mouse-specific BsAbs showed high T-cell proliferation and cytokine production levels, concluding that this system can also induce an adaptive immune response. Finally, anti-CD47 can be used in conjunction with anti-EGFR to elicit similar impacts on phagocytosis[151] while still including an Fc-functional region for macrophage-mediated uptake. This Pan- (anti-EGFR) SIRPαV-Fc bispecific antibody fusion protein and the monospecific SIRPαV-Fc protein was proven to increase macrophage phagocytic capabilities and tumor regression, with no significant difference between the two

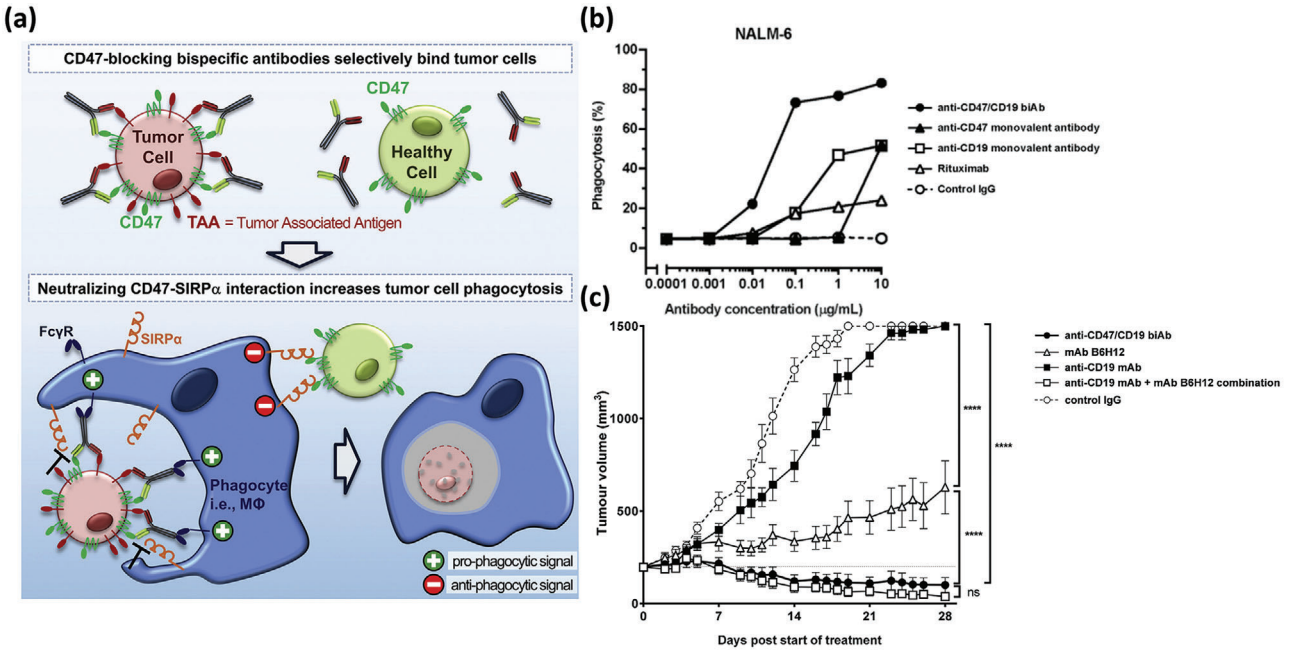

Figure 20. a) Schematic of anti-TAA/anti-CD47 bispecific antibodies increasing phagocytosis of cancer cells by macrophages. b) Phagocytosis of cancer cells is increased in the presence of the bispecific anti-CD47/CD19 bispecific antibody moreso than other controls. c) Tumor growth inhibition is significantly increased in the presence of anti-CD47/CD19 bispecific antibodies. Reproduced with permission.[150] Copyright 2017, Elsevier.

at high doses (40 mg kg^{-1}) but significance at low doses (10 mg kg^{-1}). Further safety studies with red blood cell counts show that this bispecific platform, compared to the SIRPαV-Fc has higher RBC counts, suggesting lower off-target effects like in the case of the IgG controls. While targeting CD47 is effective, there have been studies to show that it can induce hematoxicity,[174] especially when combined with effector Fc regions that can cause non-specific interactions with a wide variety of immune cells. Caution should be taken when working with this receptor and monitoring toxicity, especially with functional Fc regions, to maintain safe toxicologic profiles.

4.3.2. Fcγ Receptor and Extracellular Protein Targeting

In addition to the CD47/SIRPα axis, there is another innate receptor, the FcR, which is responsible for binding to the Fc domains of IgG (FcγRI) or IgA (FcαRI) antibodies when bound to its particular antigen. In particular, the FcγRI and FcγRIII are popular in the realms of phagocytosis and can be used to exploit the functions of macrophages and monocytes, eosinophils, and neutrophils. Macrophages, specifically, have FcγRI capabilities, which can be taken advantage of using bispecific antibodies. As early as 1992 by Chokri et al.,[175] knowing that macrophages need an influx of IFNγ to be converted to an active immune state, Chokri sought to force macrophages to "eat" RhD^+ RBCs, which inherently contained IFNγ. A bispecific antibody composed of an anti-FcγRI and anti-RhD portion allowed for erythrocytes to be coated with this BsAb, which allowed for an increase in macrophage activation more so compared to the addition of pure IFNγ, which exhibited increases in antitumoral effects. It was recently studied that using an anti-CD89/anti-CD20 bispecific antibody, there was potential to increase ADCC, phagocytosis, and inflammatory events through the known functions of a potent FcαRI, CD89.[152] This anti-CD89/CD20 BsAb was synthesized with and without a functional Fc region in order to investigate the dependence of cytotoxic activity on the FcαRI. When utilizing a specific mutation within the genetic manufacturing of this, a functional Fc region was either incorporated or left out. In comparison to an anti-CD20/IgG, both with and without a mutation, anti-CD89/CD20 was able to induce more potent tumor cell lysis when incubated with polymorphonuclear leukocytes (PMNs), showing that triggering of the FcαRI is stronger than FcγRI. Interestingly, when the experiment is conducted with monocyte-depleted PBMCs, the lack of an Fc region caused decreased tumor cell lysis, which led to the conclusion that NK cell-mediated kill resulted from having a functional Fc region. To confirm anti-tumor activity of mouse blood effector cells, an FcαR1 transgenic (Tg) mouse strain was generated which restricted CD89 expression to blood and tissue monocytes and macrophages. When conducting similar tumor cell lysis studies with monocytes, NK cells, and monocyte depleted PBMCs, most of the anti-tumor activity was derived from the monocytes when using the Fc-silenced CD89-Cd20 BsAb. Upon using this CD89-CD20 antibody and injected PBMCs in NOD/SCID mice, which have lower levels of NK cells, there was significantly greater tumor regression that treating with just PMNs. Finally, a transgenic mouse model (LLC-CD20 CD89) was generated and in comparison, to the wild type mouse, CD89-CD20 BsAb exhibited significant tumor regression than CD20-IgG controls because of the increased presence of

CD89/FcαRI. Further, TAMs (both M1 and M2) showed similarly increased killing efficiency regardless of phenotype. This research underlines the importance of CD89 and how this can be used as a potential target for macrophage and monocyte manipulation within the TME.

Not only can classical innate targeting of CD47 or CD89 be exploited, as one study has also ventured to explore more creative approaches to inducing macrophage activity, for example, through extracellular proteins. One study in glioblastoma (GBM) has used Ang-2/VEGF (A2V or CrossMab) BsAbs to reprogram macrophages from M2 to M1, increases survival.[153] Ang-2 plays a role in GBM by mediating the recruitment of Tie-2^{+} macrophages, which facilitates VEGF-induced angiogenesis and the protection of endothelial cells from VEGF withdrawal. Not only was CrossMab able to increase M1 (CD206^{low}/CD11c^{high}) to M2 (CD206^{high}/CD11c^{low}) ratios in an MGG8 GBM SCID mouse model, it dissected that this type of therapy is useful for decreasing tumor burden while maintaining microvessel integrity. By manipulating BsAbs to target surface markers other than ones for Fcγ, CD89, or CD47, toxicities and non-specificities related to those pathways have a better chance to be mitigated and cause tumor regression.

The effects of bi- and multispecific antibodies have shown a great impact on eliciting immune cell response and allowing for the redirection of immune cells toward the tumor microenvironment. By binding to immune-specific receptors that are involved in the cell-killing process and allowing for close-proximity to cellular cytotoxicity, you increase the odds of immune cell-specific attacks. Across these cells, it was shown that functional-Fc regions could increase cellular cytotoxicity, but silenced-Fc shows increased promise because of the lack of potential systemic toxicity that is inherent. Targeting of TAA, in addition to this silenced-Fc, allows for careful targeting of cancer cells, which was made even more advantageous through binding to specific portions or epitopes of those ligands. Finally, while T-cells have been the main target of bi- and multispecific antibody testing, recent studies have shown the potential that macrophages and NK cells have, opening doors for the new potential to increase targeting toolkits and making a more diverse selection of immune cells for a variety of situations and possibilities.

5. Conclusions and Future Directions

Immunotherapy is at the forefront of clinical research and is being used to revolutionize the way diseases are treated. Specifically, in cancer, the recent advances in tumor biology and immune system interactions have led to various effective therapies in vastly different tumor models. Unfortunately, intricate physiological and physical barriers as well as poor patient responses have been major obstacles and remain clinical challenges and the gatekeepers to cancer remission. Through the use of carefully designed nanotechnology and platforms, answers to these physiological questions can be found. Nanotechnology, specifically nanomaterials, can be used to trigger specific aspects of the immune system to induce tumor regression, using our own host defense to fight cancer from within.

These approaches have shown that multifunctional nano- and biological materials have the potential to address some of the most pressing challenges in cancer immunotherapy; however, very few nanomaterials have received regulatory approval for treating cancer so far, and significant limitations still remain to be solved. Currently, issues such as poor enrichment into the TME and variation in tumor regression hinder the powerful capabilities that these systems have to offer. Even though these classes of nano- and biological materials exhibit promising results, the lack of bioavailability, clearance from the body, and postdelivery side effects are crucial factors and should be considered, especially when using inorganic materials consisting of metals, silica, and quantum dots. In addition to immediate concerns, there are future challenges that need to be considered. First, the complex nature of these nano- and biological materials that carry multiple payloads have surface targeting or functionalities; the complex ratio of inactive excipients can complicate large-scale manufacturing, leading to batch-to-batch variability that can result in reproducibility concerns. While the approaches that can target and activate multiple immune cells are attractive, the effective use of these combinatorial nanomaterials would require biomarkers that can monitor the response and can enable understanding of the temporal aspects of activation of different components of the immune system. Additionally, short-term and long-term toxicities of these multifunctional nanomaterials need to be investigated, especially given that the activation of different components of immune systems might lead to long-term immune-related side-effects.

While these therapies still need improvement, the use of multifunctional materials opens a vast number of doors and points of entry where multiple angles can be used to activate specific immune responses and target cancer. In this review, we have discussed several nanomaterial-based and biological-based systems that have been deployed to enhance anticancer efficacy to immune cells such as T cell, NK cells, macrophages, and DCs, which each have their own unique ability to impact immunotherapeutic potential. These multifunctional strategies have been used to decipher the significance of combination therapy to tackle cancer treatment effectively. The strategies discussed herein shed light on the importance of meticulously designed nanomaterials that can accomplish potent immunotherapeutic potential. While side toxicities and limited efficacies impact nanotherapeutic platforms, multispecific and multifunctional approaches can be effectively used to mitigate these unwanted properties and allow for increased effectiveness, leading to increased quality of life for cancer patients and beyond.

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Conflict of Interest

The authors declare no conflict of interest.

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