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# Nanodelivery of STING agonists against cancer and infectious diseases

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## Abstract

Vaccination is a modality that has been widely explored for the treatment of various diseases. To increase the potency of vaccine formulations, immunostimulatory adjuvants have been regularly exploited, and the stimulator of interferon genes (STING) signaling pathway has recently emerged as a remarkable therapeutic target. STING is an endogenous protein on the endoplasmic reticulum that is a downstream sensor to cytosolic DNA. Upon activation, STING initiates a series of intracellular signaling cascades that ultimately generate potent type I interferon-mediated immune responses. Both natural and synthetic agonists have been used to stimulate the STING pathway, but they are usually administered locally due to low bioavailability, instability, and difficulty in bypassing the plasma membrane. With excellent pharmacokinetic profiles and versatility, nanocarriers can address many of these challenges and broaden the application of STING vaccines. Along these lines, STING-inducing nanovaccines are being developed to address a wide range of diseases. In this review, we discuss the recent advances in STING nanovaccines for anticancer, antiviral, and antibacterial applications.

### Keywords

STING; nanovaccine; immunotherapy; cancer; infectious disease

## 1. Introduction

Vaccination is an immunotherapeutic strategy focused on educating host immunity to fight off diseases. Professional antigen presenting cells (APCs) of the innate immune system are first activated in an immunostimulatory fashion, after which robust adaptive immunity against distinct antigenic targets can be achieved through a series of downstream signaling cascades. As prophylaxes, vaccines have experienced significant success and contributed largely to the eradication of major infectious diseases such as smallpox, polio, and measles (Henderson, 2011; Larson and Ghinai, 2011; Moss and Griffin, 2006). More recently, the development of therapeutic vaccines against cancer has shown much promise (Banchereau and Palucka, 2005; Hu et al., 2018; Melero et al., 2014). Unlike foreign pathogens, immune

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Declaration of Competing Interest

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stimulation against marginally mutated cancer antigens is more challenging, so immune stimuli in the form of adjuvants are routinely included. On this front, compounds that can activate the stimulator of interferon genes (STING) signaling pathway have been particularly attractive due to their pivotal role in the cancer–immunity cycle and during pathogenic invasion (Ahn and Barber, 2019; Barber, 2015; Corrales et al., 2016; Hayman et al., 2021; Marinho et al., 2017; Zhu et al., 2019).

STING is a protein embedded in the endoplasmic reticulum and acts as a downstream sensor to detect cytosolic DNA (Chen et al., 2016a; Ishikawa and Barber, 2008). Healthy cells normally do not possess cytoplasmic DNA, but it can exist as a result of pathogenic infections, cellular damage, or tumorigenesis (Li and Chen, 2018). In the cytosol, DNA binds to cyclic GMP-AMP synthase (cGAS) to produce a cyclic dinucleotide (CDN) known as cyclic GMP-AMP (cGAMP) (Barber, 2015). The cGAMP molecule will subsequently bind to and activate STING, leading to the cellular production of type I interferons (IFNs) to help regulate immune activity. Type I IFNs are key cytokines that link the innate immune system with adaptive immunity, and they can stimulate immune cells to elicit potent antitumor and antiviral responses (Ivashkiv and Donlin, 2014). While largely deployed against cancer, applications of STING agonists for treating infections have emerged in the past years (Chattopadhyay and Hu, 2020; Gall et al., 2018; Guo et al., 2015; Sali et al., 2015). Here, we will review the development of STING-targeting nanoformulations in the battle against cancer and infectious diseases (Figure 1). We begin with background on the immunological functions of STING and introduce the wide range of different natural and synthetic STING-activating compounds. Then, we will discuss advantages of nanotechnology and review applications of STING nanovaccines against cancer, viral infection and bacterial infection.

## 2. STING pathway and its immunological roles

The innate immune system can recognize specific pathogen-associated molecular patterns through pathogen recognition receptors (PRRs) (Kumar et al., 2011). STING is a PRR that senses pathogenic molecules in the cytosol and indirectly responds to cytosolic DNA through the recognition of CDNs (Barber, 2015). While some pathogens can produce CDNs that have a natural affinity for STING, detection of cytosolic DNA occurs indirectly through the cGAS-DNA sensing pathway. Interestingly, manganese ions ($Mn^{2+}$) (Zhao et al., 2020b) and β-arrestin 2 (Zhang et al., 2020c) were recently found to engage with cGAS to initiate and enhance downstream synthesis of cGAMP. Upon proper binding, STING forms a complex with TANK-binding kinase 1 (TBK1) and migrates to the perinuclear Golgi. At the site, the complex phosphorylates transcription factors such as IFN regulatory factor 3 (IRF3), nuclear factor-κB (NF-κB), and signal transducer and activator of transcription 6 (STAT6), which subsequently induce the production of type I IFNs (Cheng et al., 2020). Cytokines such as type I IFNs act as signaling molecules that alert the rest of the body to pathogenic invasions or cellular damage and help recruit immune cells in response (Ivashkiv and Donlin, 2014). Type I IFNs, among which IFN-α and IFN-β are the most common, act as mediators that link innate immunity with the specialized immune subsets from the adaptive immune system.

Type I IFNs have critical functions in both the innate immune system and the adaptive branch of immunity. During the innate phase of an immune response, the cytokines can guide phagocytes to recognize specific pathogens and stimulate infected cells to inhibit pathogen replication or restrict intracellular bacterial growth (Yan and Chen, 2012). During viral infections, type I IFNs promote the proliferation and survival of natural killer (NK) cells (Martinez et al., 2008). In the adaptive immune system, these IFNs play vital roles in activating cytotoxic T lymphocytes (CTLs), which are responsible for the selective clearance of infected and cancerous cells (Iwasaki and Medzhitov, 2015). To accomplish this function, type I IFNs upregulate the expression of proteins associated with antigen presentation in APCs and increase the production of certain proinflammatory cytokines and chemokines (McNab et al., 2015). In addition, type I IFNs were found to enhance cross-presentation and increase dendritic cell (DC) accumulation in lymphatic tissues (Le Bon et al., 2006a; Rouzaut et al., 2010; Spadaro et al., 2012). They can also act directly on the specialized T cells and B cells by providing signals for proliferation, differentiation, clonal expansion, and survival (Brinkmann et al., 1993; Le Bon et al., 2006b; Marrack et al., 1999). While production of type I IFNs can be induced through multiple signaling pathways, STING is one of the most critical mechanisms for mounting an immune response against viruses and bacteria (Ahn and Barber, 2019; Chen et al., 2011).

Highlighting the importance of STING, one study demonstrated that expansion of CTLs and other immune subsets, such as plasma cells and follicular helper T cells, was significantly reduced when primed with DCs deficient in STING (Klarquist et al., 2014). Other studies have shown that host susceptibility to vesicular stomatitis virus and herpes simplex virus 1 (HSV-1) was significantly increased in STING knockout mice (Ishikawa and Barber, 2008; Ishikawa et al., 2009). In murine models, STING was key in activating T cells and stimulating antibody production in response to HSV-1 and DNA from *Escherichia coli* and *Vibrio cholerae* (Li et al., 2013). STING-induced expression of type I IFNs was identified as a central mediator of immune responses against varicella zoster virus, hepatitis B virus, chikungunya virus (CHIKV), human adenoviruses, and cytomegaloviruses, among many others (Anghelina et al., 2016; Gall et al., 2018; Guo et al., 2015; Kalamvoki et al., 2014; Kim et al., 2017; Lio et al., 2016; Sali et al., 2015; Stempel et al., 2019). In terms of antimicrobial immunity, bacteria are known to produce CDNs as part of their colonization process; thus, during intracellular invasion, bacterial CDNs will naturally bind to STING and trigger a robust type I IFN-mediated response (Whiteley et al., 2019). Several common bacteria such as *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Shigella flexneri*, and *Neisseria gonorrhoeae* were found to trigger STING signaling in this fashion (Koppe et al., 2012; Marinho et al., 2017; Parker et al., 2011). In some bacteria such as *Mycobacterium tuberculosis*, STING activation was imperative for autophagy, an essential cellular process for degrading intracellular components and removing invasive microbes (Watson et al., 2012). STING-induced immunity can also occur during fungal and parasitic infections (Ahn and Barber, 2019; McNab et al., 2015; Sisquella et al., 2017).

More recently, the significance of the STING pathway for antitumor immunity was elucidated. STING activation was shown to induce cellular apoptosis (Tang et al., 2016), promote antigenic release (Lu et al., 2018), augment antigen presentation (Curran et al., 2016), enhance the priming and activation of CTLs (Jassar et al., 2005), improve T cell

infiltration into tumor sites (Ohkuri et al., 2014), promote immune cell proliferation and survival (Tough et al., 1996), and assist in the recognition and killing of cancerous cells (Jing et al., 2019; Lirussi et al., 2017). Since tumor cells are derived from host cells, STING activation during anticancer immunity largely stems from the detection of self-DNA through the cGAS-DNA sensing pathway. Damaged self-DNA can leak out from the nucleus or mitochondria of apoptotic cells and subsequently be processed by immune cells. Once activated, STING can induce a powerful and antigen-specific immune response, thus propagating a positive feedback loop that drives anticancer immunity. Many STING agonists have been developed for cancer immunotherapy applications (Corrales et al., 2015; Corrales et al., 2017; Corrales et al., 2016; Fu et al., 2015; Kitai et al., 2017; Li et al., 2019; Woo et al., 2014; Xia et al., 2016). Overall, STING is involved in many aspects of the immune response and is an attractive target for vaccine-based immunotherapy. Selective activation of STING can program immune cells to recognize and target invasive pathogens or educate the host immune system to identify and eradicate cancerous cells. It is important to note that the STING pathway is independent of other pathogen-sensing pathways, such as toll-like receptor (TLR), nucleotide oligomerization domain (NOD)-like receptor, and retinoic acid-inducible gene-I (RIG-I)-like receptor pathways (Kawai and Akira, 2009). Ultimately, an in-depth understanding of the wide range of different compounds that can activate this signaling pathway is vital for engineering efficacious STING-targeted therapeutics.

## 3. Types of STING agonists

STING plays an indispensable role in anticancer, antiviral, and antibacterial immunity. Owing to their broad applicability and pivotal functions in immunity, many natural and synthetic STING agonists have been utilized in the design of more effective vaccines (Ding et al., 2020; Motedayen Aval et al., 2020; Wu et al., 2020). Better mechanistic understanding of the STING biological pathway has enabled the discovery of novel compounds and helped to elucidate their structure–activity relationship. Several STING agonists are currently being investigated in clinical trials (Motedayen Aval et al., 2020; Zhu et al., 2019). STING agonists can be subdivided into several classes, which include natural CDNs, CDN derivatives, flavonoids and xanthones, and other novel and unique compounds (Figure 2).

### 3.1 Cyclic dinucleotides

CDNs are the most common STING agonists with innate binding affinity for STING (Danilchanka and Mekalanos, 2013). In the cGAS-DNA sensing pathway, mammalian cells synthesize noncanonical 2′3′-cGAMP with a mixed linkage (ML) upon detection of cytosolic DNA (Li and Chen, 2018). Canonical 3′3′-cGAMP with the classical 3′5′ phosphodiester bonds is naturally produced by bacteria (Diner et al., 2013). The potency of these two molecules for STING activation is indistinguishable; however, noncanonical 2′3′-cGAMP has a stronger binding interaction with the STING protein (Zhang et al., 2013). Alternate isoforms of cGAMP such as 3′2′-cGAMP and 2′2′-cGAMP have been evaluated as potential STING agonists, but no clear benefits were found for these synthetic molecules (Zhang et al., 2013). Besides the heterogenous cyclic nucleotide structure, natural homogenous analogs such as cyclic di-GMP (CDG) (Burdette et al., 2011; Madhun et al., 2011) and cyclic di-AMP (CDA) (Ebensen et al., 2011; Skrnjug et al., 2014) can likewise

activate the STING pathway (Jin et al., 2011). CDG and CDA are second messengers naturally produced by bacteria and have vital biological roles in pathogenesis (Corrigan and Grundling, 2013; Romling et al., 2013). In particular, CDG was found to be a promising mucosal adjuvant by many researchers (Blaauboer et al., 2015; Ebensen et al., 2017; Madhun et al., 2011; Mansouri et al., 2016). Not surprisingly, noncanonical forms of 2′3′-cyclic di-GMP (ML CDG) and 2′3′-cyclic di-AMP (ML CDA) have been chemically synthesized. While the potency of 2′3′-cGAMP and 3′3′-cGAMP is minimal, ML CDG and ML CDA can actually induce higher levels of type I IFNs in cells when compared to their canonical counterparts (Corrales et al., 2015; Fu et al., 2015).

A better understanding of the molecular interactions between natural CDNs and STING has engendered several synthetic CDN derivatives with enhanced potency, binding affinity, and stability. One such derivative is cyclic AMP-IMP (cAIMP), which is fabricated by the replacement of the guanine nucleoside with inosine in cGAMP (Lioux et al., 2016). Stimulation with 3′3′-cAIMP outperformed 2′3′-cAIMP, and both molecules were found to elicit a stronger type I IFN response than 2′3′-cGAMP. Under the same conditions, the adjuvanticity of 3′2′-cAIMP and 2′2′-cAIMP were both lower than 2′3′-cGAMP, while the difference between the two analogs was indistinguishable. Homogenous cyclic di-IMP (CDI) can be synthesized by substituting the remaining adenosine nucleoside with inosine. As an adjuvant, CDI can stimulate mucosal immunity comparable to CDG (Libanova et al., 2010), but the advantage of using synthetic CDI as opposed to natural CDG is unclear. Through observing the chemical structure of CDNs, researchers have synthesized all possible forms of canonical CDNs with the four natural ribonucleotide bases: cytosine, guanine, adenine, and uracil (Wang et al., 2017). When evaluating the immunostimulatory capability of these synthetics, 3′3′-cCAMP was found to activate STING, but its ability to induce IRF expression in a RAW264.7 reporter cell line was inferior to naturally derived bacterial CDNs. Compared with the natural STING agonists, application of these novel synthetic CDN derivatives is less common.

The chemical structure of CDNs is two nucleotides connected by phosphodiester bonds in a cyclic fashion, but the phosphodiester bonds are susceptible to degradation by phosphodiesterases and nucleases (Kato et al., 2018). These enzymes are commonly found systemically and in host cells, and they act as a barrier that lowers the effectiveness of CDNs. To overcome this obstacle and increase the stability of CDNs, phosphorothioate diester linkages have been used to connect the nucleotides. While the immunostimulatory effects of canonical CDG linked by one or two (RR-CDG) phosphorothioate diester bonds were lower than native CDG in a mucosal setting (Yan et al., 2008), similar modifications on noncanonical cGAMP, CDG, and CDA (ML RR-CDA) have shown enhanced potency (Corrales et al., 2015). In the case of cAIMP, the potency of 3′3′-cAIMP with two phosphorothioate diester linkages was likewise improved when compared to 3′3′-cAIMP (Lioux et al., 2016). Besides phosphorothioate diester modifications, CDNs have been linked with thiourea, urea, carbodiimide, guanidinium, and triazole bonds (Fujino et al., 2014; Gaffney and Jones, 2014). Stability of CDNs can be further increased through fluorination, a technique commonly utilized in medicinal chemistry (Bohm et al., 2004; Cavaliere et al., 2017; Liu et al., 2008). Interestingly, fluorination at the 2′ or 3′ nucleotide sites not only increased stability *in vivo*, but it also significantly intensified biological activity and

adjuvanticity (Corrales et al., 2015; Fu et al., 2015; Lioux et al., 2016; Smola et al., 2021; Wu et al., 2021; Zhang et al., 2020a). The phenomenon is attributed to the increased lipophilicity after fluorine modification, which allows the CDNs to better traverse the cell membrane.

### 3.2 Flavonoids and xanthones

A second major class of STING agonists are flavonoids and xanthones, which are polyphenolic compounds naturally found in plants. Flavonoids are plant metabolites that are commonly consumed for health benefits and structurally contain two phenol rings and a heterocyclic ring (Panche et al., 2016). Xanthones are molecules that are chemically similar to flavonoids, except the three cyclic rings are bound to one another. Flavone-8-acetic acid (FAA) is a flavonoid that was discovered through screening natural compounds, and it was shown to induce an immune-mediated antitumor response (Bibby et al., 1991). FAA was one of the earliest STING agonists to be uncovered prior to the discovery of the STING pathway, and recent findings have confirmed that FAA does initiate immunity through STING engagement (Zheng et al., 2020). The xanthone 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is an adjuvant derived from FAA that exhibits increased potency (Philpott et al., 1995). A single intratumoral injection of DMXAA was shown to induce systemic tumor necrosis factor (TNF-α) secretion at levels similar to FAA, but with doses 10 times lower. Another xanthone compound, 10-carboxymethyl-9-acridanone (CMA), was found to have potent antiviral effects (Guo et al., 2015). Despite promising preclinical studies, all three compounds failed clinical trials because they were later found to react only to murine STING and were unable to activate the human STING pathway (Cavlar et al., 2013; Conlon et al., 2013; Kim et al., 2013). Nevertheless, not all flavonoid and xanthone derivatives are murine STING specific, as α-mangostin was shown to activate human STING to a greater extent than murine STING (Zhang et al., 2018b).

### 3.3 Other STING agonists

High throughput drug screening has emerged as a powerful strategy to identify novel inhibitors and agonists for various therapeutic targets (Bleicher et al., 2003; Dove, 2003; Macarron et al., 2011). Many novel STING agonists, including dispiro diketopiperzine (DSDP) (Liu et al., 2017a), 6-bromo-*N*-(naphthalen-1-yl)benzo[*d*][1,3]dioxole-5-carboxamide (BNBC) (Zhang et al., 2019a), G10 (Sali et al., 2015), and C11 (Gall et al., 2018), were discovered after screening libraries of small molecules using cell reporter systems. Treatment with DSDP and BNBC elicited higher IFN-β and interleukin (IL)-29 mRNA expression in human cell lines and significantly protected THF fibroblasts from infection by dengue virus, zika virus, and yellow fever virus in a prophylactic setting. It is important to note that both DSDP and BNBC are human-specific STING agonists and do not respond to murine STING. G10 and C11 were found to increase IFN-β release through IRF3, but not by NF-κB transcription. When THF cells were pre-exposed to G10, replication of alphaviruses such as CHIKV and venezuelan equine encephalitis virus (VEEV) were inhibited by more than three orders of magnitude. Treatment with C11 was able to inhibit replication of CHIKV, VEEV, ross river virus, mayaro virus, and o'nyong-nyong virus in a similar fashion. Another STING agonist is STING-mediated interferon-inducing and cytotoxic reagent, original (SINCRO), which has dual functionalities; the

compound not only activates STING, but it also induces cellular apoptosis in cancerous cells through oxidative stress (Kimura et al., 2018). Upon intratumoral treatment, the cytocidal property of SINCRO helped to eradicate cancerous cells while concurrently activating immune cells to process tumor neoantigens in a synergistic manner.

STING agonists in free form are generally administered intratumorally, which is not always an option for cancer therapy. Dimetric amidobenzimidazole (diABZI) was identified as a STING inducer from a library of 1.8 million small molecules through a cGAMP competitive binding assay (Ramanjulu et al., 2018). Compared to 2′3′-cGAMP, diABZI is 400 times more potent for STING activation and can be safely administered systemically. In a CT26 colorectal cancer model, intravenous treatment with diABZI at 1.5 mg/kg completely eradicated tumors in 8 out of 10 mice. Several derivatives of amidobenzimidazole with comparable potency have been synthesized and identified by others (Xi et al., 2020). Another compound that can be administered systemically is SR-717, which was synthesized after screening approximately 100,000 small molecules for the induction of IRF in a THP-1 reporter cell line (Chin et al., 2020). SR-717 stimulated IFN genes at a level comparable to diABZI *in vitro*, but mice interperitoneally treated with SR-717 had plasma IFN-β levels roughly 60 times lower than with diABZI treatment. In a B16F10 melanoma model, daily intraperitoneal treatment with SR-717 at 30 mg/kg for a week was able to extend median survival from 22 days to 27 days. Benzothiophene oxobutanoic acid (MSA-2) is a compound recently identified from a library screening of 2.4 million molecules through the detection of IFN-β in a THP-1 reporter cell line (Pan et al., 2020). Treatment efficacy of MSA-2 in an MC38 tumor model was assessed after intratumoral, subcutaneous, or oral administration. While different treatment regimens and dosages were used for the three routes, complete tumor regression was achieved in 80% to 100% of all treated mice. At a 60 mg/kg dosage, administration by oral gavage achieved tumor bioavailability comparable to subcutaneous injections at 50 mg/kg. Oral delivery is particularly attractive for clinical translation and improves patient compliance due to the painless and simple administration process.

## 4. STING nanovaccines

While STING agonists have shown significant promise as vaccine adjuvants, limited bioavailability and efficacy have thwarted progress in clinical applications. On this front, nanoparticles are promising drug delivery carriers that can specifically localize drug payloads to increase efficacy and decrease nonspecific cytotoxicity (Couvreur, 2013). Nanoparticles are versatile materials with distinct advantages and can be broadly employed for many different applications. Over the past several years, a wide range of different nanoparticle formulations have been successfully developed to better facilitate the delivery of STING agonists into immune cells and increase their activity against cancers, viruses, and bacteria.

### 4.1 Advantages of nanovaccines

Nanotechnology can address many shortcomings of traditional vaccines through their unique size, shape, hydrophobicity, and surface properties. Nanovaccines can be fabricated to approximate the size of pathogens for improved cellular uptake and designed to efficiently

drain into lymphatic tissues for antigen presentation (Gheibi Hayat and Darroudi, 2019; Luo et al., 2017a). Lymphatic drainage is heavily dependent on nanoparticle size, where smaller nanoparticles accumulate significantly better than their larger counterparts (Gao et al., 2015). In one study, activation of resident DCs in the lymph nodes with 100-nm polypropylene sulfide nanoparticles was only 10% as efficient as 25-nm nanoparticles (Reddy et al., 2007). The shape of nanomaterials is another factor that needs to be considered. Spherical nanoparticles, for example, are more likely to be taken up by immune cells (Gheibi Hayat and Darroudi, 2019), whereas particles with an ellipsoid shape can interface with the surface membrane in a superior fashion (Kroll et al., 2017b; Meyer et al., 2015). On the other end of the spectrum, unique structures such as nanorods and nanostars have high cytotoxicity due to their protruding structure (Lee et al., 2019). Modulating the hydrophobicity and electrostatic properties of nanoparticles can similarly affect cellular uptake. While cationic nanoparticles are readily phagocytosed and have poor pharmacokinetic profiles, anionic nanoparticles have lower nonspecific uptake and can be engineered to specifically target immune cell subsets (Luo et al., 2017a). Modification of the nanoparticle surface with antibodies, lipids, proteins, or synthetic compounds can bestow new functionalities, alter *in vivo* fate, and extend the therapeutic window of encapsulated payloads (Ai et al., 2020; Fang et al., 2018; Gheibi Hayat and Darroudi, 2019; Liu et al., 2017b; Luo et al., 2017a).

With a wide range of different materials for selection, nanoparticles can readily incorporate both hydrophobic and hydrophilic molecules for delivery (Kroll et al., 2019; Zhou et al., 2020a). Drugs encapsulated within nanoparticles have limited exposure to the external environment, which not only protects the payloads from systemic degradation, but also reduces unwanted cytotoxicity (Zhou et al., 2020c). As ideal drug delivery vehicles, nanocarriers are attractive candidates for vaccine development, especially due to their ability to colocalize antigens and adjuvants (Zhu et al., 2017). As immune cells process nanovaccines that contain both components, they not only receive an immunostimulatory activation signal, but also have an antigenic target to direct the activation against (Fischer et al., 2013). Furthermore, nanoparticles can be stimuli-responsive, which allows them to intelligently react to pH, chemical gradients, temperature, or reactive oxygen species (Cheng et al., 2013; Ganta et al., 2008; Motornov et al., 2010). Nanocarriers can also be engineered to respond to external manipulations, such as ultrasound, magnetic fields, or irradiation (Mura et al., 2013). An advantage of nanovaccines that can respond to changes in pH is their ability to escape from lysosomal degradation and enhance major histocompatibility complex (MHC) I-mediated antigen presentation (Kim et al., 2019). Traditional vaccines struggle with this process because a large majority of the antigenic material is degraded within the endosomes, while only a small portion is processed through endogenous cross-presentation pathways that lead to the activation $CD8^+$ T cells (Joffre et al., 2012).

### 4.2 STING nanovaccines for cancer immunotherapy

The STING signaling pathway plays critical roles in the cancer–immunity cycle, and as such, many researchers have identified STING as a good therapeutic target to exploit for cancer treatment. On this front, an impressive number of STING nanovaccines have been developed to treat a wide range of different cancers.

**4.2.1 Polymeric nanoparticles—**Polymeric nanoparticles are commonly employed in the development of STING nanovaccines for cancer therapy due to their ease of synthesis, scalability, and biocompatibility. One study reported on a polymeric nanovaccine using antigen-loaded PC7A nanoparticles that enabled safe and effective delivery to the peripheral lymph nodes (Figure 3) (Luo et al., 2017b). When mixed with the model antigen ovalbumin (OVA), PC7A polymers naturally self-assembled into 29-nm nanoparticles. Besides OVA, PC7A nanoparticles can be formulated with personalized cancer neoantigen peptides for clinical applications (Wilhelm et al., 2021). In addition to its facile fabrication procedure, the platform also facilitated the loading of the peptide antigens onto MHC I to prime a strong $CD8^+$ T cell response. While certain subsets of APCs can naturally present exogenous antigens on MHC I through natural cross-presentation mechanisms (Embgenbroich and Burgdorf, 2018), PC7A nanoparticles expedited the process through cytosolic delivery. Phagocytosed PC7A nanoparticles escaped from the endosome by the proton sponge effect, where the endosomes were ruptured from osmotic pressure buildup due to an influx of protons (Smith et al., 2019). Once the contents were released into the cytosol, the PC7A naturally bound to and activated the STING signaling pathway for potent immunity. PC7A nanoparticles loaded with the appropriate tumor antigens demonstrated effective tumor growth inhibition in B16-OVA, B16F10 melanoma, MC38 colon cancer, and human papilloma virus (HPV) TC-1 mouse models. Combination of the PC7A nanoparticles with anti-PD-1 immune checkpoint blockade (ICB) led to enhanced efficacy against both the B16-OVA and the TC-1 tumor models. The effectiveness of the treatment was immune mediated and resulted in long-term memory, as mice with previously eradicated TC-1 tumors were resistant to tumor rechallenge 82 days later.

In a subsequent study, PC7A nanovaccines were found to be ineffective against solid tumors once an immunosuppressive tumor microenvironment (TME) had been established (Luo et al., 2019). In combination with radiation therapy (RT), however, efficacy could be improved, and the growth of large tumors was controlled. The synergism was driven by the enhanced local antitumor immunity resulting from RT, while PC7A nanovaccines helped to elicit a systemic immune response. PC7A nanovaccines have also been combined with existing STING agonists such as 2′3′-cGAMP (Li et al., 2021). Since PC7A binds to STING in a uniquely different site, synergistic treatment of MC38 tumors with 2′3′-cGAMP, which was loaded in the nanovaccine, completely eradicated tumors in 4 out of 7 mice. In comparison, monotherapy with 2′3′-cGAMP rescued only 1 out of the 6 mice, while all mice undergoing PC7A treatment alone succumbed to the disease. In another study, a PC7A nanovaccine was used in a combinational approach to produce an *in situ* cancer vaccine (Patel et al., 2019). Rather than targeting just the STING pathway, PC7A was encapsulated in a polyplex core along with CpG oligodeoxynucleotides to concurrently stimulate the TLR9 pathway. Furthermore, the nanoparticle core was coated with bacteria-derived outer membrane vesicles as an additional broad spectrum immune stimulus. Maleimide functional groups were embedded onto the membrane surface to generate the final formulation. Rather than preloading antigens into the formulation, the maleimides captured tumor antigens *in situ* after local RT (Min et al., 2017). In murine B78 melanoma and NXS2 neuroblastoma models, the nanovaccine in combination with RT eliminated a significant portion of the treated tumors.

A more common application of polymeric nanoparticles is STING agonist delivery, an example being the utilization of biodegradable poly(beta-amino ester) (PBAE) nanoparticles to carry CDNs into the cytosol (Wilson et al., 2018). Like PC7A, the cationic nature of PBAE allows for direct cytosolic delivery through the proton sponge effect. Cellular uptake of RR-CDG-loaded PBAE nanoparticles was significantly higher in THP-1 human monocytes and RAW264.7 murine macrophages when compared to B16 cancer cells. The selectivity can be attributed to the versatility of PBAE, where endcap modifications of the polymer can result in vastly different cellular uptake profiles (Sunshine et al., 2009). In a B16 tumor model, the more potent ML RR-CDA-loaded PBAE nanoparticles controlled tumor growth significantly better than free CDN at the same dosage. When used in combination with anti-PD-1 checkpoint inhibitors, the nanoformulation achieved similar efficacy at a ten times lower dosage compared to free CDN alone. Other examples of delivery using polymeric nanocarriers include methoxy poly(ethylene glycol)-poly(lactide) nanoparticles to deliver α-mangostin (Zheng et al., 2018) and poly(l-glutamic acid)-g-methoxy poly(ethylene glycol) nanoparticles to deliver SN38 and induce DNA damage for indirect STING activation (Zhao et al., 2021).

Endosomolytic polymerosomes, which are amphiphilic polymers that assemble into a liposome-like structure, were developed to carry STING agonists directly to the cytosol (Shae et al., 2019; Wang-Bishop et al., 2020). The polymerosomes were composed of a hydrophilic core for high CDN loading, a pH-responsive vesicle membrane for endosomal escape, and an outer polyethylene glycol (PEG) shell for prolonged circulation. In an erythrocyte hemolysis assay, noncanonical cGAMP-loaded polymerosomes rapidly disassembled and induced hemolysis at lower pH, implying that the platform could facilitate the cytosolic delivery of cGAMP through endosomal disruption. Intratumoral treatment with the formulation in a B16F10 model elicited higher numbers of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells when compared to free cGAMP. In a therapeutic setting, tumor growth was significantly controlled, with complete responses observed in 3 out of 9 mice. When used in combination with anti-PD-1 and anti-CTLA-4 ICBs, intratumoral injections of the nanoformulation resulted in profound tumor regression in both the treated tumor and a contralateral tumor. Combination with ICBs likewise boosted the potency of nanoparticles that were administered intravenously, resulting in strong antitumor responses.

Peptide neoantigens have been loaded into CDN-containing endosomolytic polymerosomes to strengthen vaccine efficacy through antigen–adjuvant colocalization (Shae et al., 2020). Upon cytosolic delivery, the nanoparticles boosted presentation of cancer neoantigens on MHC I to elicit stronger $CD8^+$ T cell immunity. Mice treated with a formulation loaded with SIINFEKL, an MHC I-restricted peptide derived from OVA, had the highest number of OVA-specific $CD8^+$ T cells compared to various controls. Similarly, mice treated with an MC38-specific cocktail loaded with either Reps1 or Adpgk peptide epitopes had the highest proportion of IFN-$\gamma^+$TNF-$\alpha^+$$CD8^+$ peripheral T cells upon *ex vivo* restimulation. In murine tumor studies, cocktail nanovaccine treatment with anti-PD-1 inhibited MC38 and B16F10 tumor growth. Another pH-responsive polymerosome platform employed a PEG block copolymer with poly(2-(diisopropanol amino) ethyl methacrylate) to deliver DMXAA and peptide antigens (Zhou et al., 2020b). The polymersomes had mannose as an additional DC-targeting moiety, which contributed to increased lymph node localization, higher DC

uptake, better MHC I presentation, and stronger antigen-specific $CD8^+$ T cell activation. In both a B16-OVA and a 4T1 orthotopic breast cancer model, subcutaneous vaccination at the tail base with the polymerosomes slowed tumor growth.

Instead of relying on the delivery of tumor antigens, STING agonists can also work synergistically with chemotherapy by utilizing antigens generated *in situ* (Figure 4). One such strategy involved the mixture of 2′3′-cGAMP-loaded hollow poly(lactic-*co*-glycolic acid) (PLGA) nanoshells with CT26 cells that were treated with irinotecan (CPT-11), a frontline chemotherapeutic (Chattopadhyay et al., 2020). When incubated with JAWS II murine DCs *in vitro*, the mixture induced significant upregulation of DC maturation markers and type I IFN release. In an animal study, CT26 tumor-bearing mice treated with the combined formulation had the slowest tumor growth, with 1 out of 8 mice becoming tumor-free. The strategy was tested in other animal models with different chemotherapy combinations, including B16 plus cisplatin and B16F10 plus doxorubicin (DOX) in combination with anti-CTLA-4. In the former, 5 out of 10 mice had a complete response to the therapy, whereas in the highly aggressive B16F10 model, only 1 out of 7 mice survived until day 90 despite the triple combination. In another example of chemoimmunotherapy with STING agonists, CDA was loaded with camptothecin (CPT) into nanotubes that self-assembled into a hydrogel structure (Wang et al., 2020a). The hydrogel encouraged the retention of CDA and CPT, allowing a gradual and continuous release of the vaccine components as a substitute for booster doses (Jiang et al., 2020a). A single intratumoral dose of the hydrogel resulted in strong efficacy in GL-261 glioblastoma, CT26, and 4T1 models.

**4.2.2 Liposomes and lipid nanoparticles—**Liposomes consist of phospholipids assembled into a spherical nanostructure and are one of the most popular drug delivery platforms. Due to their facile synthesis and biocompatibility, liposomes have been utilized to effectively deliver STING agonists. In one study, PEGylated phosphatidylcholine liposomes enhanced delivery of CDG into APCs and improved targeting into the draining lymph nodes (dLNs) (Hanson et al., 2015). Enhanced delivery of the liposomal formulation directly translated to better immune activation and therapeutic efficacy. EG.7-OVA tumor-bearing mice vaccinated with a mixture of the adjuvant-loaded nanoparticles and OVA on days 6, 13, and 20 had a nearly 3-fold higher number of OVA-specific $CD8^+$ T cells when compared to vaccination with free CDG and OVA. Treatment using the nanoformulation effectively extended the median survival time from around 16 days to 29 days, while no noticeable efficacy was observed in mice treated with the free CDG mixture. Similar effects were seen in a B16F10 tumor model using the melanoma-specific gp100 antigen.

YSK05 is a pH-sensitive cationic synthetic lipid that promotes fusion with endosomal membrane (Sato et al., 2012). By incorporating the lipid into liposomes, YSK05-modified liposomes have been utilized for siRNA and CDG delivery (Miyabe et al., 2014). In RAW264.7 cells, a CDG-loaded formulation induced high levels of IFN-β secretion and upregulated the maturation markers CD80, CD86, and MHC I. Mice challenged with E.G7-OVA a week after immunization with the adjuvanted liposomes mixed with OVA showed a noticeable reduction in tumor growth. Antitumor efficacy in this model was primarily mediated by CTLs due to the high expression of SIINFEKL-MHC I on the tumor surface. Interestingly, antitumor efficacy of these liposomes was also observed in B16F10 cells with

downregulated MHC I expression (Nakamura et al., 2015). Upon closer inspection, the immunity originated from the activation of NK cells. Splenocytes derived from mice 8 hours after intravenous immunization had a significantly smaller proportion of NK cells, indicating that they had been recruited away in response to CDG. In a B16F10 lung metastatic tumor model, mice treated with the formulation had obvious reduction in lung nodules, but such effects were abrogated in mice with NK cells depleted by anti-asialo GM1.

Another example employed cationic liposomes fabricated from cholesterol and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) to treat B16F10 tumors (Koshy et al., 2017). The liposomes were loaded with 2′3′-cGAMP and coated with 5% or 10% PEG to improve stability (Figure 5). The cationic nature of the liposomes allowed binding to the cell membrane with high affinity and facilitated the endosomal release of cGAMP into the cytosol. Compared to free 2′3′-cGAMP and CpG controls, the liposomal formulations elicited better gene expression of *Ifnb1*, *Cxcl10*, *Cxcl9*, and *Tnf* in mice. In an orthotopic tumor model, mice intratumorally treated with free cGAMP and the liposomal formulations all had the same survival rate, but upon rechallenge 60 days later, the long-lasting immune memory elicited by the liposomes could be discerned. While 50% of the mice in the free cGAMP group succumbed to the rechallenge, 100% of the mice treated with the 2′3′-cGAMP-loaded liposomes coated with 10% PEG survived. Liposomes have also been leveraged to deliver canonical cGAMP against more aggressive tumor models, such as triple-negative breast cancer (TNBC) (Cheng et al., 2018). TNBC is especially resistant against existing treatments due to the absence of three common breast cancer markers. When used in combination with anti-PD-L1, treatment with the cGAMP-loaded liposomes effectively eradicated the tumors, leading to a 100% survival rate. In addition, an inhalable 2′3′-cGAMP-loaded liposomal formulation has been recently proposed as a treatment for metastatic lung cancer through the activation of pulmonary APCs (Liu et al., 2019c).

Lipid nanoparticles (LNPs) have been designed to directly activate STING without the need for a separate agonist payload. In one case, a lipid nanoformulation was screened from a library of ionizable lipid-like materials, and an optimal nanovaccine was identified from more than 1,000 formulations (Miao et al., 2019). The identified formulation was found to efficiently deliver mRNA into cells and *in vivo*, activate innate and adaptive immunity through the STING pathway, and potently induce anticancer immunity in a therapeutic setting (Figure 6). The STING activation was found to be due to the presence of unique cyclic amino head groups. In a B16-OVA model, a single dose of OVA-encoding mRNA-loaded LNPs prolonged survival and rescued 3 out of 11 mice. Using mRNA encoding for TRP2, a B16F10 antigen, three doses of the LNPs significantly retarded tumor growth, with more than 60% of the mice still alive on day 40. In contrast, all untreated mice bearing B16F10 tumors succumbed to the disease by day 25. Comparable therapeutic efficacy was observed with a TC-1 tumor model after a single dose of LNPs in combination with anti-PD-1.

**4.2.3 Inorganic nanoparticles—**The toxicity of inorganic nanoparticles to humans and the environment is a common concern in the field of nanotechnology (Sengul and Asmatulu, 2020; Zhou et al., 2021). However, when employed carefully, the inherent properties of inorganic nanoparticles can be leveraged against cancerous cells. In one example, cationic

silica nanoparticles were used to induce necrotic tumor cell death and locally deliver CDG for vaccination (An et al., 2018). To prepare the particles, negatively charged CDG was electrostatically complexed with the cationic, amine-modified silica nanoparticles. Intratumoral administration induced destruction of the tumor from the intrinsic cytotoxicity of the nanoparticles, and the dying cancer cells were subsequently phagocytosed by APCs activated with the CDG. Tumor tissues from treated mice had noticeable areas of necrosis. In a melanoma model, treatment with the CDG-loaded nanoformulation significantly inhibited tumor development in mice compared to free CDG and free CDG co-administered with unloaded silica nanoparticles. The latter control group demonstrated the importance of incorporating CDG with the nanoformulation, since free STING agonists have difficulty bypassing the plasma membrane and can rapidly diffuse out of the tumor and into the bloodstream. A single dose of the CDG-loaded nanoparticles rescued 3 out of 8 mice in a B16F10 model and helped to establish long-lasting immunity, as all mice survived a tumor rechallenge 60 days later.

Metal nanoparticles have been explored for treating aggressive cancers such as head and neck squamous cell carcinoma (HNSCC) (Tan et al., 2018). HNSCC is challenging to treat with ICBs and has low CTL responses. In addition, SOX2, a common HNSCC oncogene, was discovered to degrade STING through autophagy and inhibit STING-mediated IFN production. To combat HNSCC, a nanosatellite vaccine composed of iron oxide nanoparticle cores coated with a biodegradable copolymer was utilized to deliver 2′3′-cGAMP to tip the immune balance away from immunosuppression. The cores were attached with gold nanoparticles as satellites and contained peptide antigens conjugated onto the surface. The nanosatellites significantly enhanced intracellular delivery of cGAMP into the cytosol as measured by increased mRNA levels in *IFNA4*, *IFNB1*, *ISG15*, *ISG54*, *CXCL9*, and *CXCL10*. In addition, the expression of CD86 and MHC II increased in bone marrow-derived dendritic cells (BMDCs). In an engineered MOC2-E6/E7 mouse oral squamous cell carcinoma model, the nanosatellites achieved a reduction in tumor growth and increased survival when compared to free peptide antigens, free 2′3′-cGAMP, and anti-PD-L1.

Manganese nanoparticles themselves have the propensity to activate the STING pathway through the release of $Mn^{2+}$ (Zhao et al., 2020b). The manganese does not activate STING directly, but rather induces cGAMP synthesis by binding to cGAS and concurrently promotes stronger binding affinity between cGAMP and STING (Wang et al., 2018). In an example work, amorphous porous manganese phosphate nanoparticles were loaded with DOX and coated with phospholipids (Hou et al., 2020). DOX is a common chemotherapeutic that not only eradicates tumor cells, but also promotes DNA damage to further enhance STING activation and generate immunogenic cell death (Casares et al., 2005), while phospholipid modification can help extend *in vivo* stability and circulation (Figure 7). It was shown that the formulation was pH responsive and could release DOX in response to the mildly acidic TME, thus preventing undesirable systemic drug exposure. In addition, phospholipase is overly expressed inside aggressive tumor cells, which further strengthened the specificity to the tumor site. In a 4T1 tumor model, intravenously administration of the DOX-loaded nanoparticles synergistically inhibited tumor growth compared to various controls. Tumor growth reduction was similarly seen in a distant tumor implanted post-treatment, indicating the generation of systemic antitumor immunity.

**4.2.4 Microparticles—**Although microparticles have limited lymphatic drainage due to their large size (Reddy et al., 2007), the application of microparticles for cancer therapy have been reported due to their ability to carry more payload and release the cargo over an extended period of time. To avoid frequent administrations and address the issue of patient compliance, a PLGA microparticle platform was developed to release STING agonists with varying kinetics (Lu et al., 2020). The microparticles were fabricated by soft lithography and contained a hollow rectangular base filled with 3′3′-cGAMP, which was then sealed with another PLGA cap. Individual microparticles could load up to 4 nL of solution, but with alternating dry and fill cycles, up to 10 μg of cGAMP could be loaded into a single particle. The release kinetics were fine-tuned by varying the lactide to glycolide ratio or the average molecular weight of the polymers, and a combination injection of 3 different polymeric microparticles achieved a pulsed release on days 4, 8, and 12. In a B16F10 model, a single intratumoral injection of the cGAMP-loaded microparticles showed tumor inhibition on par with four separate administrations of soluble 3′3′-cGAMP. The advantage of a single dose, pulsed release formulation was clearly shown in a pancreatic cancer allograft model due to the high difficulty in performing multiple injections. Compared to a bolus injection of free cGAMP at the same dosage, treatment with cGAMP-loaded microparticles resulted in significantly smaller tumors and fewer metastatic nodules in the lungs.

Microparticles have also been used to co-deliver STING and TLR agonists to the lymph nodes. In an example, acetalated dextran microparticles were incorporated with resiquimod (R848), a TLR7/8 agonist, and 3′3′-cGAMP (Collier et al., 2018). The acetal groups provided the particles with pH sensitivity, which facilitated rapid release of the payload inside the lysosomes after cellular uptake. BMDCs treated with the microparticles stimulated higher levels of IL-6, TNF-α, IL-1β, and IFN-β compared to free cGAMP and R848, as well as a mixture of microparticles loaded individually with either cGAMP or R848. Overall, the results suggested that codelivery of the two adjuvants was more effective than delivering them separately. The acetalated dextran microparticles also outperformed PLGA microparticles at the same dosage due to more rapid degradation and release. Intramuscular vaccination with the formulation using OVA as the antigen produced the highest levels of antibody titers, even slightly outperforming the alum positive control. Splenocytes from mice vaccinated with the microparticles that were restimulated *ex vivo* also had the highest amounts of IL-2 and IFN-γ secretion. In a follow up study on the same platform, it was shown that microparticles loaded with only 3′3′-cGAMP had the best antitumor activity in B16F10 tumors compared to microparticles loaded individually with poly(I:C), murabutide, or imiquimod, which are agonists for TLR3, NOD2, and TLR4, respectively (Watkins-Schulz et al., 2019). The results highlighted the advantages of cGAMP as an adjuvant for antitumor immunotherapy when compared to other commonly employed adjuvant systems. Efficacy was also observed in a E0771 TNBC tumor model.

### 4.3 STING nanovaccines for infectious diseases

STING agonists have been largely exploited as adjuvants in anticancer vaccines due to the difficulty in achieving proper immune activation against tumor antigens that originate from endogenous proteins. In the context of infectious diseases, generating immunity against foreign pathogens generally does not require the use of sophisticated adjuvant systems.

Nevertheless, adjuvants can be leveraged to help amplify the immune response and efficacy, and thus nanovaccines employing STING agonists have been developed against both viruses and bacteria.

**4.3.1 Antiviral nanovaccines—**Middle East respiratory syndrome coronavirus (MERS-CoV) is a respiratory virus closely related to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that caused the 2019 pandemic (de Wit et al., 2016). Treatments for both SARS-CoV-2 and MERS-CoV are limited (Zhang et al., 2020b; Zhou et al., 2021). As a measure to prevent future coronavirus pandemics, a hollow PLGA nanovaccine with CDG loaded inside and MERS-CoV receptor-binding domain (RBD) antigens on the surface was developed (Lin et al., 2019). The capsid-like nanoparticle structure mimics that of the actual virus and can evoke immune response similar to natural viruses (Figure 8). The polymeric shell contained an outer PEG layer for better pharmacokinetics and was acid-sensitive, allowing for burst release of the payload at lower pH values. Footpad injection of the formulation elevated IFN-β cytokine levels in the dLNs while no noticeable increase in TNF-α was found in the serum, which demonstrated the local immune priming capability of the nanoparticles while minimizing systemic inflammation. Subcutaneous vaccination on days 0 and 21 elicited significant antibody titers against the RBD antigen, and they remained elevated for at least 300 days. Higher numbers of functional $CD4^+IFN\text{-}\gamma^+$ T cells and $CD44^+CD62L^+$ central memory T cells were detected in the spleen, and more importantly, antigen-specific $CD8^+IFN\text{-}\gamma^+$ T cells were increased by nanoparticle vaccination. Profound protective efficacy was shown in a human DPP4 transgenic mice model, where prophylactic vaccination with the nanovaccine protected 100% of the mice from a lethal MERS-CoV challenge.

Polymeric nanovaccines have also been formulated against human immunodeficiency virus (HIV) (Aroh et al., 2017). Here, noncanonical cGAMP was loaded into PC7A polymeric nanoparticles, and the resulting formulation was able to inhibit replication of HIV-BaL, HIV-1 (IIIB), and HIV-1 (LAI) in peripheral blood mononuclear cells (PBMCs), a phenomenon that was not observed with the adjuvants poly(I:C), R848, and CpG. Efficacy appeared to be STING-specific, with type I IFNs playing a major role. More in-depth studies elucidated that monocyte-depleted PBMCs did not confer any protection, whereas B cell and NK cell depletion had minimal effects. Another STING-inducing nanovaccine for HIV treatment utilized liposomes as the carrier (Hanson et al., 2015). The formulation was loaded with CDG and included the membrane proximal external region (MPER) from HIV gp41 and gp120 tethered onto the surface. MPER is a lowly immunogenic antigen, so the incorporation of the STING agonist was used to help boost the immune response. Indeed, vaccination with the final formulation elicited 4-fold higher DC activation and 3-fold higher macrophage activation as compared to empty MPER liposomes administered with free CDG. Furthermore, a robust humoral response was generated while minimizing the induction of systemic inflammatory cytokines. However, despite the elevated antibody titer levels, sera from vaccinated mice failed to neutralize HIV.

Influenza virus is a highly mutative pathogen that mandates the development of new flu vaccines annually. To address this issue, several universal influenza vaccines have been proposed (Boyoglu-Barnum et al., 2021; Kanekiyo et al., 2019; Kanekiyo et al.,

2013). One formulation utilized pulmonary surfactant biomimetic liposomes encapsulating 2′3′-cGAMP as a mucosal adjuvant (Wang et al., 2020b). When the liposomes were intranasally administered along with inactivated A/California/7/2009 (CA09) H1N1 virus, IgG antibodies in the serum and IgA antibodies in the bronchoalveolar lavage fluid (BALF) were elevated by 10,000-fold and 60-fold, respectively, compared to immunization with the inactivated virus alone. Mechanistic studies elucidated that the negatively charged nanoformulation was taken up by alveolar macrophages with the assistance of surfactant proteins A and D. Upon cytosolic release, cGAMP not only activated the alveolar macrophages, but was also transferred into alveolar epithelial cells through gap junctions for activation (Figure 9). In addition to humoral responses, the nanoparticles augmented cellular immunity by activating $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells. A major benefit of this approach was that the vaccine formulation conferred protection against the influenza virus as early as 2 days after immunization. Early immunity was not mediated by innate immune responses, as the nanoparticles alone lacked efficacy, but rather due to rapid induction of $CD8^+$ T cells in both the lungs and BALF. Immunization provided broad protection against the seven heterosubtypic influenza viruses that were tested. Vaccinated mice had nearly 100% survival when challenged by the seven different substrains, whereas almost all unvaccinated mice succumbed to the infections.

Another influenza vaccine was developed to target the aging population, where vaccine potency tends to diminish due to immunosenescence (Ross et al., 2019). Polyanhydride nanoparticles loaded with the hemagglutinin and nucleoprotein antigens were synthesized through a double emulsion process. Concurrently, a pentablock copolymer micelle was fabricated to facilitate sustained antigenic release and promote drug delivery into the cytosol. While subcutaneous immunization with the two nanoparticles produced high levels of antibody titers and lowered viral loads in young mice, vaccine potency was significantly compromised in aged mice. Incorporation of RR-CDG into the formulation increased antibody titer levels by an order of magnitude and protected 60% of the animals from a lethal H1N1 challenge. Another unique platform against influenza consisted of acetalated dextran microparticles loaded with 3′3′-cGAMP (Junkins et al., 2018). For the antigenic target, HA protein derived from influenza strain A/Puerto Rico/8/1934 H1N1 (PR8) was adsorbed onto the microparticle surface. Intramuscular injection induced antibody titers 41-fold higher compared to free 3′3′-cGAMP, 600-fold higher than alum, and more than 5 orders of magnitude higher than HA alone. Furthermore, germinal center B cells in the dLNs and central memory $CD4^+$ and $CD8^+$ T cells in the spleen were significantly increased 14 days after immunization. The expanded immune cell populations helped to protect 12 out of 13 mice from a lethal challenge of PR8 influenza virus one month post vaccination, and all mice challenged seven months later.

**4.3.2 Antibacterial nanovaccines—**Even though CDNs originate from bacteria, the use of STING agonists in bacterial vaccines is much less common than with other applications. Polyanhydride nanoparticles loaded with F1-V antigens were combined with ML RR-CDG to treat pneumonic plague (Wagner et al., 2019). Polyanhydride nanoparticles are naturally immunostimulatory and have been frequently employed as adjuvant systems (Torres et al., 2011), so the combinatorial approach with ML RR-CDG presented a dual-

adjuvant system to further bolster the immune response. The F1-V antigen is derived from the V antigen and the F1 capsule of *Yersinia pestis*, a gram-negative bacterium that is responsible for the plague. Subcutaneous administration of the combination nanovaccine showed higher antibody titers against the F1-V antigen and strongly protected the animals from a lethal dose of *Y. pestis* CO92 challenge. Immunization with the antigen-loaded nanoparticles alone did not confer sufficient protection. Interestingly, co-administration of ML RR-CDG with soluble F1-V antigens elicited similar levels of antibodies and protected approximately 90% of mice from the challenge. However, when the dose of the bacteria was increased by two-fold, almost all mice vaccinated with the soluble antigens succumbed to the disease, while mice vaccinated with the nanoparticles maintained complete protection. A single dose of the combination nanovaccine induced long-lasting immunity, with a 75% survival rate in mice challenged 182 days after vaccination.

### 4.4 Nanovaccines outlook

STING-activating nanovaccines have shown considerable promise as therapeutics against cancer and as prophylaxes against infectious pathogens. However, almost all the examples discussed have focused on a single antigenic target; as such, mutations and antigenic escape can render the vaccine formulations ineffective (Denamur and Matic, 2006; Petrova and Russell, 2018; van der Burg et al., 2016). Multivalent vaccines can circumvent this issue by producing immunity against a broad range of relevant antigens and have a higher possibility to completely eradicate or prevent the targeted disease (Angsantikul et al., 2015; Fang et al., 2015; Singh, 2021). Along these lines, cell membrane coating technology is an emerging biomimetic technique utilized to fabricate immunocompatible and multivalent nanovaccines (Fang et al., 2018; Hu et al., 2015; Hu et al., 2011). The membrane from live cells can be isolated and coated onto diverse substrates, including nanoparticles (Fang et al., 2014; Hu et al., 2013b; Wei et al., 2016), nanofibers (Chen et al., 2016b), micromotors (Esteban-Fernández de Ávila et al., 2018; Esteban-Fernandez de Avila et al., 2018; Wei et al., 2019a), and even two dimensional nanomaterials (Gong et al., 2019; Kumar et al., 2019), to bestow new biological functionalities. Cell membrane coating technology has proven successful with a wide range of different membrane sources, including immune cells (Thamphiwatana et al., 2017; Wei et al., 2018; Zhang et al., 2018a), stem cells (Bose et al., 2018; Gao et al., 2016a; Gao et al., 2016b), exosomes (Liu et al., 2019a; Yong et al., 2019; Zhao et al., 2020a), bacterial membranes (Chen et al., 2020a; Gao et al., 2015; Zhang et al., 2019b), and fusion membranes (Chen et al., 2020b; Dehaini et al., 2017; Liu et al., 2019b). Because the entire cellular membrane is employed, nanovaccines produced in this fashion are naturally multiantigenic and can evoke broad immune protection without the need for labor-intensive studies to identify and fully characterize individual antigens. Multivalent cell membrane-coated nanovaccines have been successfully implemented against cancer (Jiang et al., 2020b; Kroll et al., 2017a; Yang et al., 2018) and bacteria (Gao et al., 2015; Hu et al., 2013a; Wang et al., 2016; Wei et al., 2017; Wei et al., 2019b). Combined with STING agonists, these nanovaccines have the potential to effect more powerful, long-lasting, and multivalent immunity against various diseases. In addition, membrane-cloaked platforms have been engineered for effective cytosolic delivery through mechanisms such as endosomal escape (Zhuang et al., 2020) or direct fusion with the plasma membrane (Gong et al., 2021).

An example application of the cell membrane coating technology with STING agonists was very recently reported (Yang et al., 2021). The nanovaccine leveraged manganese dioxide nanoparticles and DiR, a photothermal agent, encapsulated inside B16F10 cancer cell membrane vesicles. Besides providing a multivalent source of tumor antigens, cancer cell membrane has natural homotypic targeting properties (Fang et al., 2014). Upon reaching the TME, the manganese dioxide nanoparticles, which can stimulate STING responses through $Mn^{2+}$ release, were rapidly degraded by hydrogen peroxide and hydronium ions (Figure 10). Reaction of the manganese dioxide nanoparticles in the TME restored pH levels back to normal and generated oxygen to alleviate tumor hypoxia. The remaining DiR-loaded membrane vesicles were exploited for photothermal therapy to further enhance tumor killing and local antigen release. Intravenous administration of the nanoparticles resulted in the best antitumor efficacy in a primary tumor model when combined with laser irradiation. At the tumor site, a significant number of $CD4^+$ and $CD8^+$ T cells infiltrated into the tumor, while the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells was low. In a surgical resection recurrence model, large tumors were initially treated with the formulation before the primary tumor was removed in its entirety. When rechallenged 12 days later after surgery with a secondary tumor on the contralateral flank, 3 out of 5 mice that received the nanoformulation along with photothermal therapy did not exhibit tumor growth. Similar effects were observed in a bilateral tumor model where only the primary tumor was subjected to laser treatment. In a metastatic melanoma model, the treatment significantly inhibited tumor nodule formation in the lungs. The impressive results achieved by this biomimetic nanoplatform provides a glimpse into the rationale combination of cell membrane coating nanotechnology with STING agonists to achieve broad immune responses.

## 5. Conclusions

In this review, we have summarized the recent advances in nanovaccines that exploit the STING signaling pathway to combat cancer, viral infection, and bacterial infection. STING is a PRR with numerous roles in the cancer–immunity cycle and during pathogenic infections. Proper activation of the pathway can potently evoke type I IFN-mediated innate and adaptive immune responses, which can be leveraged in disease treatment. While research in the field is still at its infancy, a wide range of different STING agonists have been discovered and synthesized, greatly expanding the toolkit available for research and development. However, soluble agonists currently suffer from low bioavailability and have difficulty traversing the plasma membrane. On this front, nanoparticles have been utilized to more effectively deliver STING agonists for vaccine applications. Because STING is located in the cytosol, nanocarriers offer the ability for enhanced intracellular delivery while also improving safety. Nanovaccines applied in this fashion have demonstrated considerable potential against cancers and infectious diseases. Ultimately, continued research along these lines will lead to the development of innovative immunotherapeutic platforms that can reshape how we approach the clinical management of a wide range of diseases.

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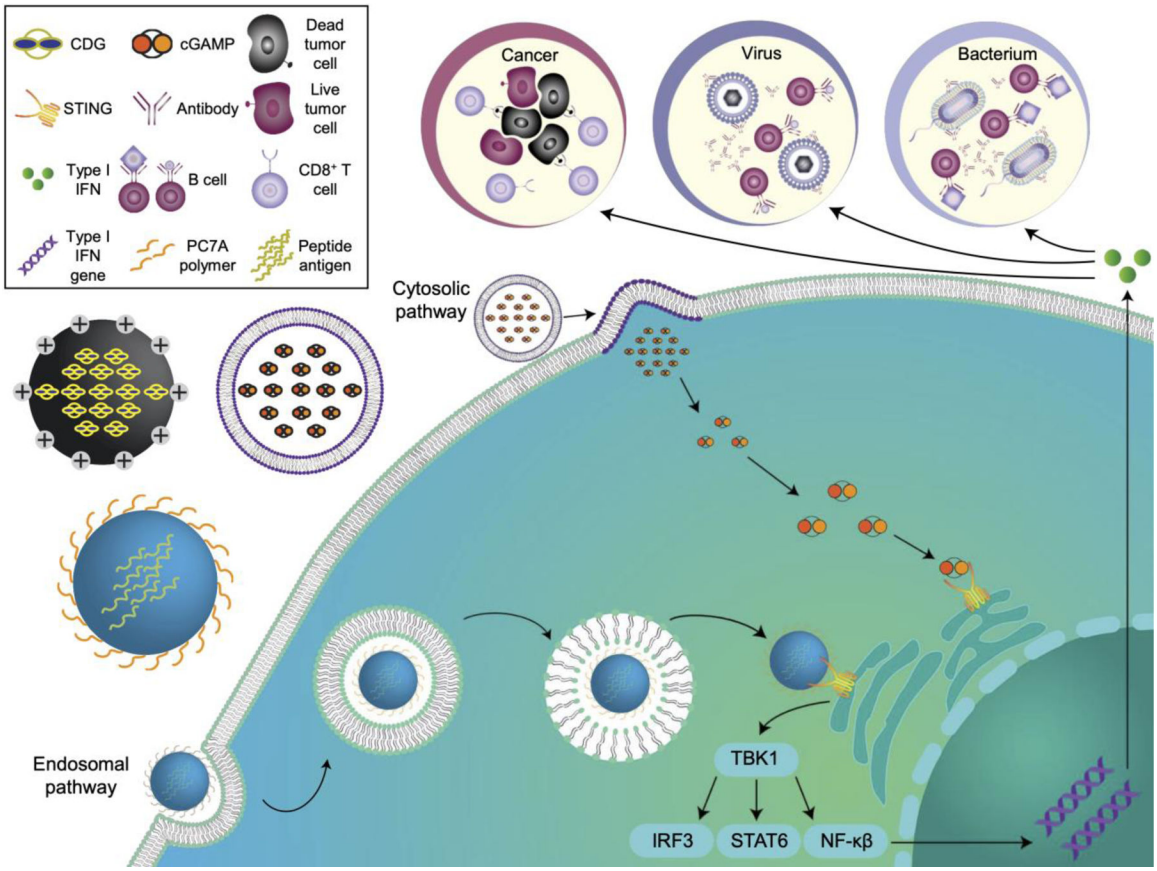


**Figure 1.**

STING-activating nanovaccines against cancer, virus and bacterium. STING nanovaccines can be synthesized from various nanomaterials to deliver payloads intracellularly. After endocytosis, pH-responsive nanovaccines can escape from the endosome and engage with STING in the cytosol. In the cytosolic pathway, the nanovaccines can fuse directly with the plasma membrane to release the encapsulated payload into the cytosol. Once activated, STING complexes with TANK-binding kinase 1 (TBK1) and phosphorylates interferon regulatory factor 3 (IRF3), signal transducer and activator of transcription 6 (STAT6), or nuclear factor-ϰB (NF-ϰB) to stimulate the production of type I interferons (IFNs).

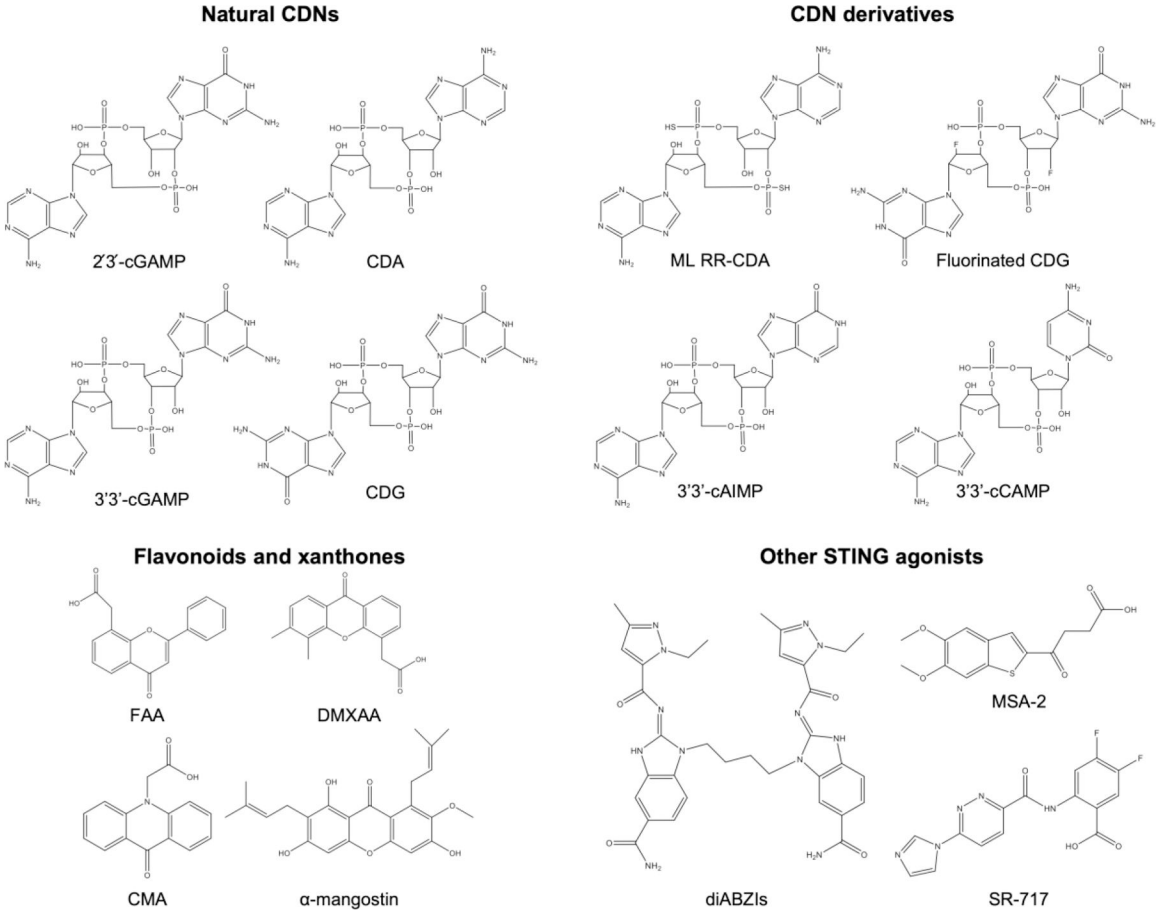


**Figure 2.**

Chemical structures of various STING agonists. STING agonists can be generally divided into four main groups: natural cyclic dinucleotides (CDNs), derivatives of CDNs, flavonoids and xanthones, and other STING agonists.

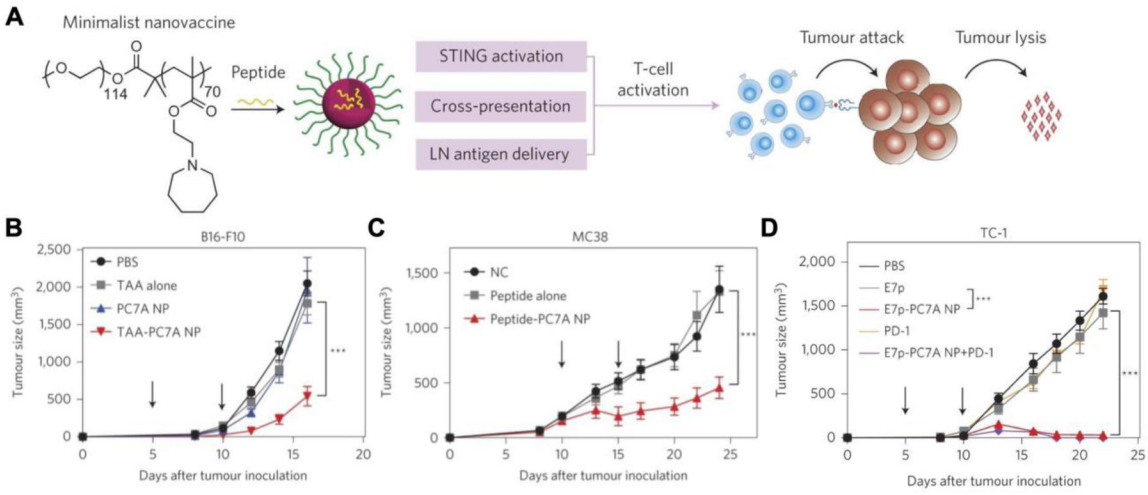


**Figure 3.**

PC7A polymer nanovaccines for anticancer therapy. A) Neoantigen peptides mixed with PC7A polymers self-assemble into nanovaccines. Once administered, the PC7A nanovaccine can deliver the peptides to the cytosol, and the polymers can directly activate STING to generate antigen-specific T cells. B) Treatment with a PC7A nanovaccine loaded with tumor associated antigens (TAAs) slows the growth B16F10 melanoma tumors. C) A neoantigen peptide-loaded PC7A nanovaccine effectively controls tumor growth in an MC38 colorectal cancer model. D) Combination of anti-PD-1 checkpoint blockade with an antigen peptide-loaded PC7A nanovaccine inhibits tumor growth in a TC-1 model. Reproduced with permission (Luo et al., 2017b). Copyright 2017, Springer Nature.

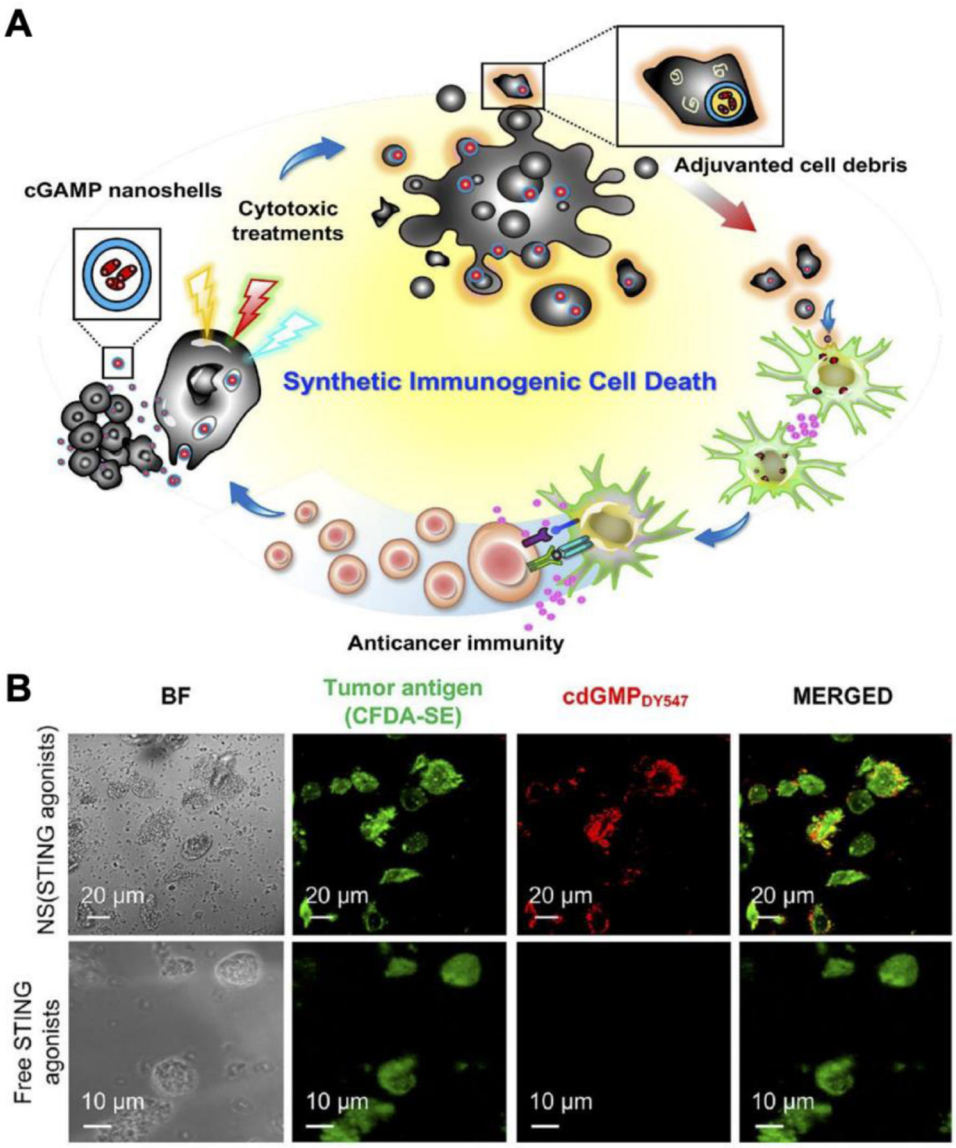


**Figure 4.**

Nanoshells loaded with cGAMP to induce synthetic immunogenic cell death for chemoimmunotherapy. A) cGAMP-loaded nanoshells can be readily loaded into dying tumor cells treated with chemotherapeutics. Upon phagocytosis, nanoparticle-laden cancer cells can stimulate an antitumor immune response. B) Fluorescently labeled nanoshells are significantly colocalized with dead tumor cells compared to free CDG. Reproduced with permission (Chattopadhyay et al., 2020). Copyright 2020, American Chemistry Society.

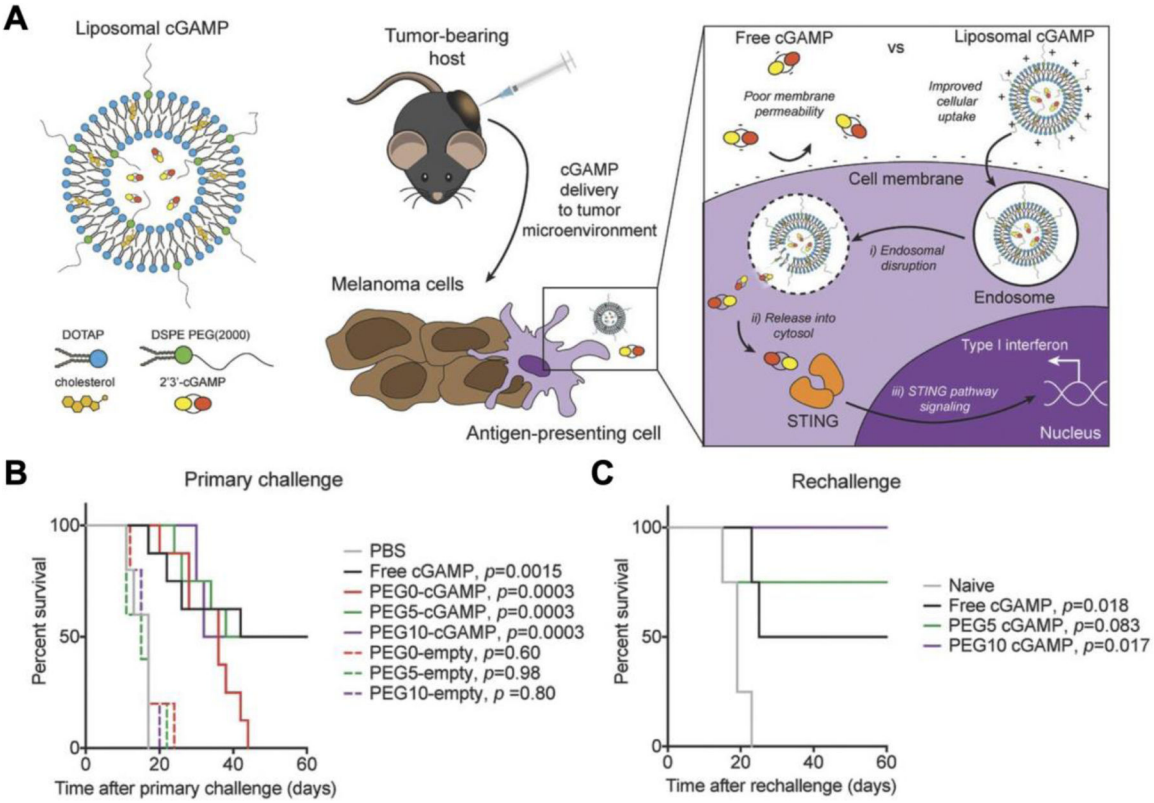


**Figure 5.**

Cationic liposomes enhance cytosolic delivery of cGAMP for melanoma treatment. A) Noncanonical cGAMP is encapsulated inside liposomes fabricated from cholesterol and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). The liposomes more readily facilitate intracellular delivery of cGAMP to activate the STING pathway. B) Treatment with PEGylated liposomal formulations prolongs survival in a B16F10 melanoma model. C) After treatment with cGAMP-loaded liposomes, a significant portion of mice are protected from a tumor rechallenge. Reproduced with permission (Koshy et al., 2017). Copyright 2017, Wiley-VCH.

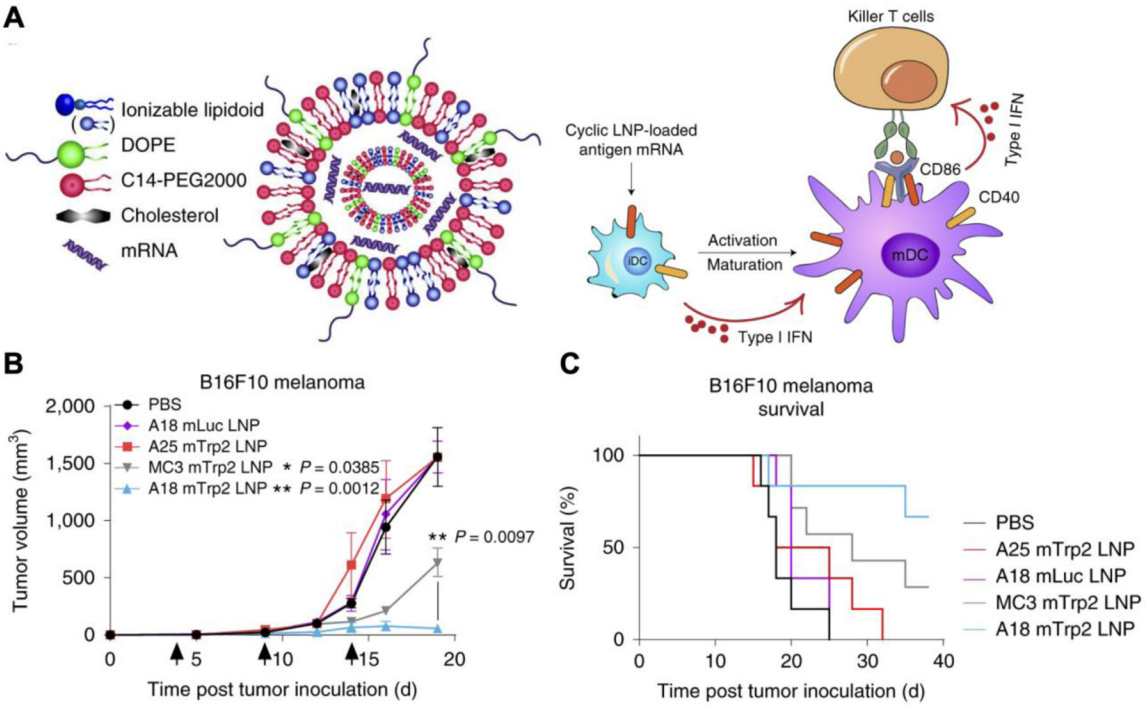


**Figure 6.**

Liposomal mRNA vaccine directly activates the STING pathway to treat melanoma. A) Heterocyclic lipid nanoparticles (LNPs) are composed of an ionizable lipidoid for STING activation and mRNA encoding for tumor antigens. Cellular uptake of the LNPs induces dendritic cell (DC) maturation and potentiates STING-mediated immunity against tumor cells via type I interferon (IFN) production. B,C) A18 LNPs with mRNA encoding the melanoma antigen TRP2 control tumor growth (B) and extend survival (C) in a B16F10 melanoma model. Reproduced with permission (Miao et al., 2019). Copyright 2019, Springer Nature.

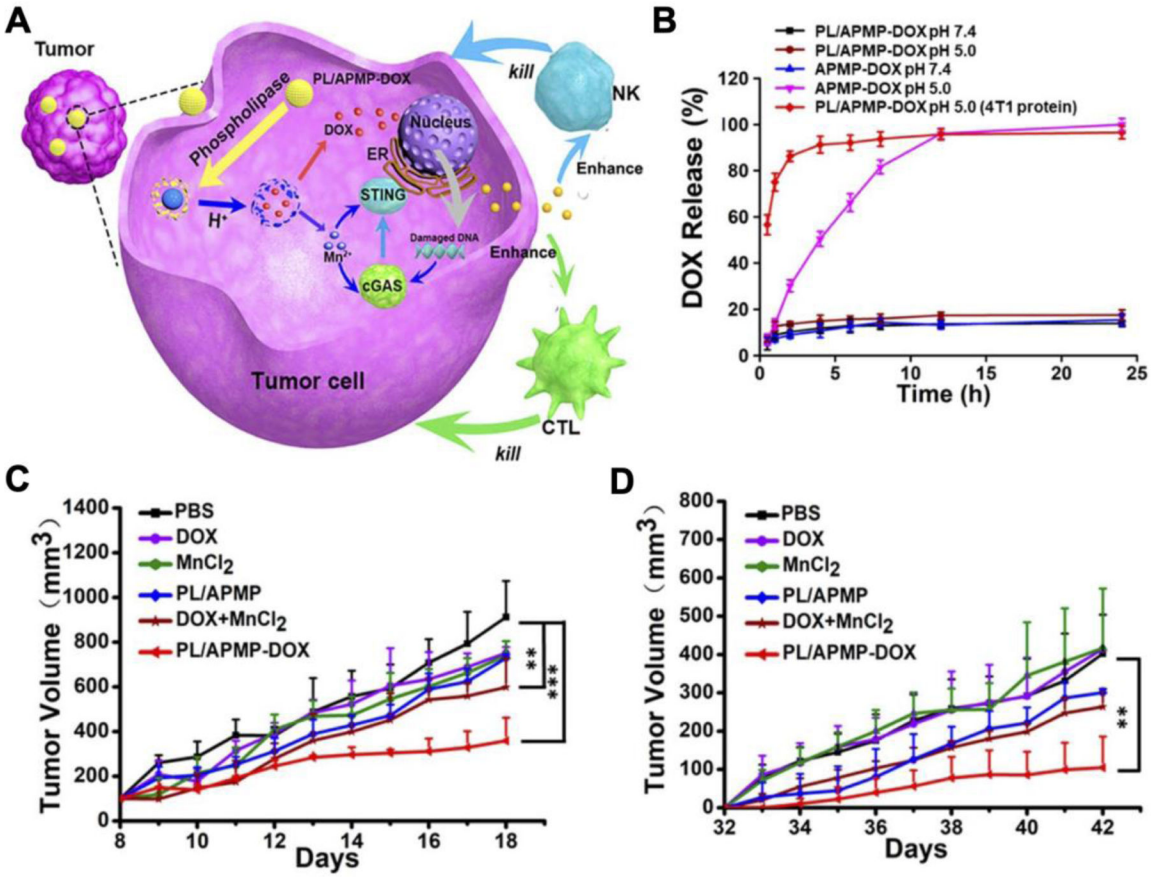


**Figure 7.**

Manganese nanoparticles loaded with doxorubicin (DOX) for synergistic immunotherapy against 4T1 breast cancer. A) The degradation of DOX-loaded amorphous porous manganese phosphate nanoparticles coated with phospholipid (PL/APMP-DOX) is facilitated by phospholipase within tumor cells. DOX causes damaged DNA to release from the nucleus, while manganese ions ($Mn^{2+}$) facilitate STING activation to promote immune responses mediated by natural killer (NK) cells and cytotoxic T lymphocytes (CTL). B) DOX is rapidly released at low pH and in the presence of phospholipase, which are significantly upregulated in cancerous cells. C,D) Intravenous treatment with PL/APMP-DOX reduces growth in both the primary tumor (C) and a distant secondary tumor (D) inoculated post-treatment. Reproduced with permission (Hou et al., 2020). Copyright 2020, American Chemistry Society.

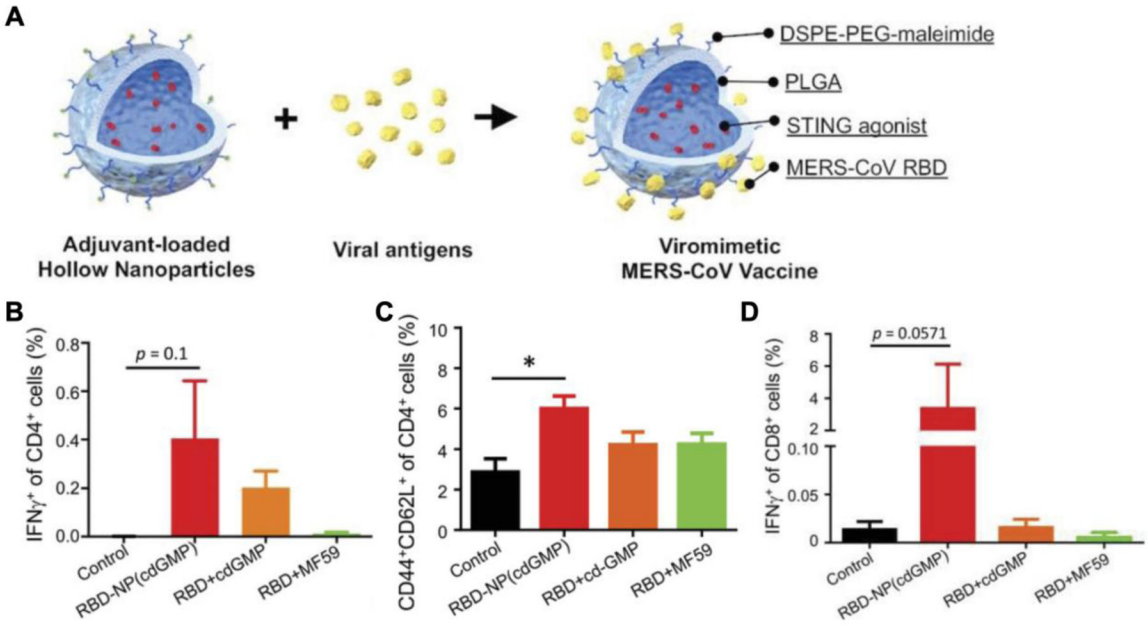


**Figure 8.**

Virus-like particles loaded with CDG as a vaccine against Middle East respiratory syndrome coronavirus (MERS-CoV). A) Hollow PLGA nanoparticles are loaded with CDG as an adjuvant and engineered to display recombinant MERS-CoV antigens on the surface to mimic the structure of natural viruses. B-D) Vaccination with the viromimetic nanoparticles induces high proportions of $CD4^+IFN\text{-}\gamma^+$ functional T cells (B), $CD4^+CD44^+CD62L^+$ central memory T cells (C), and $CD8^+IFN\text{-}\gamma^+$ effector T cells (D). Reproduced with permission (Lin et al., 2019). Copyright 2019, Wiley-VCH.

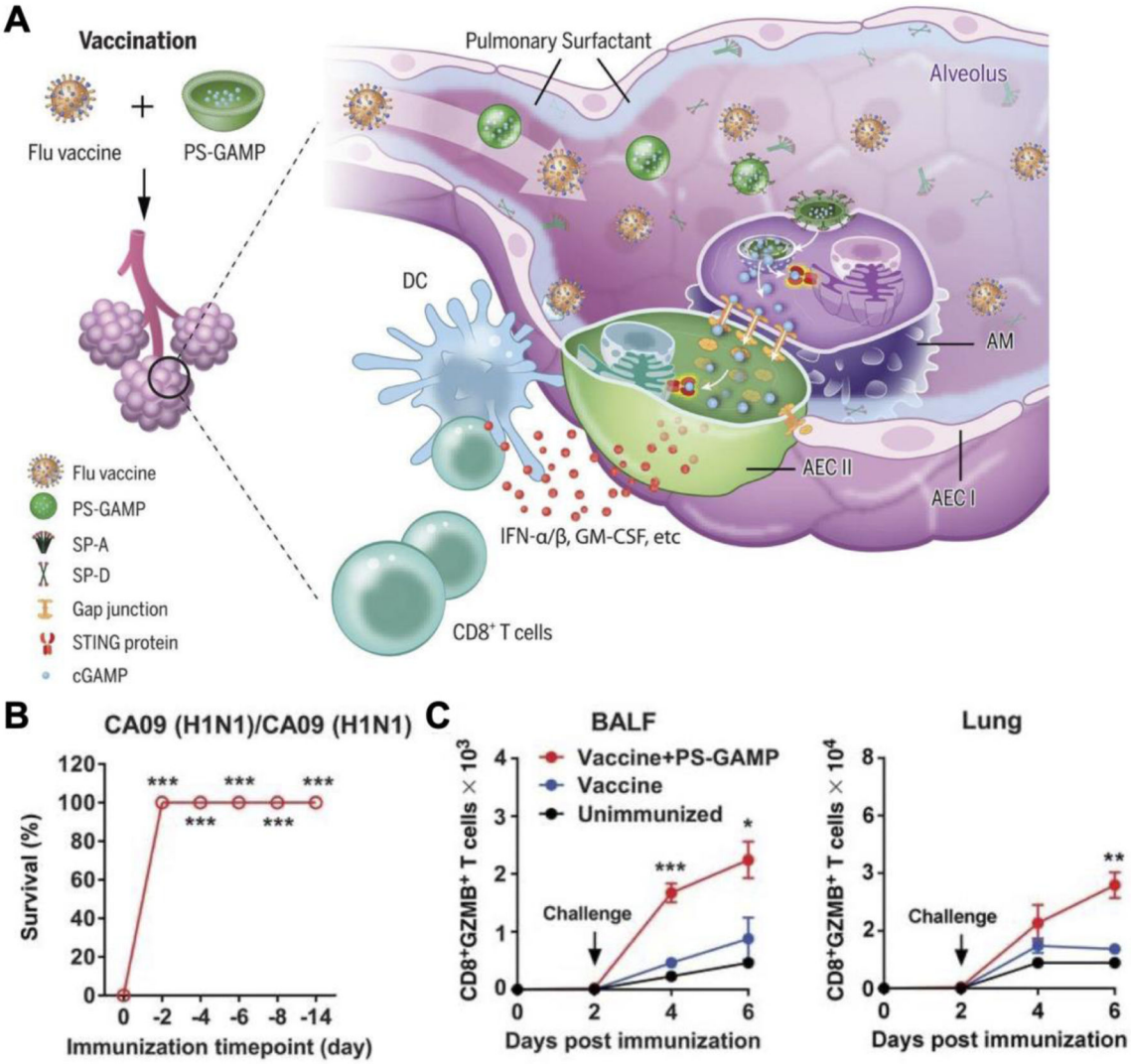


**Figure 9.**

Biomimetic liposomal cGAMP co-administered with a flu vaccine to protect against influenza. A) Liposomes modified with biomimetic surfactants and loaded with cGAMP are mixed with inactivated influenza virus as a mucosal vaccine. The surfactant aids adjuvant transport across the epithelium and into alveolar macrophages to induce a rapid and broad immune response against different substrains of influenza. B) Immunized animals are rapidly protected from lethal viral challenges. C) The adjuvanted vaccine promotes high numbers of $CD8^+$Granzyme $B^+$ (GZMB) effector T cells in the bronchoalveolar lavage fluid (BALF) and the lungs shortly after immunization. Reproduced with permission (Wang et al., 2020b). Copyright 2019, American Association for the Advancement of Science.

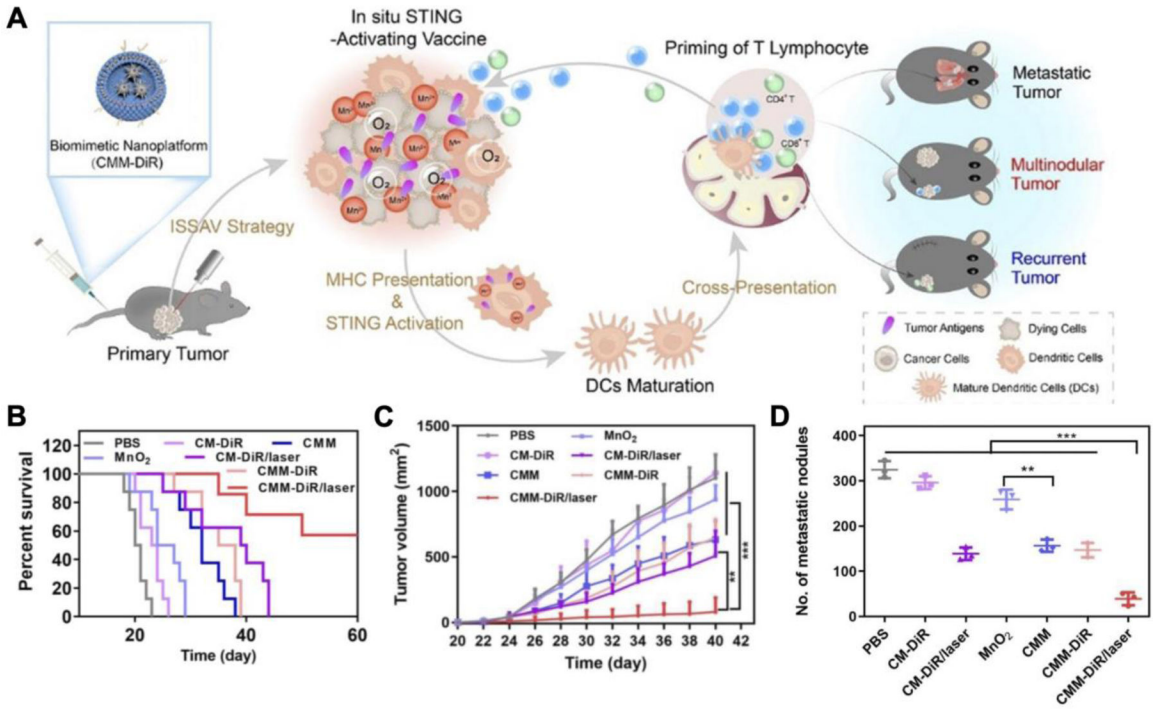


**Figure 10.**

Cancer cell membrane-coated manganese dioxide ($MnO_2$) nanoparticles loaded with DiR (CMM-DiR) as a multivalent nanovaccine platform. A) CMM-DiR preferentially accumulates at the tumor site after intravenous injection due to homotypic targeting. At the tumor, the $MnO_2$ nanoparticles are rapidly degraded into manganese ions ($Mn^{2+}$) for STING activation, while the remaining nanovesicles can undergo photothermal therapy to amplify antigenic release. Activated dendritic cells (DCs) can take up tumor neoantigens locally and trigger an antigen-specific immune response. B) Combination of CMM-DiR with laser treatment increases survival rates in a primary melanoma model. C) Growth of secondary tumors implanted after the removal of treated primary tumors is significantly controlled by the combinatorial therapy. D) Metastatic nodules in the lungs of mice intravenously challenged with B16F10 after primary tumor treatment are significantly reduced by the combination treatment. Reproduced with permission (Yang et al., 2021). Copyright 2021, Elsevier.