CHEMICAL REVIEWS

Chemical and Biomolecular Strategies for STING Pathway Activation in Cancer Immunotherapy

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Abstract

The stimulator of interferon genes (STING) cellular signaling pathway is a promising target for cancer immunotherapy. Activation of the intracellular STING protein triggers the production of a multifaceted array of immunostimulatory molecules, which, in the proper context, can drive dendritic cell maturation, antitumor macrophage polarization, T cell priming and activation, natural killer cell activation, vascular reprogramming, and/or cancer cell death, resulting in immune-mediated tumor elimination and generation of antitumor immune memory. Accordingly, there is a significant amount of ongoing preclinical and clinical research towards further understanding the role of the STING pathway in cancer immune surveillance as well as the development of modulators of the pathway as a strategy to stimulate antitumor immunity. Yet, the efficacy of STING pathway agonists is limited by many drug delivery and pharmacological challenges. Depending on the class of STING agonist and the desired administration route, these may include poor drug stability, immunocellular toxicity, immune-related adverse events, limited tumor or lymph node targeting and/or retention, low cellular uptake and intracellular delivery, and a complex dependence on the magnitude and kinetics of STING signaling. This review provides a concise summary of the STING pathway, highlighting recent biological developments, immunological consequences, and implications for drug delivery. This review also offers a critical analysis of an expanding arsenal of chemical strategies that are being employed to enhance the efficacy, safety, and/or clinical utility of STING pathway agonists and lastly draws attention to several opportunities for therapeutic advancements.

Key Words: biomaterials, cancer immunotherapy, cyclic guanosine monophosphate–adenosine monophosphate synthase, drug delivery, immuno-engineering, immuno-oncology, nanomedicine, stimulator of interferon genes.

Contents

1. Introduction

The stimulator of interferon genes (STING) cellular signaling pathway has profound importance for the health and survival of a large diversity of organisms (*e.g.* humans, sea anemones, fruit flies, *etc.*)[1], due to its critical role in the immune-mediated elimination of numerous pathogens and diseases[2]. Accordingly, elements of the STING pathway have been evolutionarily conserved within metazoans for over 600 million years through natural selection[3-5]. Since the relatively recent scientific discovery of the STING protein in 2008, the pathway has been extensively characterized, and a growing number of infectious pathogens and diseases have been found to stimulate host immune responses by initiating STING signaling[6-10].

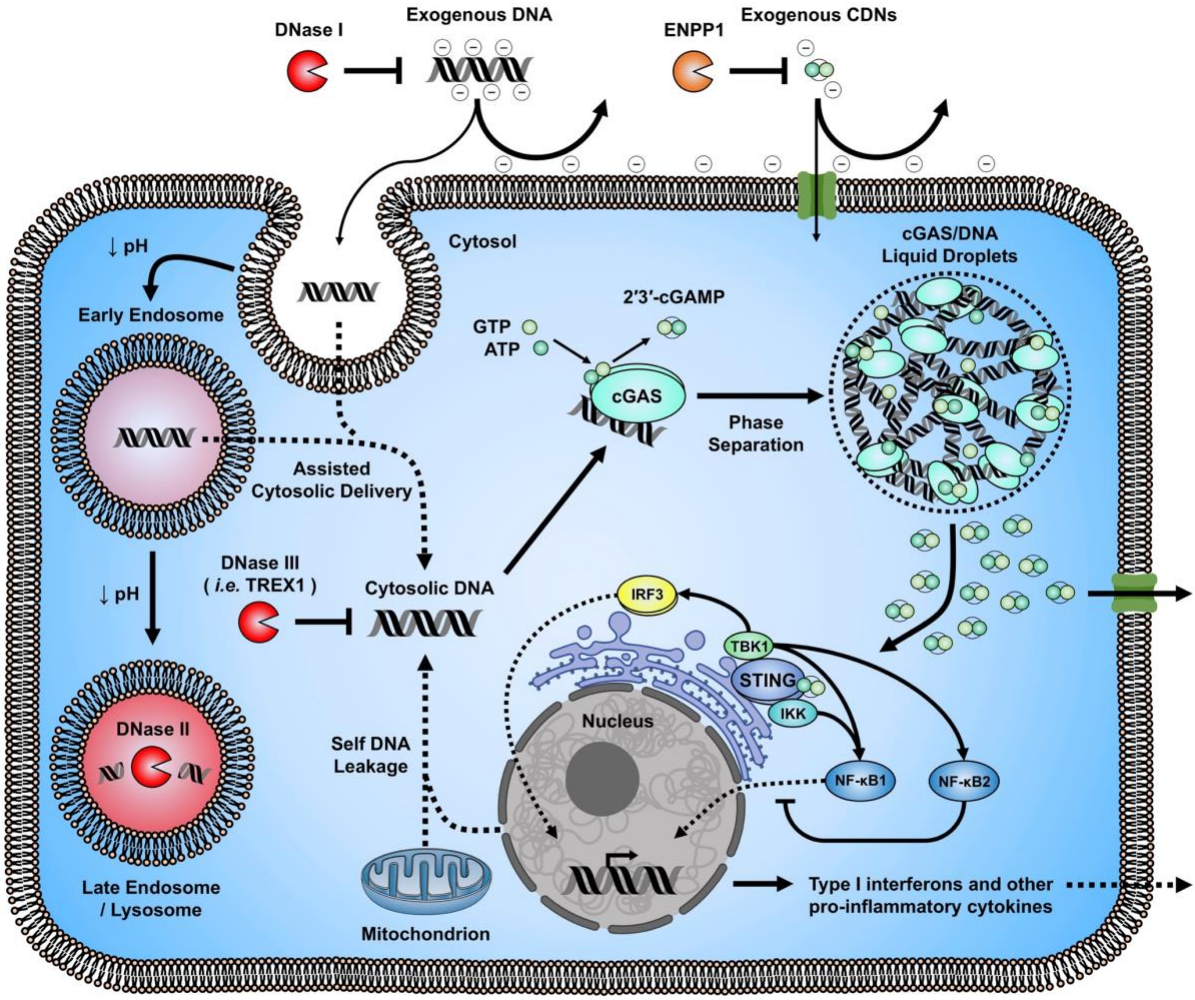

Figure 1: *The stimulator of interferon genes (STING) cellular signaling pathway*.

The cGAS enzyme surveils the cytosol of cells for the accumulation of double-stranded DNA, which serves an indicator of cellular malfunction or infection. Notably, cytosolic double-stranded DNA may arise intrinsically (*e.g.* self-DNA leakage from nucleus or mitochondria) or extrinsically (*e.g.* pathogen-derived). Upon recognition (*i.e.* binding) of double-stranded DNA in the cytosol,

cGAS oligomerizes into liquid-like droplets and catalyzes the production of 2′3′-cGAMP, which can bind and activate the STING protein on the endoplasmic reticulum to initiate downstream signaling, primarily through TBK1 and IKK. Notably, STING activation typically leads to the activation of the transcription factors, IRF3 and NF-κB1 as well as NF-κB2, which is known to partially inhibit the activity of NF-κB1. STING signaling results in the production of IFN-I and various other proinflammatory cytokines, the profile of which largely depends on context. Lastly, 2′3′-cGAMP can also vacate its cell of origin through various transport mechanisms and function as an immunotransmitter that can locally propagate STING signaling in neighboring cells. To pharmacologically activate the signaling pathway, STING pathway agonists (*i.e.* cGAS agonists and STING agonists) must cross the cell membrane, access the cytosol, and evade degradation by various deoxyribonucleases (DNases) and phosphatases. Due to its relatively large size and negative charge, exogenous DNA requires assistance (*e.g.* pathogen-mediated delivery) to penetrate cellular membranes and gain access the cytosol. Furthermore, DNA is highly susceptible to degradation by DNase I in the extracellular space, DNase II (*i.e.* Acid DNase) during natural endolysosomal trafficking, and DNase III (*i.e.* TREX1) in cytosols. Alternatively, CDNs can utilize various membrane channels and transporters to access the cytosol, though the use of such transfer modalities is relatively inefficient and typically requires high local concentrations of CDNs. Moreover, certain naturally occurring CDNs, including 2′3′-cGAMP, are highly susceptible to degradation by ENPP1 in the extracellular space. Figure created with *biorender.com*.

The STING pathway continuously monitors the cytosol of cells for certain “danger signals” (*i.e.* anomalies that are indicative of cellular distress) as part of a network of cytosolic pattern recognition receptors of the innate immune system – referred to as cytosolic immune surveillance. Molecular recognition of such irregularities within the cytosol initiates STING signaling (***Figure 1***), which then propagates a coordinated distress signal that is primarily directed by the cellular production of various proinflammatory cytokines[1, 11, 12]. The distress signal ultimately summons an innate immune response that can galvanize the immune system to address a myriad of potential threats. Notably, the immunostimulatory attributes of STING signaling distinguish the pathway as a prime target for applications in cancer immunotherapy (*i.e.* therapies that either involve or use components of the immune system for the treatment of cancer patients).

The specific downstream effects of STING pathway activation can be largely variable, as they depend heavily on cellular context as well as signal intensity and duration[13]. However, a distinctive feature of mammalian STING signaling is the secretion of interferons (IFNs)[14], especially type I IFNs (IFN-I) such as IFN-β[15, 16], which is known to exhibit pleiotropic effects on cell function[17-19]. Notably, the type I IFN signature of STING activation has been linked to enhanced antigen-specific T cell responses[14, 17, 18] and natural killer (NK) cell responses[20] that collectively drive cell-mediated immunity. In certain settings, STING signaling can also induce

various forms of programmed cell death, such as autophagy, apoptosis, necroptosis, and lysosomal cell death[21, 22]. Thus, the versatile nature of downstream STING signaling imparts cells with the ability to elicit a context-dependent immune response that can ultimately result in the clearance of diseased cells[15, 23, 24].

In 2012, it was discovered that the therapeutic efficacy of the small molecule cancer therapeutic, 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) was STING-dependent, establishing that pharmacological activation of STING signaling in solid tumors could promote antitumor responses in mice with established cancer[25]. Shortly thereafter, in 2014, the STING pathway was found to have a central role in preventing the onset of cancer in mice through tumor immune surveillance[26]. The STING pathway was thus identified as a promising target for cancer immunotherapy owing to its natural role in initiating and propagating endogenous immune responses to cancer. Moreover, it has now also been shown that many standard-of-care cancer treatments (*e.g.* DNA-damaging chemotherapies and radiotherapy) may promote additional therapeutic benefits through iatrogenic STING pathway activation[27-29]. Collectively, these findings have inspired the development of synthetic STING pathway agonists for cancer immunotherapy. Preclinical research using STING agonists to treat cancer has been exceptionally successful for generating antitumor immunity against a wide range of cancer types, which has prompted numerous clinical trials, many of which are ongoing (***Table 1***).

While STING pathway agonists offer considerable promise for cancer immunotherapy as both a monotherapy and an adjunct to current standard-of-care cancer treatments, none have yet reached the pharmaceutical market. As we will describe, the clinical landscape of STING pathway agonists is rapidly evolving with a number of promising candidates in clinical trials that may soon yield the first approval of a STING agonist for cancer immunotherapy by the US Food and Drug Administration (FDA). Nonetheless, both the efficacy and safety of STING-activating therapeutics are restricted by many drug delivery and pharmacological challenges, including poor drug stability, immunocellular toxicity, immune-related adverse events, limited tumor or lymph node (LN) targeting and/or retention, low cellular uptake and intracellular delivery, and a complex dependence on the magnitude and kinetics of STING signaling[30, 31]. In this review, a detailed summary of the STING pathway as well as a synopsis of chemical strategies to enhance the efficacy, safety, and/or clinical utility of STING pathway agonists are presented.

Table 1: *Clinical Trials of STING agonists for Cancer Therapy*.

Phase 2 Clinical Trials:	**Active Compound**	**Route of Delivery**	**Sponsor and *Collaborators***	**Trial Identifier**	**Status**
MIW815 +/- Pembrolizumab in Head and Neck Cancer	MIW815 (ADU-S100): Synthetic CDN STING Agonist	Intratumoral	Aduro Biotech, Inc.	NCT03937141	Active; Not Recruiting
MK-1454 +/- Pembrolizumab in Head and Neck Cancer	MK-1454: Synthetic CDN STING Agonist	Intratumoral	Merck Sharp & Dohme Corp.	NCT04220866	Active; Not Recruiting
Phase 1/2 Clinical Trials:	**Active Compound**	**Route of Delivery**	**Sponsor and *Collaborators***	**Trial Identifier**	**Status**
CDK 002 in Advanced/Metastatic, Recurrent, Injectable Solid Tumors	CDK 002 (exoSTING): PTGFRN-Targeted Exosome containing Synthetic CDN STING Agonist	Intratumoral	Codiak BioSciences	NCT04592484	Recruiting
Phase 1 Clinical Trials:	**Active Compound**	**Route of Delivery**	**Sponsor and *Collaborators***	**Trial Identifier**	**Status**
MIW815 +/- Spartalizumab in Advanced Solid Tumors or Lymphomas	MIW815 (ADU-S100): Synthetic CDN STING Agonist	Intratumoral	Novartis Pharmaceuticals	NCT03172936	Completed
MIW815 +/- Ipilimumab in Advanced Solid Tumors or Lymphomas	MIW815 (ADU-S100): Synthetic CDN STING Agonist	Intratumoral	Aduro Biotech, Inc. *Novartis Pharmaceuticals*	NCT02675439	Active; Not Recruiting
E7766 in Non-muscle Invasive Bladder Cancer	E7766: Synthetic CDN STING Agonist	Intravesical	Eisai Inc. *H3 Biomedicine Inc.*	NCT04109092	Withdrawn
E7766 in Advanced Solid Tumors or Lymphomas	E7766: Synthetic CDN STING Agonist	Intratumoral	Eisai Inc. *H3 Biomedicine Inc.*	NCT04144140	Recruiting
MK-1454 +/- Pembrolizumab in Advanced Solid Tumors or Lymphomas	MK-1454: Synthetic CDN STING Agonist	Intratumoral	Merck Sharp & Dohme Corp.	NCT03010176	Active; Not Recruiting
MK-2118 +/- Pembrolizumab in Advanced Solid Tumors or Lymphomas	MK-2118: STING Agonist	Intratumoral / Subcutaneous	Merck Sharp & Dohme Corp.	NCT03249792	Recruiting
SB 11285 +/- Nivolumab in Advanced Solid Tumors	SB 11285: Synthetic CDN STING Agonist	Intravenous	Spring Bank Pharmaceuticals, Inc.	NCT04096638	Recruiting
GSK3745417 in Advanced Solid Tumors	GSK3745417: Small Molecule STING Agonist	Intravenous	GlaxoSmithKline	NCT03843359	Recruiting
BMS-986301 +/- Nivolumab or Ipilimumab in Advanced Solid Cancers	BMS-986301: Small Molecule STING Agonist	Intratumoral / Intramuscular	Bristol-Myers Squibb	NCT03956680	Recruiting
SYNB1891 +/- Atezolizumab in Advanced Solid Tumors and Lymphoma	SYNB1891: *E. coli* STING Agonist	Intratumoral	Synlogic *IQVIA Biotech*	NCT04167137	Recruiting
BI 1387446 +/- Ezabenlimab in Advanced Solid Tumors	BI 1387446 (BI-STING): Synthetic CDN STING Agonist	Intratumoral	Boehringer Ingelheim	NCT04147234	Recruiting
TAK-676 +/- Pembrolizumab in Advanced Solid Tumors	TAK-676: Small Molecule STING Agonist	Intravenous	Takeda	NCT04420884	Recruiting
SNX281 +/- Pembrolizumab in Advanced Solid Tumors	SNX281: Small Molecule STING Agonist	Intravenous	Stingthera, Inc.	NCT04609579	Recruiting
IMSA101 +/- Immune Checkpoint Inhibitor in Advanced Treatment-Refractory Malignancies	IMSA101: Synthetic CDN STING Agonist	Intratumoral	ImmuneSensor Therapeutics Inc.	NCT04020185	Recruiting

2. Biochemistry and Biology of the STING Pathway

There are a number of ways through which STING signaling can be initiated. However, activation of the intracellular STING protein, or more specifically, translocation of STING to the Golgi is invariably required for the downstream STING signaling that can trigger innate immune activation[32-35]. In its resting state, the STING protein is localized on the surface of the endoplasmic reticulum[36] and is canonically activated by cyclic dinucleotides (CDNs)[37]. Alternatively, STING can also be directly bound and activated by several other chemical agents, many of which will be discussed in detail in this review.

Endogenous activation of the STING protein is largely dependent upon the recognition (*i.e.* binding) of the self-derived CDN, 2′3′-cyclic guanosine monophosphate – adenosine monophosphate (2′3′-cGAMP)[38, 39]. At the forefront of the STING pathway, 2′3′-cGAMP is produced intracellularly by cGAMP synthase (cGAS) after the enzyme detects the aberrant presence of double-stranded DNA (dsDNA) in the cytosol of cells. Thus, both cGAS and STING act as general sensors (*i.e.* pattern recognition receptors) for pathogens and pathologies that induce the cytosolic accumulation of such danger signals[40].

2.1 Recognition of Cytosolic DNA by cGAS

Under normal conditions, the cytosol of cells is largely DNA free, and any nominal amount of DNA that may be present is rapidly degraded by cytosolic nucleases. Accordingly, the accumulation of DNA within the cytosol is indicative of pathogenic threats or compromised cellular states. Mammals express numerous DNA sensors that are capable of detecting and communicating such breaches in cellular homeostasis. Many of these DNA sensors can provoke IFN-I responses to activate innate immunity in response to the abnormal accumulation of either extrinsic or misplaced-self dsDNA within the cytosol[41]. Extrinsic DNA can infiltrate the cytosol through a variety of mechanisms (*e.g.* tumor-derived exosomes, viral infection, *etc.*), while intrinsic, self-DNA derived from mitochondria, chromosomes, or endogenous retroelements can accumulate in the cytosol in response to cellular stress or genetic mutation (***Figure 1***) [10, 42-45]. Notably, many cancerous cells have an established capacity for releasing endogenous nuclear DNA into the cytosol[46-48], which likely contributes to the natural role of the cGAS/STING pathway in both tumor immune surveillance and spontaneous antitumor immunity.

Upstream of STING in the pathway, cGAS is considered to be the predominant contributor to endogenous STING activation following the detection of cytosolic DNA. However, some of the

other cytosolic DNA sensors (*e.g.* DDX41, IFI16, DAI, RNA pol III, LRRFIP1, *etc.*) can also initiate IFN-I responses through STING signaling[49], either in conjunction with cGAS or even in the absence of cGAS[50]. Notably, cGAS is itself an IFN-stimulated gene (ISG)[51], and therefore, in cells with low baseline cGAS expression, cytosolic DNA can initially trigger other DNA sensors. The resultant IFN-I response can then lead to local cGAS production and subsequent cGAS activation if the DNA persists long enough within the cytosol (*e.g.* prolonged viral challenge), thereby increasing the magnitude of the IFN-I response in a positive feedback manner.

The activation of cGAS by dsDNA has been extensively characterized through many structural and biochemical studies[52-57]. Briefly, cGAS exhibits an autoinhibited conformation in its unbound, monomeric form. Positively charged sites on the C-terminal domain (CTD) of cGAS bind the sugar-phosphate backbone of dsDNA. Steric interactions between cGAS and the bound DNA induce conformational transitions in cGAS that open the nucleotide binding pocket, which is also located on the CTD. The DNA strands serve as natural crosslinkers to promote cGAS oligomerization[54]. The dsDNA/cGAS oligomeric complexes undergo liquid–liquid phase separations within the cytosol, forming liquid-like droplets that function as intracellular microreactors for 2′3′-cGAMP production[12, 58]. The activated cGAS enzymes catalyze the production of 2′3′-cGAMP from intracellular adenosine triphosphate (ATP) and guanosine triphosphate (GTP)[38, 39]. The enzymatic synthesis occurs in a stepwise manner through the initial generation of 5′-pppG(2′,5′)pA prior to cyclization to c[G(2′,5′)pA(3′,5′)p][57]. Notably, 2′3′-cGAMP has mixed 2′,5′ and 3′,5′ phosphodiester bonds (c[G(2′,5′)pA(3′,5′)p]) in contrast to bacteria-derived CDNs, which exclusively have two uniform 3′,5′ phosphodiester bonds[57, 59, 60] (***Figure 2***). The biological consequences of CDN linkage orientation are discussed in detail in ***Section 4.1***.

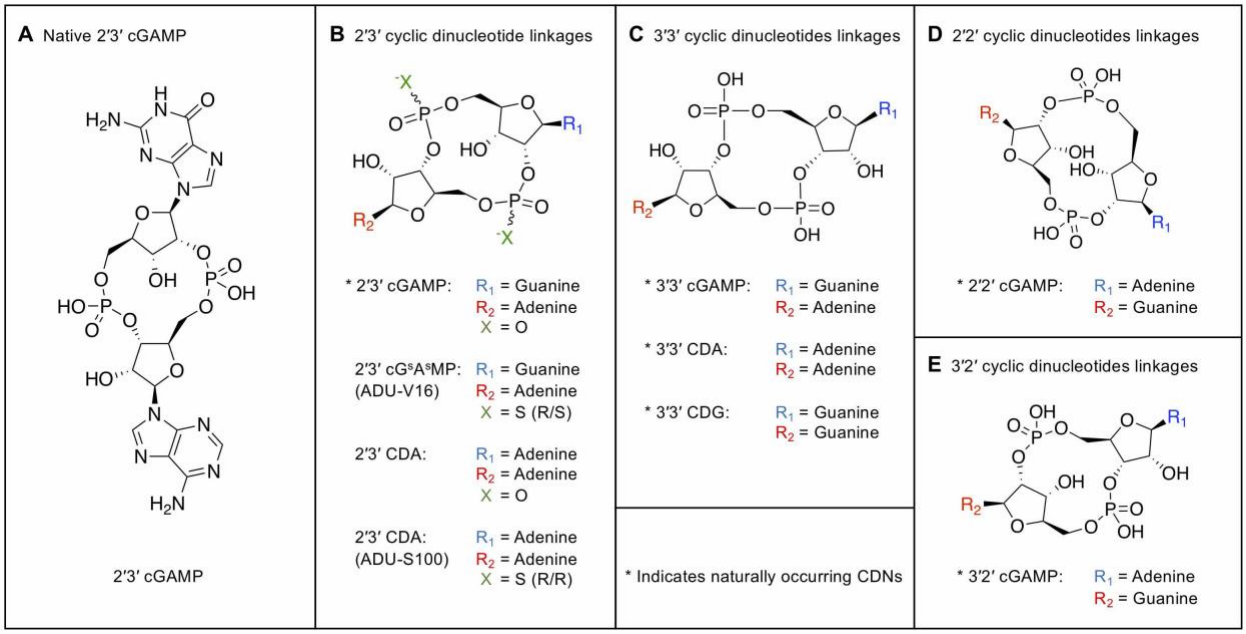

Figure 2: *Chemical structures of cyclic dinucleotide (CDN) STING agonists*.

(A) Mammalian 2′3′-cGAMP. **(B)** Various naturally occurring or synthetic CDNs with the noncanonical 2′3′ linkage orientation that is produced by mammals. **(C)** Various naturally occurring CDNs with the canonical 3′3′ linkage orientation that is produced by bacteria. **(D)** Synthetic 2′2′-cGAMP with the noncanonical 2′2′ linkage orientation that has not yet been found in nature. **(E)** Naturally occurring 3′2′-cGAMP with the noncanonical 3′2′ linkage orientation that is produced by *Drosophila melanogaster* (*i.e.* fruit flies).

The recognition of dsDNA by cGAS is largely sequence-independent, and the length of dsDNA that is empirically required *in vitro* for minimal cGAS activation in cell-based assays varies by species (*e.g.* ~ 45 base pairs (bp) in humans, ~ 20 bp in mice)[61, 62]. With only a few exceptions[63], short strands of dsDNA under these length thresholds cannot activate cGAS in any meaningful way, as they are unable to induce the formation of the liquid-like droplets that stabilize the dsDNA/cGAS complex through multivalent interactions[58]. This is largely due to the relatively low affinity of dsDNA for cGAS, the dissociation constant (K_D) of which has been estimated to be ~ 1−2 μM[52, 54]. Notably, the phase-separation of the liquid-like droplets stabilizes the dsDNA/cGAS complexes through more than just enhanced colocalization. The liquid-like droplets sequester the cGAS and dsDNA molecules, thereby providing a barrier that limits the physical access of DNA nucleases that would otherwise degrade the dsDNA ligands[64]. Prolonged protection from such negative regulators is especially important for cGAS, as it is considered an unusually slow enzyme with one round of cGAMP synthesis taking ~ 20 seconds[65].

The cGAS enzyme is allosterically activated by dsDNA in a length-dependent manner, such that binding longer strands of dsDNA increases the presence and stability of the active dsDNA/cGAS biocondensates and thereby increases the local production of 2′3′-cGAMP[66, 67]. Accordingly, the length of cytosolic dsDNA is a critically important determinant of both the magnitude and profile of the resultant immune response. The length-dependent cGAS activation is most pronounced at physiologically relevant low dsDNA concentrations that are comparable to that of self dsDNA sensing and viral infection (*e.g.* ~ 17 fg/cell for herpes simplex virus 1)[13, 66]. At low dsDNA concentrations (*e.g.* 15 ng/mL), which are representative of natural exposure, dsDNA that is technically above the length threshold for activation (*e.g.* 100 bp) fails to induce a measurable response, while much longer dsDNA (*e.g.* 2000 bp) is still capable of efficiently inducing STING signaling[66]. Notably, at the high dsDNA concentrations (*e.g.* 1 µg/mL or greater) that are often assessed *in vitro*, cGAS activity can be saturated using a relatively low molecular weight dsDNA (*e.g.* ~ 60 kDa), likely through substrate exhaustion (*i.e.* depletion of cellular ATP and/or GTP)[66].

Cytosolic dsDNA can also activate the protein known as absent in melanoma 2 (AIM2)[68], which has noteworthy implications for STING signaling. AIM2 is another prominent pattern recognition receptor for cytosolic dsDNA and is known to modulate STING signaling[69-74]. Activation of AIM2 characteristically results in pyroptosis-mediated cell death and the release of IL-1β and IL-18 via the AIM2 inflammasome. Concurrent activation of AIM2 and cGAS in antigen presenting cells (APCs) broadens the resultant cytokine response, but it also reduces the magnitude of STING-specific cytokines produced[70]. The dampened STING signaling caused by simultaneous AIM2 activation is largely due to the pyroptosis induced by AIM2. At the onset of AIM2-induced pyroptosis, gasdermin D pokes small holes in the cellular membrane. The pores in the cellular membrane enable a potassium efflux from the cell, which then inhibits cGAS activation prior to cell death[74].

AIM2 evolved as an innate immune sensor much more recently than cGAS (*i.e.* ~ 110 million years ago[75] versus ~ 600 million years ago[3]) and is entirely orthologous between murine and human species[76]. Notably, AIM2 is minimally activated by relatively longer dsDNA (*i.e.* ~ 80 bp)[77, 78]; robust activation of cGAS and AIM2 at *in vitro* concentrations of ~ 1 µg/mL generally requires dsDNA lengths of at least ~ 100 bp and ~ 200 bp, respectively[67, 79-83]. Though, as previously stated, dsDNA length thresholds for *in vitro* activation do not necessarily directly correspond with thresholds for *in vivo* activation, because cells within a living organism do not

naturally experience such high cytosolic dsDNA concentrations even under stressed cellular conditions. Future research investigating the interplay between cGAS/STING signaling and the AIM2 inflammasome in a cancer setting will be necessary to define the impact of such dual activation on antitumor immunity.

The primary effector function of AIM2 activation is to induce cell-death, which is a digital (non-tunable) process that does not depend on an allosteric equilibrium[84, 85]. The AIM2 inflammasome does not disassemble after it has formed on sufficiently long cytosolic dsDNA, and the assembly of the AIM2 inflammasome is reinforced by multiple positive feedback loops, which supports a binary signaling response[83]. Conversely, cGAS activation is tunable and the downstream response can be quite variable and setting specific[13, 86, 87]. STING signaling can evoke diverse stress responses that range from the suppression of viral replication to apoptosis depending on signal strength, signaling duration, and cellular context[10, 13, 15, 88-90].

2.2 Regulation of cGAS

Mammalian DNA is primarily packaged and compartmentalized inside the nuclei and mitochondria of cells and therefore typically avoids contact with cGAS[91]. However, nominal amounts of self dsDNA routinely enter the cytosol under normal cellular conditions[10, 42-45]. Mammals have evolved to locally restrict intrinsic activation of pattern recognition receptors to a baseline level by constitutively expressing deoxyribonucleases (DNases)[10, 92, 93]. DNase I, DNase II, and TREX1 (*i.e.* DNase III) actively degrade dsDNA in systemic circulation, lysosomes, and cytosols, respectively[44, 94-96]. The cytosolic exonuclease, TREX1 directly affects the length, concentration, and persistence of dsDNA within the cytosol, and consequently, is critically important for negatively regulating cGAS activity[97-100].

TREX1 deficiency has been linked to many type I interferonopathies caused by overactive STING signaling. Most notably, mutations in the *TREX1* gene cause Aicardi-Goutières syndrome (AGS) and have also been associated with many other autoimmune diseases, including both familial chilblain lupus and systemic lupus erythematosus[100]. Interestingly, the genes encoding cGAS and TREX1 are both prominent ISGs and thus they contribute to local regulatory feedback loops that can either amplify or restrict the subsequent immune response in various settings[51, 101]. Recently, the intratumoral inhibition of TREX1 has even been proposed as a novel immunotherapeutic strategy to promote local STING signaling for the treatment of cancer[102]. Notably, radiotherapy-induced tumor immunogenicity is strongly negatively regulated by TREX1

at high doses of radiation (*i.e.* 12−18 gray)[103, 104]. It has been shown that reactive oxygen species (ROS), a biproduct of ionizing radiation[105] can oxidize intracellular DNA bases[106], which can then partially inhibit TREX1-mediated degradation through steric hindrance to perpetuate STING signaling during radiotherapy[107, 108]. However, TREX1 inhibition via oxidized bases is contingent upon low TREX1 concentrations (*e.g.* ~ 50 nM or less); high concentrations of TREX1 (*e.g.* ~ 200 nM or greater) can efficiently degrade DNA containing oxidized bases[109]. Thus, the observed dose-dependent regulation of radiotherapy-induced tumor immunogenicity by TREX1 may be explained by dose-dependent ISG expression, where higher doses of radiation lead to higher concentrations of TREX1, which can then degrade oxidized dsDNA and thereby limit the extent of cGAS activation. In support of this theory, it was determined that consecutive low doses of radiation (*i.e.* 3x 8 gray) could circumvent TREX1-mediated cGAS inhibition[103]. Nevertheless, TREX1 represents a formidable obstacle for all DNA-based cGAS-activating cancer therapies and must therefore be given careful consideration when designing such therapeutic approaches.

In addition to TREX1, there are numerous other factors that can significantly influence the intensity of STING signaling in a particular tissue and therefore alter the nature of the resultant immune response. The activity of cGAS is known to be intricately regulated by many different post-translational modifications of cGAS, such as acetylation, glutamylation, phosphorylation, sumoylation, and ubiquitination[110-115]. Post-translational modifications are heavily dependent on environmental conditions and therefore likely contribute to cell-type specific STING signaling. cGAS activation is also vitally dependent on the ability of cGAS to encounter its dsDNA substrate, which is undoubtedly a function of the protein's spatiotemporal distribution within cells.

The subcellular localization of cGAS is currently a subject of controversy and seems to be quite dynamic in nature depending on cell cycle phase, cell type, and environmental conditions[116]. Until recently, cGAS has generally been regarded as a strictly cytosolic protein[38]; however, recent studies have challenged this theory. In murine bone marrow-derived macrophages (BMDMs) and in human THP1 monocytes, it was determined that cGAS primarily resides on the interior of the plasma membrane due to the electrostatic interactions of the N terminus of cGAS with the membrane-bound $PI(4,5)P_2$ phospholipid[117]. The intracellular localization of cGAS to the plasma membrane was found to limit the recognition of self dsDNA by spatial segregation from the nucleus and simultaneously maximize the potential response to viral infection by allowing for a more rapid encounter with exogenous DNA.

cGAS has also been identified within the nuclei of mammalian cells[38, 118-120]. Outside of the canonical STING signaling axis, cGAS has an established secondary function, where it operates as a negative regulator of DNA repair, inhibiting homologous recombination in the nucleus[121, 122]. Several research groups currently contend that cGAS is constitutively present in the nuclei of cells at steady state[122-124]. One study found that the non-catalytic N terminal domain of cGAS was responsible for an association of cGAS with the centromeres of chromosomes within the nuclear compartment[123]. More recently, another study has asserted that cGAS is predominantly a nuclear protein that is tethered tightly to intact chromatin by a salt-resistant interaction in its resting state[124]. The researchers found that cGAS was resistant to standard salt-based elution, requiring relatively high salt concentrations for complete solubilization (*e.g.* 0.75 M NaCl compared to the 420 mM NaCl that is typically used to isolate nuclear proteins). They have suggested that the observed tight interactions of cGAS in the nucleus cannot be explained by its relatively low intrinsic affinity for DNA (*e.g.* K_D ~ 1−2 μM).

These disparate findings indicated that the N terminus of cGAS was dispensable for nuclear localization; instead, the core of human cGAS, composed of a bilobed nucleotidyltransferase structure bridged by an alpha-helical spine, was required for the observed nuclear tethering. It was noted that the amino acid residues, which are important for nuclear tethering, partially overlap with one of the DNA-binding surfaces of cGAS. Consequently, a model of “regulated desequestration” was proposed, which proclaims that cGAS is inactive while chromatin-bound and that there exists an unknown regulated step prior to the assembly of cGAS onto dsDNA that enables its release from chromatin and subsequent activation.

Chromatin tethering is indeed one of several regulatory mechanisms that can inhibit cGAS activation at times where immune activation is unnecessary (*e.g.* cell division)[110, 125, 126]. Specifically, chromatin tethering can prevent the oligomerization of cGAS that is necessary for liquid-like droplet formation and efficient 2′3′-cGAMP synthesis[126]. Accordingly, the tethering of cGAS to chromatin actually increases during mitosis when the nuclear envelope breaks down, so as to prevent spurious activation of cGAS while DNA is exposed to the cytosol[119, 127]. Further research in this area may lead to the discovery and characterization of the aforementioned unknown regulatory mechanism that is responsible for the release of cGAS from nuclear chromatin, which may thereby enable targeted strategies for controlling the degree of cGAS activation to enhance cancer therapies.

2.3 Regulation of STING

After cGAS catalyzes the synthesis of 2′3′-cGAMP, the CDN acts as a second messenger that binds and activates STING proteins on the endoplasmic reticulum[36, 39, 59]. STING comprises four transmembrane helices coupled to a cytoplasmic ligand-binding and signaling domain[128]. The transmembrane and cytoplasmic regions naturally interact to form a domain-swapped homodimer in its resting form[129]. Two intertwined STING molecules take the shape of an opened butterfly with the head toward the membrane (***Figure 3A***)[130]. Upon binding 2′3′-cGAMP, the STING homodimer undergoes extensive conformational rearrangements. While 2′3′-cGAMP induces closure of the ligand-binding domain, it is important to note that not all agonists of STING provoke a closed lid confirmation (***Figure 3B***). Indeed, several STING agonists (*e.g.* the bacteria-derived CDN, cyclic di-guanosine monophosphate (c-di-GMP)) promote STING oligomerization and exhibit immunostimulatory activity without rearrangement of the lid region (***Figure 3C***)[131-133].

Activated STING proteins oligomerize, are ubiquitinated, and then traverse the Golgi apparatus, whereupon they are palmitoylated and traffic to submicrometer-sized perinuclear vesicles (*i.e.* STING translocators)[32, 134-138]. Following translocation though the Golgi body, TANK-binding kinase 1 (TBK1) binds and phosphorylates STING[139, 140]. Notably, TBK1 recruitment to STING has been identified as essential for STING-mediated antitumor immunity[141]. The STING/TBK1 complex phosphorylates interferon regulatory factor 3 (IRF3), which then homodimerizes and navigates into the nucleus to induce target gene expression[136, 142-144].

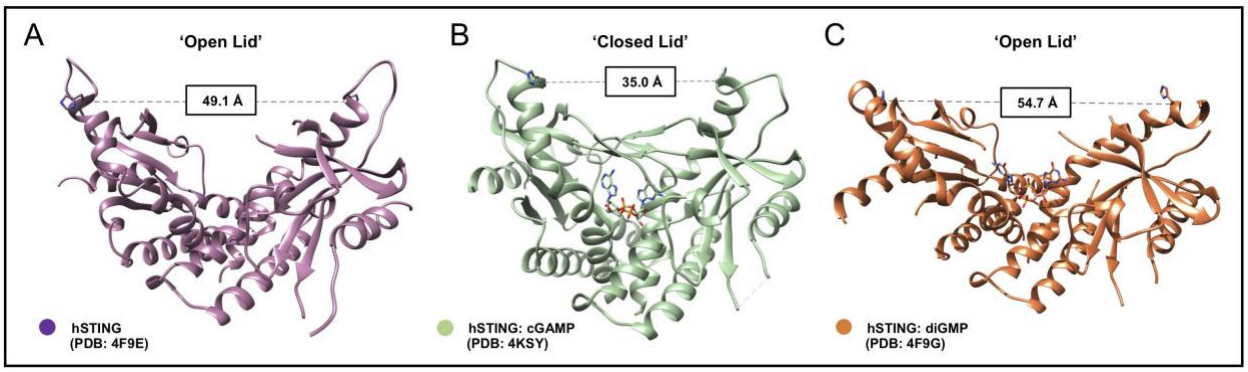

Figure 3: *Crystal Structures of symmetrical human STING dimers*.

(A) The resting 'Open Lid' configuration of an apo (*i.e.* unbound) human STING dimer. Adapted with permissions from reference[145]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc. (PDB ID: 4F9E)[131]. Copyright © 2012 Elsevier Science & Technology Journal; permission conveyed through Copyright Clearance Center, Inc. **(B)** The 'Closed Lid' configuration of a holo (*i.e.* ligand bound) human STING dimer bound to 2′3′-cGAMP. Adapted with permissions from reference[145].

Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc. (PDB ID: 4KSY)[59]. Copyright © 2013 Elsevier Science & Technology Journal; permission conveyed through Copyright Clearance Center, Inc. **(C)** The 'Open Lid' configuration of a holo (*i.e.* ligand bound) human STING dimer bound to 3′3′-diGMP. Adapted with permissions from reference[145]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc. (PDB ID: 4F9G)[131]. Copyright © 2012 Elsevier Science & Technology Journal; permission conveyed through Copyright Clearance Center, Inc.

Similar to the liquid phase condensation of cGAS that is triggered by activation of the endogenous STING pathway[58], untranslocated ER-resident STING can also undergo a liquid-liquid phase separation[146]. However, unlike the liquid-like droplets of activated cGAS that enhance STING signaling, the STING condensates contain inactive STING proteins and negatively regulate the pathway by preventing the translocation of STING that is necessary for downstream signaling[147]. When intracellular 2′3′-cGAMP concentrations reached a certain threshold (*e.g.* 1 μg/mL *in vitro*), which is above the threshold for the initial activation of STING by 2′3′-cGAMP (*e.g.* K_D ~ 4.59 nM), STING condensates form as micrometer-sized granules that colocalize with the ER. Additionally, when present in an exceptionally high concentration (*e.g.* 6 μg/mL *in vitro*), 2′3′-cGAMP also further induces a fluid-to-gel transition of the STING condensates that significantly decreases their internal molecular mobility. Notably, the STING condensates also formed in response to the bacterial CDN, c-di-GMP. It is currently unclear whether the phase separation of STING occurs in response to all of the known STING agonists or just CDNs. It is also unknown if constitutively active STING mutants trigger the assembly of the STING phase-separator.

While most hydrogels of biocondensates formed by protein liquid-liquid phase separation are largely disordered or assemble into polymeric fibrils[148, 149], the STING condensates, now termed the STING phase-separator, surprisingly comprise a highly organized membranous structure that resembles jigsaw puzzles. Following DNA virus infection, formation of active STING translocators occurred 3 hours post infection and peaked at 8 hours, whereas inhibitory STING condensates peaked at 20 hours. Thus, formation of the STING phase-separator is a partially delayed response and serves to prevent overactivation of STING and inhibit excessive innate immune signaling.

Additional transcription factors synergize with IRF3 to direct context-dependent antiviral gene expression[150]. In various settings, STING signaling has been associated with the activation of canonical and non-canonical nuclear factor κB (NF-κB), mitogen-activated protein (MAP)

kinases, and signal transducer and activator of transcription (STAT) transcription factors[36, 151-154]. Notably, efficient production of IFN-β, a hallmark of STING signaling, relies on the cooperative assembly of the enhanceosome, a higher order transcription enhancer complex[155, 156]. Individual transcription factors of the enhanceosome, such as IRF3 and canonical NF-κB, cannot initiate IFN-β gene expression by themselves[157, 158]. Instead, they must work in conjunction with each other and several other enhancer components for maximal gene transcription[16]. Indeed, a 50% decrease in IFN-β production was observed in primary mouse embryonic fibroblast cells when canonical NF-κB expression was partially silenced via RNA interference (RNAi)[151].

The intricacy of the enhanceosome elegantly highlights the importance of synergy between multiple inducible transcription factors[159]. Thus, in addition to post-translational modifications of STING pathway constituents, the combinatorial regulation of gene transcription likely contributes to cell-type specific STING signaling, as it is largely responsible for the selective protein expression that occurs in various environmental conditions. Accordingly, a better understanding of the transcriptional regulation that ensues STING activation in various cell types could lead to more efficacious cancer immunotherapies designed to differentially regulate the expression of certain STING-stimulated proteins to enhance antitumor effects and minimize unnecessary off-target effects.

Single nucleotide polymorphisms in the STING protein are responsible for existence of distinct human STING (hSTING) isoforms that exhibit variable intrinsic activity as well as distinctive reactivity to various STING agonists[57, 160, 161]. The five most prominent haplotypes of hSTING are known as **WT** (R232), **HAQ** (R71H, G230A, R293Q), **REF** (R232H), **AQ** (G230A, R293Q), and **Q** (R293Q), and their allelic frequencies in the human population are 57.9%, 20.4%, 13.7%, 5.2%, and 1.5%, respectively[15, 160]. Relative to the other major variants, hSTING^{HAQ} generally exhibits lower intrinsic IFN-I and NF-κB activity, which has been attributed to the R71H substitution that likely affects the protein's resting localization to the endoplasmic reticulum[160, 162, 163].

There are many agonist-specific differences in the recognition and activation of the various STING isoforms that can be attributed to the unique chemical structures of the STING agonists. While bacteria-derived CDNs can activate murine STING (mSTING) and certain hSTING variants, they do not appreciably activate the hSTING^{REF} or hSTING^{Q} isoforms[15, 160, 161]. Alternatively, endogenous 2′3′-cGAMP can activate mSTING as well as all 5 of the major hSTING variants[15, 59].

However, whether 2′3′-cGAMP is a weak agonist for certain hSTING isoforms is currently a controversial topic. Some researchers have reported that for hSTING^{REF}, 2′3′-cGAMP is weaker agonist, exhibiting reduced IFN-I activity, despite generating comparable NF-κB activity[160, 164]. Conversely, others have shown that 2′3′-cGAMP engenders no significant difference in its inducible IFN-I activity with the hSTING^{REF} isoform[15]. Furthermore, the small molecule, DMXAA potently activates mSTING, but is unable to activate any of the hSTING variants[165]. Thus, the isoforms of STING represent a crucial design consideration for the clinical development of any STING agonist, as translatability will favor universal STING agonists.

2.4 Regulation of cGAMP

In addition to binding STING within the cell of origin, endogenous 2′3′-cGAMP can also vacate the native cell and thereby function as an immunotransmitter to neighboring cells[166, 167]. The accumulation of intracellular cGAMP that follows robust cGAS activation creates a strong electrochemical gradient that promotes cGAMP expulsion[168, 169]. The distribution of cGAMP to nearby cells can occur in several different ways, either directly (*e.g.* cell-to-cell) or indirectly (*e.g.* secretion followed by proximal cellular uptake). Direct cell-to-cell transfer of cGAMP may occur through connexin-dependent intercellular gap junctions, cellular fusion, and phagocytosis of dead or dying cells[47, 170-176]. Notably, the predominant gap junction protein involved in cGAMP transfer, connexin-43 (Cx43) is also established as a tumor suppressor in many types of cancer[177-179]. Although cGAMP transfer has not yet been directly linked to the anticancer role of Cx43, facilitating STING signaling in a time of cellular stress could potentially support a tumor suppressor function via the activation of innate immunity. In contrast to the direct transfer of cGAMP, indirect transfer may be mediated by ion channels, transport proteins, virions, and extracellular vesicles released from infected or apoptotic cells[167-169, 180-187].

Many of these cGAMP transfer modalities have limited functionality in various settings, as several are largely dependent on cellular context, viability, and/or infection status. Indeed, the unidirectional cell membrane transporter, SLC19A1 was shown to be important for cGAMP import in U937 monocyte-derived cells and monocytic THP1 cells, but was also found to be minimally expressed in many other cell types[167, 181]. Additionally, SLC46A2 has more recently been identified as the dominant cGAMP importer in primary human monocytes and monocyte-derived macrophages[187]. Conversely, gap junctions containing Cx43 and volume-regulated anion channels (VRACs) have important roles in cell survival and are therefore ubiquitously expressed in human cells[179, 188-190].

Gap junctions form intercellular channels in appositional cellular membranes and thereby promote direct cellular communication and nutrient exchange, both of which are essential to cellular physiology[179]. VRACs also help maintain cellular homeostasis, though they do so by counteracting dynamic cytoplasmic pressures[190-192]. Gap junctions and VRACs are both capable of two-way molecular transit, unlike some transporters that are simply unidirectional (*e.g.* the cell-specific cGAMP importers, SLC19A1 and SLC46A2)[167-169, 181, 187]. Thus, gap junctions and VRACs represent the main cGAMP transfer mechanisms in humans, though the contribution of each is likely tissue specific. Gap junctions were recently found to be essential for cGAMP transfer in lungs upon nanoparticulate STING agonist administration and also in livers following alcohol-induced hepatocyte injury[173, 174]. Alternatively, VRACs were identified as the dominant cGAMP importer in human microvascular endothelial cells, which are characteristic of many tumor microenvironments (TMEs)[169].

Notably, gap junctions enable transfer of cGAMP to a limited number of connected cells, while VRACs allow for secretion into the extracellular space and likely enable cGAMP transmission to a larger number of cells via paracrine signaling. Indeed, VRACs were found to be responsible for ~ 50−70% of cGAMP uptake in a wide variety of cell types[168]. While cGAMP and other CDN STING agonists may enter cells through these portals, the efficiency of cellular import appears to be quite low for these compounds. Notably, when cells are treated *in vitro* with cGAMP or other CDNs, dose-response studies for STING pathway activation typically yield values for the half-maximal effective concentration (EC_{50}) in the high micromolar range, suggesting inefficient CDN entry into the cytosol via the membrane transporters as well as poor cell membrane permeability due to their polar nature and negative charge[193]. This cytosolic delivery barrier has inspired the development of nanotechnology to enhance the intracellular delivery of exogenous STING agonists[194], which we discuss in detail below (***Figure 4***).

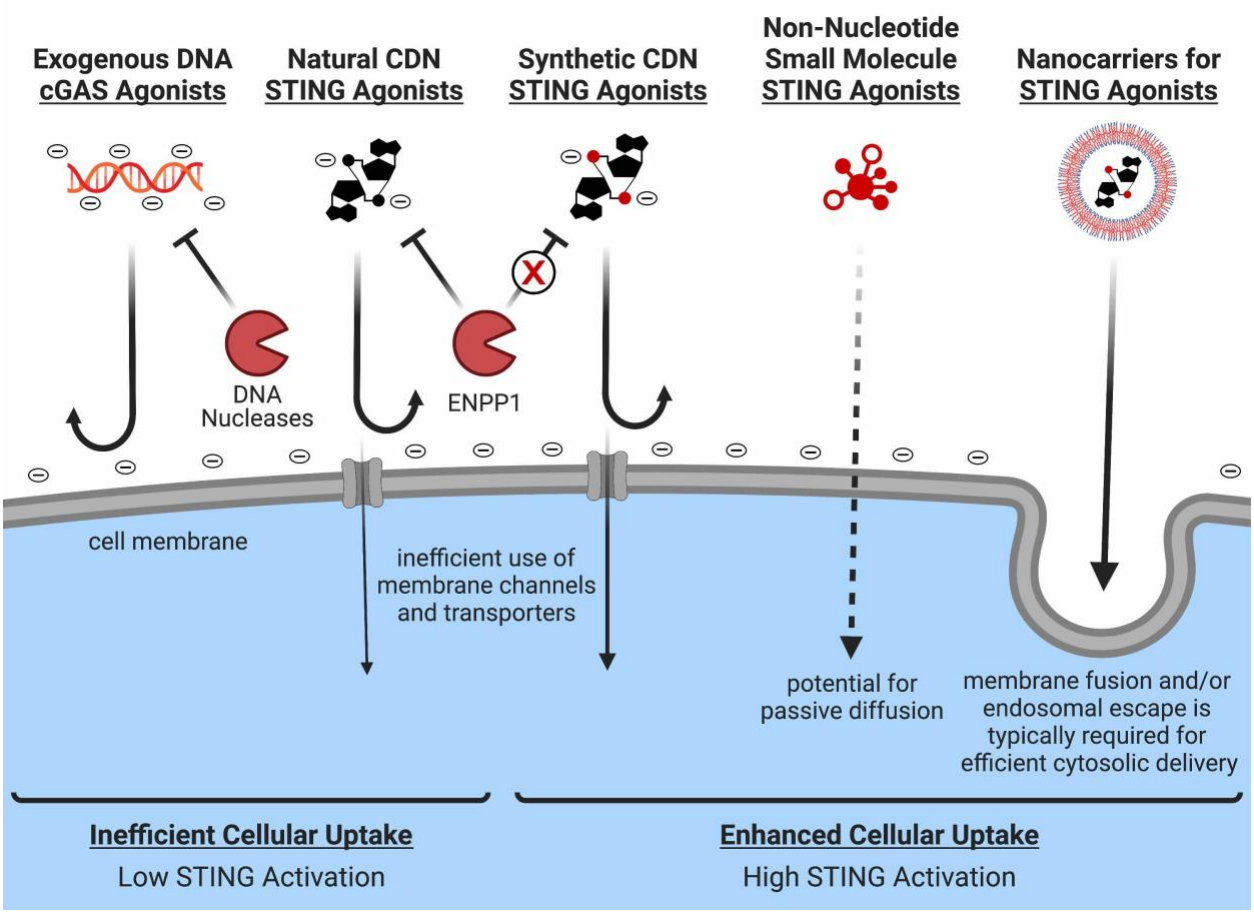

Figure 4: *Intracellular delivery challenges for STING pathway agonists*.

Exogenous DNA and CDNs are negatively charged and hydrophilic and consequently cannot readily access the cytosol to activate the STING pathway. While both natural and synthetic CDNs are small enough to infiltrate the cytosol through the use of membrane channels and transporters, these transport modalities are inefficient. Furthermore, extracellular nuclease and phosphatases quickly degrade exogenous DNA and natural CDNs, respectively. Accordingly, relatively high concentrations of CDNs are required to elicit measurable STING activation. Non-nucleotide, small molecule agonists of the STING pathway have potential to passively diffuse across the cell membrane and therefore are an attractive alternative to the natural agonists. Lastly, certain nanocarriers can improve the efficacy and safety of STING pathway agonists by promoting intracellular delivery. Figure created with *biorender.com*.

Currently, there is no indication that extracellular cGAMP preferentially spreads into any particular cell type, since gap junctions and VRACs are so broadly expressed. Rather, cGAMP likely distributes indiscriminately, but predominantly enters local cells due to the presence of ecto-nucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) in the extracellular space[166, 195]. ENPP1 hydrolyzes extracellular cGAMP and thus prevents extensive spread[195, 196]. Elevated expression of ENPP1 has even been correlated with tumor development in several cancer

types[166, 197, 198]. Accordingly, inhibitors of ENPP1 are currently being developed for cancer immunotherapy[166, 199]. Synthetic STING agonists without phosphodiester bonds have also been engineered to avoid degradation by ENPP1 and thereby enhance drug stability. Phosphorothioate modifications are commonly employed as they are resistant to ENPP1 degradation and may even enhance cellular uptake and cytosolic delivery[15, 195, 200, 201]. Though the development of nonhydrolyzable analogs of cGAMP has circumvented the issue of extracellular degradation, evading ENPP1 remains an important design criterion for therapies that exploit natural cGAMP from endogenous STING signaling (*e.g.* radiotherapy).

The manner in which cGAMP is transferred also uniquely affects the mechanism of action for subsequent STING signaling. Unlike intracellular CDNs that trigger classical cGAS/STING signaling, extracellular CDNs can activate an alternative cGAS/STING signaling pathway[202]. Liu *et al.* found that cells primarily endocytose extracellular CDNs in a clathrin-dependent manner. Endocytosed CDNs were released into the cytosol through an unidentified mechanism that required endosome maturation and acidification, whereupon the internalized extracellular CDNs bound cGAS directly. A CDN/cGAS/STING complex was subsequently formed and ultimately activated IRF3. Exceptionally similar downstream effects have been observed between this alternative pathway and the classical pathway, though overall protein expression seems to differ in magnitude with intracellular CDNs and the classical pathway evoking a greater response[194, 202]. *In vivo* cancer therapies that use CDN STING agonists without a cytosolic delivery agent likely activate this alternative STING signaling pathway and consequently may not maximize their immunostimulatory potential.

The duration of STING pathway stimulation is also a critically important consideration, as it can dramatically influence the balance between immunological outcomes (***Figure 5***). While, acute and localized activation of the STING pathway generally supports an appropriate level of immune activation for disease eradication, chronic STING signaling can elicit many inflammation-driven diseases. Such diseases include monogenic autoinflammatory syndromes (*e.g.* STING-associated vasculopathy with onset in infancy (SAVI), AGS, familial chilblain lupus, *etc.*), autoimmune diseases (*e.g.* systemic lupus erythematosus and rheumatoid arthritis), neurological disorders (*e.g.* ischaemic brain injury, Parkinson disease, Huntington disease, age-dependent macular degeneration, *etc.*), metabolic diseases (*e.g.* nonalcoholic steatohepatitis (NASH), alcoholic liver disease, *etc.*), inflammatory diseases (*e.g.* sepsis), cardiovascular diseases (*e.g.* myocardial infarction), cancer (*e.g.* metastases), as well as senescence and aging[203].

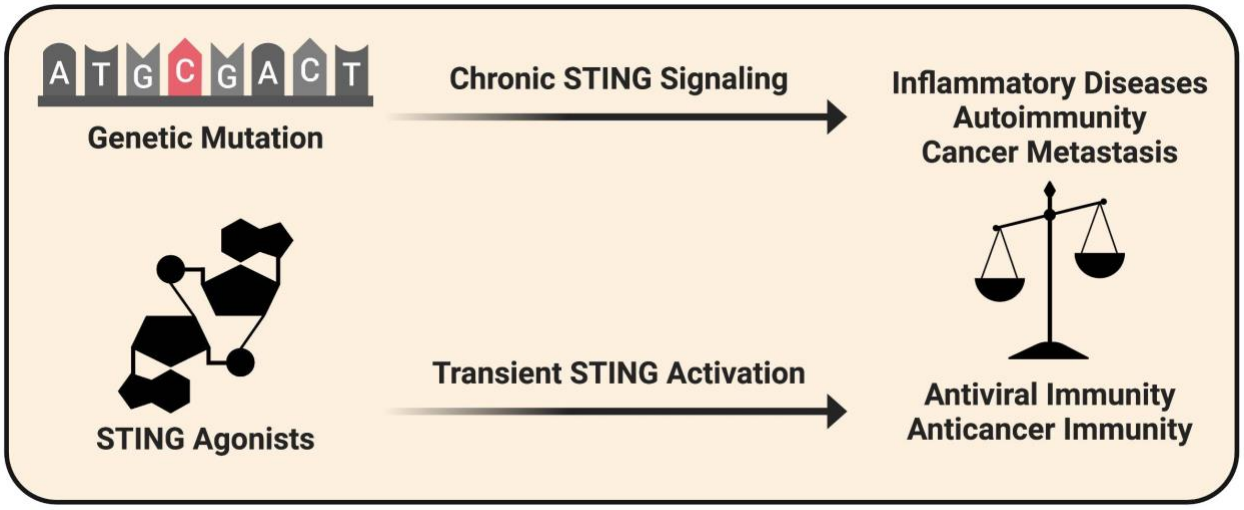

Figure 5: *The importance of STING signaling kinetics*.

The distinct outcomes of STING activation are balanced by signal persistence. Chronic STING signaling, which is quite often the result of genetic mutations, can lead to numerous IFN-driven inflammatory diseases, autoimmunity, and even cancer metastasis. Conversely, transient STING signaling, which can be induced by the acute STING activation from STING pathway agonists, can galvanize robust antiviral and/or anticancer immunity. Figure created with *biorender.com*.

Since prolonged stimulation of the STING pathway can lead to lethal inflammatory disease[151] as well as cancer development and metastasis in certain settings[204-206], the degree and persistence at which cGAMP is able to spread and activate STING in neighboring cells can play a large role in disease pathogenesis. Indeed, STING-induced metastasis in the context of brain cancer has been observed and attributed to the continuous transfer of cGAMP from cancerous cells to neighboring astrocytes via gap junctions[47]. Therefore, in order to avoid promoting disease progression, careful thought should be given to treatment regimen and the cellular context of the treatment location when designing cancer therapies that exploit cellular transfer of cGAMP.

3. STING and the Cancer Immunity Cycle

3.1 Intrinsic STING Signaling and Innate Antitumor Immunity

The main process through which the immune system recognizes and eliminates cancer has been described as the *Cancer Immunity Cycle* (CIC)[207]. The CIC summarizes how antitumor cellular immune responses are initiated and propagated through cooperation between the innate and adaptive immune systems. In principal, the cycle perpetually functions to inhibit cancer formation and growth through the following major steps: 1) Antigen Processing and Presentation,

2) Lymphatic Trafficking, 3) T Cell Priming and Activation, 4) Systemic Trafficking of T Cells, 5) Infiltration of T Cells into Tumors, 6) Immune Recognition of Cancer Cells, and 7) Killing of Cancer Cells / Antigen Release.

Spontaneous CIC operations that prevent the immune escape of pre-cancerous cells can be largely dependent on STING signaling[208, 209]. Mechanistic studies using genetically engineered mouse models of immunodeficiencies have identified STING signaling as an integral mechanism for innate immune sensing of immunogenic cancers. Notably, wildtype mice with functional STING signaling exhibited attenuated tumor growth relative to mice that were deficient in various STING pathway components[23, 26]. In accordance with the CIC, the innate antitumor effects of intrinsic STING signaling have been primarily attributed to enhanced tumor antigen-specific T cell responses[210, 211]. While the STING pathway was found critical to the spontaneous priming of antitumor T cells in certain murine tumor models, several other pattern recognition receptor pathways, including RIG-I and various Toll-like receptors (TLRs) were less essential for generating cell-mediated antitumor immunity despite their conserved ability to induce the production of type I IFNs[26]. Additionally, in accordance with the dependence of immune checkpoint blockade (ICB) on spontaneous T cell responses, it has been established that functional STING signaling is critical for the maximal efficacy of ICB in murine tumors[26, 212, 213].

The development of cancer is often the result of immunosuppression that impedes the favorable progression of the CIC. Indeed, selective pressure can lead to the deregulation of STING signaling, a prevalent mechanism by which cancer cells evade tumor immune surveillance[6, 7]. In two seminal reports that characterized the functionality of STING signaling in human colon cancer and human melanoma, Barber and colleagues discovered that cGAS and/or STING expression was absent in ~ 54% of colon cancers examined (*i.e.* 21/39 patient samples)[6] and ~ 54% of melanomas examined (*i.e.* 30/56 patient samples)[7] as determined by immunohistochemistry analysis, and greater silencing of cGAS and/or STING expression was observed in the late stages of both cancers relative to their respective earlier stages. Interestingly, the genes encoding cGAS and STING were found to be seldom mutated in pan-cancer (*i.e.* less than 1% of documented human tumors exhibit missense, nonsense, or frame shift mutations in the *cGAS* or *STING* gene)[214, 215]. Instead, epigenetic silencing of cGAS and/or STING is considered the predominant cause of the STING signaling dysfunction that is observed in the immune escape of various cancers[6, 7, 11, 214]. Accordingly, epigenetic modifications (*e.g.* hypermethylation of promoter regions, histone modifications, *etc.*) of the cGAS and/or STING loci,

in addition to the possible deregulation of essential signaling partners downstream of STING activation, are likely responsible for poor expression of cGAS and/or STING in as much as 50% of human tumors, though the exact frequency of tumors that have effectively silenced the STING pathway has not yet been reported for pan-cancer and is likely to be tumor-type specific. Furthermore, while many cancers can deregulate STING signaling in the cancer cell compartment, immune cells that are present in those tumors are unlikely to lose their capacity for STING signaling and therefore make ideal targets for STING pathway agonists in such cancers. Thus, cancers with deregulated STING are not necessarily precluded from the therapeutic benefits of STING pathway agonists.

The cellular transfer of cGAMP and/or tumor-derived dsDNA to stromal cells (*e.g.* myeloid cells, endothelial cells) becomes particularly important for tumor immune surveillance when STING signaling becomes deregulated in cancer cells.[104, 176]. Extrinsic STING signaling may then be employed to promote immune recognition, generation of antitumor immunity, and subsequent immune-mediated elimination of such cancer cells. Notably, it has been suggested that the STING protein may facilitate the intracellular clearance of cGAMP[93]. Therefore, cGAMP could be prone to accumulate more rapidly in the cytosol when expression of the STING protein is suppressed in cancer cells. Such accumulation of cGAMP in tumor cells could generate high intracellular concentrations that would promote cGAMP transfer to surrounding cell populations. Thus, tumorigenesis could be prevented by activation of antitumor immunity, provided the degree of cGAMP spread was sufficiently high to stimulate innate immune activation. However, this is clearly insufficient to prevent the development of all cancers, since deregulated STING is a common feature of many immune-evasive tumors. Factors such as ENPP1 may critically inhibit the degree of extrinsic STING signaling despite an increased efflux of cGAMP from cancer cells. Restricted cGAMP transfer in such cases might even contribute to the development of tumors with deregulated STING, as sustained low-level STING signaling may actually promote tumor growth and metastasis[47, 152, 204, 216], especially for tumors with low antigenicity[217].

3.2 Therapeutic Effects of Type I Interferons

As previously mentioned, generation of antitumor innate and adaptive immunity is considered the primary mechanism by which STING activation can combat cancers[155]. Indeed, in response to STING agonist treatment, antitumor immunity is mainly responsible for the tumor regression observed in murine tumor models as well as the sustained protection against disease recurrence demonstrated by efficacy in tumor rechallenge experiments[23, 24, 218]. Such therapeutic

responses have been largely attributed to type I IFN signaling in addition to other proinflammatory cytokines (*e.g.* TNF-α) downstream of STING activation.

Type I IFNs (*i.e.* IFN-α and IFN-β) are signature cytokines of STING activation and are considered a primary effector induced by STING signaling[14]. Type I IFNs directly regulate the transcription of over 100 genes that influence protein synthesis, autophagy, apoptosis, angiogenesis, and immunity[17]. Notably, the direct administration of type I IFNs into solid tumors has demonstrated clinical efficacy, and in 1986, recombinant IFN-α2 became the first immunotherapeutic approved by the FDA for the treatment of cancer. Many mechanisms of action have been proposed for the therapeutic effect of type I IFNs in the treatment of cancer, including both immune-mediated and immune-independent mechanisms. In various settings, type I IFNs have been found to directly inhibit tumor cell proliferation[219-221], disrupt tumor vasculature[222, 223], prompt the maturation of various APCs[224-226], induce CTL responses[227, 228], and activate NK cells[221, 229, 230].

For any IFN-driven cancer therapy (*e.g.* targeted STING pathway activation), the dosing of type I IFN and/or type I IFN inducers is a critically important therapeutic design consideration, as they can directly influence the mechanism of antitumor activity[18, 231]. Cancer treatments that implement high levels of intratumoral type I IFN can result in significant tumor regression that is largely independent of host adaptive immunity and instead depends heavily upon disruption of the tumor vasculature[222]. This high-dose ablative effect on tumors has also been observed with STING agonists[232] and may be related to the type I IFN component of downstream STING signaling. Similar to the dose-dependence of type I IFN treatment, robust antitumor T cell responses are achieved in murine tumor models with lower, more immunogenic doses of STING agonists, and excessive STING activation fails to sufficiently generate the antitumor immunity that can prevent tumor growth upon rechallenge.

In addition to dosing, timing of intratumoral type I IFN administration and/or induction can affect the development of antitumor immunity, which is essential for durable responses and long-term survival[18, 231]. As stated previously, type I IFNs induce DC maturation[224-226]. When DCs undergo maturation, they lose their phagocytic ability, thereby preventing the capture of new antigens in favor of an increased ability to cross-prime naïve $CD8^+$ T cells that are specific for antigens previously internalized by the DCs[233]. Accordingly, Tzeng *et al.* found that generation of

antigenic tumor debris must precede the induction of type I IFNs in order to efficiently prime long-term antitumor immunity[234].

Though type I IFNs have demonstrated efficacy in the treatment of cancer, they have also been associated with systemic adverse effects, which have limited their clinical use. The observed side effects for type I IFN therapies included fever and chills upon initial administration and fatigue, depression, and anorexia with continued treatment[235, 236]. While the production of type I IFNs is a critically important component of STING signaling for promoting antitumor immunity, other IFN-independent signaling pathways downstream of STING activation (*e.g.* NF-κB signaling) are also important for immune regulation[237] and can act to balance and resolve the resultant immune response[238].

3.3 Immunological Effects of STING Activation

The CIC can be considered as the "central dogma" of cancer immunotherapy; in order to work effectively, cancer immunotherapies must harness the CIC and promote it, either by pushing the cycle forward or by removing the restraints that impede the proper operation of the cycle. Accordingly, the great potential of STING pathway agonists for cancer immunotherapy arises from their exceptional capacity to bolster antitumor immune responses by promoting each phase of the CIC (***Figure 6***). Indeed, STING has been described as a master regulator of the CIC[239].

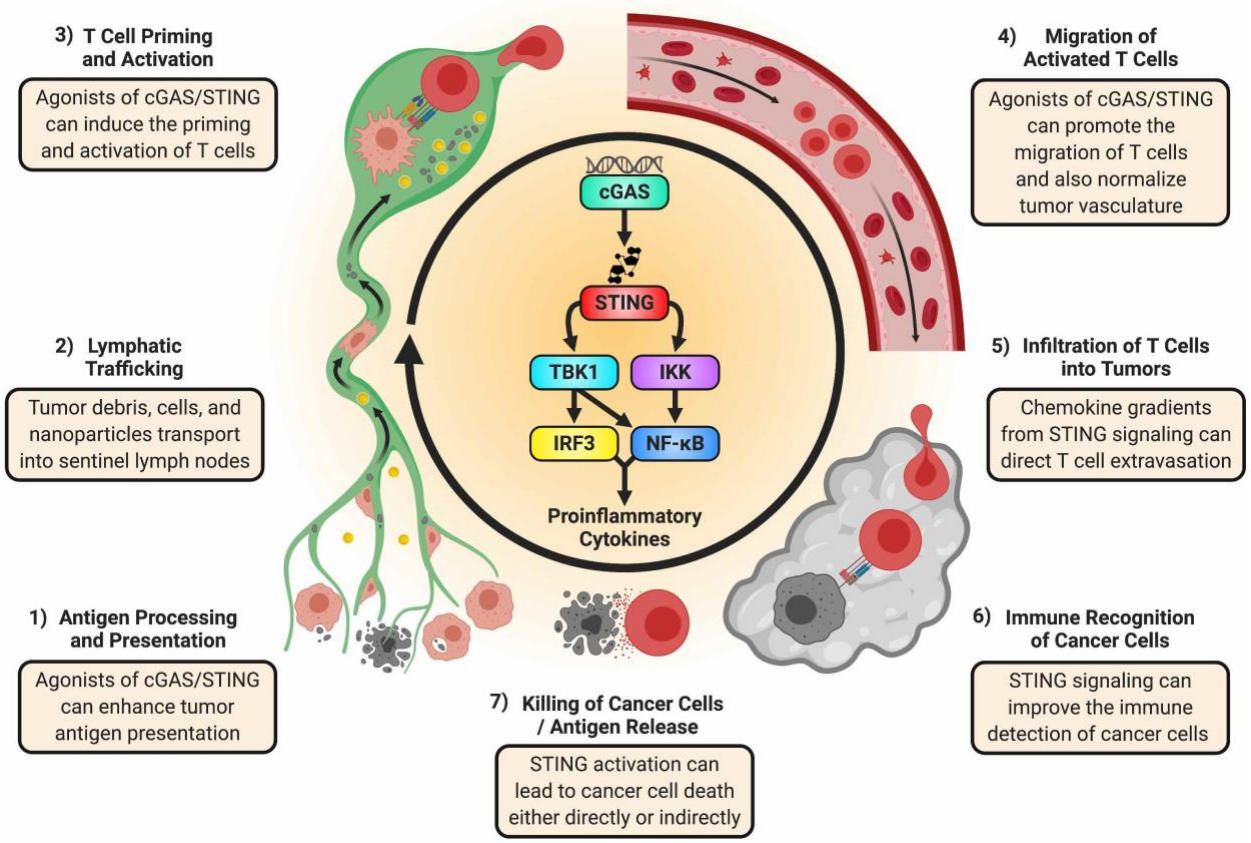

Figure 6: *STING and the Cancer Immunity Cycle*.

STING can promote antitumor immunity via the Cancer Immunity Cycle by promoting each of the following steps: 1) Antigen processing and presentation, 2) Lymphatic trafficking, 3) T cell priming and activation, 4) Systemic trafficking of T cells, 5) Infiltration of T cells into tumors, 6) Immune recognition of cancer cells, and 7) Killing of cancer cells / antigen release. Figure created with *biorender.com*.

3.2.1 Tumor Antigen Processing and Presentation

The production of type I IFNs is essential for the STING-mediated propagation of the CIC. Type I IFNs prompt the maturation of various APCs, promoting the expression of major histocompatibility complex (MHC) molecules, costimulatory molecules, and various other proinflammatory cytokines that are required for T cell priming and activation[240]. Indeed, STING signaling has been found to stimulate antigen processing and presentation in a manner that is dependent on type I IFN[241, 242].

A particular subset of DCs known as CD8α$^+$ Batf3 DCs have been described as the main APC responsible for generating antitumor T cells[243, 244]. CD8α$^+$ Batf3 DCs typically reside in

secondary lymphoid tissues and are characterized by an exceptional capacity for antigen cross-presentation (*i.e.* the process of antigen internalization and subsequent antigen presentation in complex with MHC-I to $CD8^+$ T cells). Notably, type I IFN production within solid tumors, like that induced by STING activation, promotes the intratumoral accumulation of $CD8\alpha^+$ Batf3 DCs from surrounding tissues[228]. Additionally, interferon-alpha/beta receptor (IFNAR) signaling within tumor-infiltrating $CD8\alpha^+$ Batf3 DCs is required for successful cross-priming of tumor antigen–specific $CD8^+$ T cells and subsequent immune control of tumor growth[228, 245].

Matured APCs, especially matured DCs, upregulate CC-chemokine receptor 7 (CCR7), causing them to enter the lymphatic vasculature, which expresses the CCR7 ligand, CC-chemokine ligand 21 (CCL21)[246, 247]. The APCs then further migrate to the tumor draining lymph nodes (tdLNs), where they can interact with naïve T cells. While T cell activation is thought to primarily occur in tdLNs, it has been suggested that intratumoral expression of type I IFNs may also prompt tumor-infiltrating $CD8\alpha^+$ Batf3 DCs to cross-prime $CD8^+$ T cells within the TME, thus bypassing the need for migration to the tdLNs[243]. Indeed, the direct activation of naïve T cells in tumors has been observed in mice that were treated with a T cell recirculation blocker[248] as well as in mice that were devoid of LNs and spleens[249]. Furthermore, targeted STING activation within B16-F10 murine melanoma tumors has been reported to induce the intratumoral formation of tertiary lymphoid structures, which may also serve as a local site for T cell priming to occur[250].

In addition to the type I IFN effects of STING signaling, there are other downstream effects of STING activation that can also enhance tumor antigen processing and presentation. Notably, STING activation typically results in the production of other proinflammatory cytokines (*e.g.* IL-6 and TNF-α) and reactive oxygen and nitrogen species that can promote M1-like polarization of macrophages[251, 252]. Moreover, STING signaling can even repolarize the phenotype of existing tumor resident macrophages from M2 to M1[252, 253]. While M2 macrophages tend to be immunosuppressive and protumor, M1 macrophages are more conducive to effective cancer treatments, as they can inhibit the proliferation of surrounding cells via paracrine signaling[251] and also induce lysis in various types of cancer cells[254, 255].

3.2.2 T Cell Priming and Activation

Generally, three signals are required from APCs to activate naïve T cells: peptide antigen displayed on MHC molecules for recognition by the T cell receptor (TCR), co-stimulatory molecules, and certain proinflammatory cytokines, all of which can be enhanced by STING

activation as just described. Thus, T cell priming and activation in the tdLNs naturally follow the STING-mediated APC response.

The two major types of effector T cells are MHC-I–restricted $CD8^+$ T cells, which are known as *cytotoxic T lymphocytes* (CTLs) and MHC-II–restricted $CD4^+$ T cells, which are known as *helper T lymphocytes*. A main function of CTLs is to directly kill diseased cells that express and present their cognate antigen[256], while helper T lymphocytes tend to regulate the function of other immune cells via paracrine signaling[257]. Notably, functional APC responses from STING signaling can enhance the activation of both CTLs[228, 242] and helper T lymphocytes[241, 258]. The CTLs are generally considered to be the primary driver of the antitumor immune responses that are stimulated by STING signaling[210, 211]. However, the helper T lymphocytes are known to support CTL function and cytolytic activity. Indeed, in response to STING signaling, the helper T lymphocytes exhibit a balanced Type 1 / Type 2 (Th1/Th2) phenotype, with slightly greater Th1 activity[259], which promotes M1 macrophage polarization[260, 261].

In addition to stimulating T cell responses through the activation of innate immunity, STING signaling can also directly influence antitumor T cell function. STING signaling within T cells has been shown to have varied effects depending on the degree and duration of the stimulus. Hyperactivation of STING can drive antiproliferative and apoptotic signaling within T cells[86, 262, 263]. Some lesser degree of STING signaling within T cells does however maintain $CD8^+$ T cell stemness, which can improve T cell-mediated tumor clearance[211]. In light of this dichotomous role of STING signaling in T cells, careful evaluation of how STING pathway agonists impact antitumor T cell viability and effector function will be critical to maximizing immunotherapeutic responses.

3.2.3 Systemic Trafficking and Tumor Infiltration of T Cells

Before CTLs can recognize and kill cancer cells, they must egress the tdLNs and traffic to tumor sites. Like matured DCs, naïve T cells are largely attracted to and retained within LNs through their expression of CCR7[264]. Activated T cells migrate out of LNs and into systemic circulation by downregulating CCR7 and simultaneously upregulating the receptor for sphingosine1-phosphate (S1P)[265], which is a signaling sphingolipid that is present in the blood at much higher concentrations than in lymphoid organs[266, 267]. Once activated T cells accumulate in the bloodstream, they require additional signals for direction to their effector site. STING signaling generates a chemokine gradient (*e.g.* CXCL9 and CXCL10) that can guide T cell extravasation into solid tumors[228, 244, 268]. Notably, CXCL9 and CXCL10 are also capable of driving NK cell

recruitment, activation, and maturation[269]. Moreover, activated NK cells can augment adaptive antitumor immunity by recruiting additional DCs to the TME[270].

Despite the powerful effects of chemokine gradients, dysfunctional tumor vasculature, a common feature of many cancers, can still act as a major barrier to immune cell infiltration and function[271]. However, vascular normalization, a reversal of tumor vessel abnormalities, has been shown to increase T cell infiltration and restore T cell function[271]. In addition to promoting chemokine gradients, STING activation can also normalize tumor vasculature and thereby further enhance T cell infiltration into tumors. Specifically, the direct injection of STING agonists into solid murine tumors results in reduced blood vessel density and vascular sprouts as well as an increase in pericyte coverage and an upregulation of endothelial-leukocyte adhesion molecules[272]. The normalized tumor vasculature that ensues STING activation has been found to facilitate the intratumoral trafficking of effector T cells across the endothelial barrier and condition the TME to enhance antitumor immunity[250, 272]. Notably, while other agents can also normalize tumor vasculature, STING-activating therapeutics offer the potential for coordinating vascular remodeling with reprogramming of the immune microenvironment, which can allow T cells to more efficiently home to tumor sites and perform their effector function.

3.2.4 Recognition and Killing of Cancer Cells / Cancer Antigen Release

STING signaling can trigger tumor elimination either by directly inducing cell death programs in cancer cells[273] or indirectly via mechanisms involving the immune system, particularly CTLs[26] and NK cells[20, 274]. Notably, the direct induction of cell death programs in cancer cells appears to be most pronounced in hematopoietic malignant cells, such as B cell and T cell lymphomas[86]. As demonstrated by numerous murine tumor models where immune cells have been knocked out or inhibited, antitumor immune responses are the primary cancer elimination mechanism promoted by STING signaling[155].

Antitumor immunity can be enhanced by intratumoral STING signaling in a multitude of ways. STING signaling can promote the expression of MHC-I on the surface of cancer cells to enhance the recognition of cancer cells by CTLs, which promotes CTL-mediated cancer cell death[275]. Some tumors can however evade this cellular response through loss of MHC-I expression or lack of tumor antigens[276-278]. NK cells can act to overcome such evasion mechanisms by recognizing stress-induced cells, particularly those that have lost MHC-I, and eliciting a cytotoxic response[276, 277]. NK cells have also been reported to drive tumor cell killing in

cancers with poor antigenicity[19]. Indeed, it has recently been described that NK cells mediate the clearance of CTL-resistant tumors in response to STING agonists[279]. Furthermore, STING signaling within cancer cells has also been shown to upregulate ligands for the NK cell-specific immunoreceptor, NKG2D, which increases NK cell recognition and elimination of cancer cells[280]. Cancer cell death can result in the release of additional tumor antigens, which leads to epitope spreading and recommencement of the CIC.

3.4 Iatrogenic STING Activation by Classical Cancer Therapies

As previously mentioned, indirect STING activation is a consequence of many classical cancer treatments (***Figure 7***), including many DNA-damaging chemotherapies (*e.g.* cisplatin[281], camptothecin[282], doxorubicin[283], paclitaxel[127, 284], etoposide[285-288], *etc.*), radiotherapy[289], and therapies that compromise the DNA damage response (*e.g.* poly(ADP)-ribose polymerase 1 (PARP) inhibitors[290-294], ataxia telangiectasia and Rad3-related protein (ATR) inhibitors[295], *etc.*). The inadvertent STING activation within tumor cells from such cancer therapies is induced by cellular dsDNA that becomes accessible for cGAS recognition. Many researchers have suggested that the recognition of dsDNA by cGAS in such cases is primarily mediated by micronuclei formation[27, 28]. However, recent work refutes micronuclei as the primary source of dsDNA for the cGAS activation that ensues drug-induced mitotic errors and instead finds that chromatin bridges are mainly responsible for the associated cGAS activation[296]. Notably, inhibitors of DNA methyltransferases are another class of cancer therapeutics, which have been approved for the treatment of acute myeloid leukemia[297] and are known to work well in combination with radiation and various chemotherapies in preclinical cancer models[298]. Recent findings suggest that the pharmacological inhibition of DNA methylation caused by a DNA methyltransferase inhibitor (*i.e.* 5-aza-2'-deoxycytidine) can also promote STING signaling by reversing the epigenetic silencing of both cGAS and STING that is commonly observed in a variety of cancer types[299].

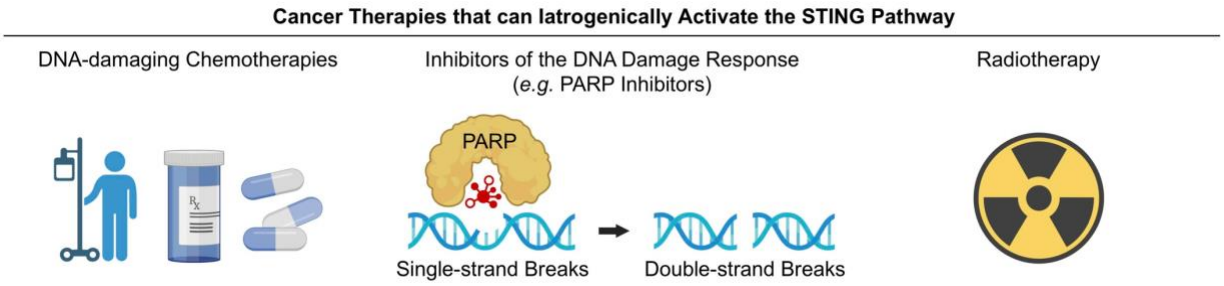

Figure 7: C*ancer therapies that can iatrogenically activate the STING pathway*.
STING activation is a known biological consequence of many classical cancer treatments, including DNA-damaging chemotherapies, therapies that compromise the DNA damage response, and radiotherapy. While the effects of classical cancer treatments are multifaceted, therapies that also induce STING signaling have potential to enhance overall therapeutic efficacy by providing a supportive inflammatory context for generating antitumor immunity. Figure created with *biorender.com*.

While the effects of classical cancer treatments are multifaceted, therapies that also induce STING signaling have potential to enhance overall therapeutic efficacy by providing a supportive inflammatory context for generating antitumor immunity. Indeed, it has been reported that STING signaling actively contributes to immune-mediated tumor growth inhibition in murine tumor models treated with a growing number of cancer treatments, notably including topotecan[300], viral oncolytic therapy[6], PARP inhibition[290, 292, 301], and radiotherapy[289]. Additionally, STING agonists were found to synergize well with radiotherapy in murine pancreatic tumors by promoting inflammatory pathways following tumor antigen release by radiotherapy[302].

STING signaling has also been implicated in the response to classical cancer treatments even in the absence of immune-mediated mechanisms. STING activation in cancer cells induced by antimitotic chemotherapies (*e.g.* taxane drugs) has been shown to trigger a proapoptotic secretory phenotype, which promotes BCL-xL-dependent apoptotic priming in untreated cancer cells[284]. It was confirmed that the STING-dependent apoptotic effects are required for the antitumor response to paclitaxel *in vivo*. Additionally, autophagy caused by STING-activating chemotherapies can clear diseased cells directly in addition to promoting desirable antitumor immune responses by triggering ATP release and immunogenic cell death (ICD)[303, 304]. In the context of radiotherapy, the cGAS protein can also directly contribute to cancer cell clearance by initiating cell death programs and accelerating γ-irradiation-induced cell ablation[122].

The functional significance of iatrogenic STING activation in human cancer patients is currently unclear. As previously discussed, the magnitude and context of STING signaling are critically important determinants of antitumor immune responses, and therefore iatrogenic STING activation may not be optimal for maximizing therapeutic impact. Furthermore, many classical cancer treatments target tumors indiscriminately and thus likely also impact immune cells within the TME. Therefore, the balance of STING activation, degree and type of tumor cell death, and the effect of the treatment on immune cells are all important variables to consider, as they will likely influence therapeutic outcomes[305]. Nevertheless, research has already begun to explore the employment of nanotechnology for enhancing STING-activating chemotherapies, strategies that not only address drug delivery challenges but also seek to simultaneously reinforce antitumor immunity within the TME[306].

4. STING Pathway Agonists

The development of STING pathway agonists as a cancer therapy long preceded the discovery of the STING pathway, beginning with the therapeutic characterization of flavone acetic acid (FAA). FAA was initially described as a vascular-disrupting agent and showed promise as a potential cancer therapeutic, inducing hemorrhagic necrosis in murine tumor models[307-309]. However, the narrow therapeutic window and poor pharmacokinetic properties of FAA led to the chemically-optimized design of 5,6-Dimethylxanthenone-4-acetic acid (DMXAA)[310-313]. Over a decade after their initial discovery, both FAA and DMXAA were identified as potent mSTING agonists[25, 314]. The robust antitumor activity of DMXAA in murine tumor models, which is now known to involve STING activation, advanced the compound to clinical testing. However, DMXXA failed in late-stage clinical trials for the treatment of non-small cell lung cancer due to a lack of efficacy[315, 316].

The negative results from the clinical trials involving DMXAA have since been largely attributed to the species-specific differences in the STING protein that render DMXAA incapable of binding (*i.e.* activating) any of the major hSTING isoforms[165]. Chimeric molecules comprised of mSTING with a hSTING CTD did not respond to DMXAA, while a chimeric hSTING molecule with a mSTING CTD resulted in signaling[317]. Indeed, a specific isoleucine residue of mSTING that is not present in any of the hSTING isoforms is critically involved in the recognition of DMXAA[318]. Nonetheless, studies involving DMXAA have significantly contributed to a fundamental understanding of the STING pathway in cancer therapy. DMXAA served as the first direct evidence for the existence of non-nucleotide, small molecule STING agonists and also

demonstrated their potential for immunotherapy as alternatives to CDNs. A comparable molecule, 10-carboxymethyl-9-acridanone (CMA) was also developed as a STING-targeting antiviral drug, but similarly suffered from an inability to activate hSTING, further guiding the field to develop hSTING agonists[319].

4.1 Cyclic Dinucleotide STING Agonists

As the biology of the STING pathway became more defined, the focus of STING-related cancer research and therapeutic development shifted to CDNs (***Figure 2***), the natural ligands of STING[37, 320, 321]. Canonical CDNs, originating in bacteria, comprise a 3′3′ linkage orientation (*i.e.* two uniform 3′,5′ phosphodiester bonds) and can activate certain hSTING variants[60, 208]. Since they do not activate all hSTING isoforms, canonical CDNs have been largely dismissed as potential drug candidates for lack of translatability [15, 160, 161]. Alternatively, noncanonical CDNs possess 2′2′, 3′2′, or 2′3′ linkage orientations. 2′2′-cGAMP, which contains two uniform 2′,5′ phosphodiester bonds, is a synthetic CDN that has not yet been found in nature[322]. 3′2′-cGAMP, which contains mixed 3′,5′ and 2′,5′ phosphodiester bonds, has recently been discovered in *Drosophila melanogaster* (*i.e.* fruit flies) as an intracellular product of cGAS-like receptors that recognize cytosolic double-stranded RNA[323, 324]. 2′3′-cGAMP, which contains mixed 2′,5′ and 3′,5′ phosphodiester bonds, is produced intracellularly by mammalian cGAS and exhibits some level of affinity for all of the major hSTING variants[15, 59]. Notably, the relative hSTING-binding affinities for the various CDN linkage orientations are 2′3′-cGAMP > 2′2′-cGAMP > 3′3′-cGAMP ∼ 3′2′-cGAMP[59, 322]. However, as discussed earlier, all natural CDNs are poor drug candidates as they experience inefficient cytosolic delivery and are susceptible to hydrolytic degradation by ENPP1.

Due to the poor drug-like properties of natural CDNs, second-generation STING agonists are now being developed. Both chemically-modified CDNs and non-nucleotide, small molecules are being explored as synthetic hSTING ligands. These compounds have enhanced drug-like qualities and are currently navigating the pharmaceutical pipeline (***Table 1***). The majority of these new STING agonists are synthetic, non-hydrolyzable CDN analogues with mixed 2′,5′ and 3′,5′ phosphorothioate bonds[205]. For example, ML RR-S2 CDA (now known as ADU-S100) is a dithio-substituted cyclic di-adenine with mixed phosphorothioate bonds that has been designed to increase stability and lipophilicity and therefore promote enhanced STING signaling. By substituting the non-bridging oxygen atoms at the phosphate bridge with sulfur atoms, the CDN is less susceptible to degradation by phosphodiesterases (*e.g.* ENPP1) and may even promote cellular uptake and cytosolic delivery[15, 195, 200, 201]. Additionally, the phosphate bridge configuration,

containing both 2′,5′ and 3′,5′ bonds, mimics that of noncanonical CDNs (*e.g.* endogenous 2′3′-cGAMP), which can activate all of the major hSTING isoforms[15]. This drug, and similar CDNs, are currently being investigated in clinical trials as an intratumorally administered treatment for head and neck squamous cell carcinoma and other solid tumors as well as lymphomas[325, 326]. Macrocyclized STING agonists, such as E7766, have also been developed and are now being implemented in phase I clinical trials. These aim to improve therapeutic efficacy by utilizing transannular macrocyclic bridges to lock the CDN in its bioactive, "U" conformation[327].

4.2 Non-nucleotide, Small Molecule STING Agonists

Although DMXAA lacked a capacity for hSTING activation, it inspired the development of non-nucleotide, small molecule STING agonists (***Figure 8***). Non-nucleotide, small molecules have potential to exhibit advantageous drug-like properties as well as improved access to the cytosol compared to anionic and highly water-soluble CDNs. Sali *et al.* developed a small molecule innate immune activator as an antiviral drug for the alphaviruses, Chikungunya (CHIKV) and Venezuelan Encephalitis (VEEV)[328]. Both viruses are lethal with little to no treatment options, but have been found to be sensitive to the antiviral effects of type I IFN responses[329, 330]. After high throughput screening, they arrived at the molecule, 4-(2-chloro-6-fluorobenzyl)-N-(furan-2-ylmethyl)-3-oxo-3,4-dihydro-2H-benzo[b][1,4]thiazine-6-carboxamide, denoted "G10." G10 demonstrated high potency and low toxicity in human fibroblasts. Using reporter cell assays and quantitative PCR (qPCR), the authors demonstrated G10's ability to trigger IRF3-dependent IFN signaling. The molecule also reduced replication of both CHIK and VEEV *in vitro* (IC_{90} values of 8.01 and 24.57 μM, respectively). Interestingly, STING deletion eliminated the G10-mediated IRF3 S386 phosphorylation and the ability to block viral replication, indicating that G10 acts through a STING-dependent pathway. However, this molecule does not directly bind to STING, but activates STING in an indirect manner, which is still to be explored. G10 demonstrated minimal activity in murine myeloid-derived cells, categorizing it as a hSTING-specific agonist.

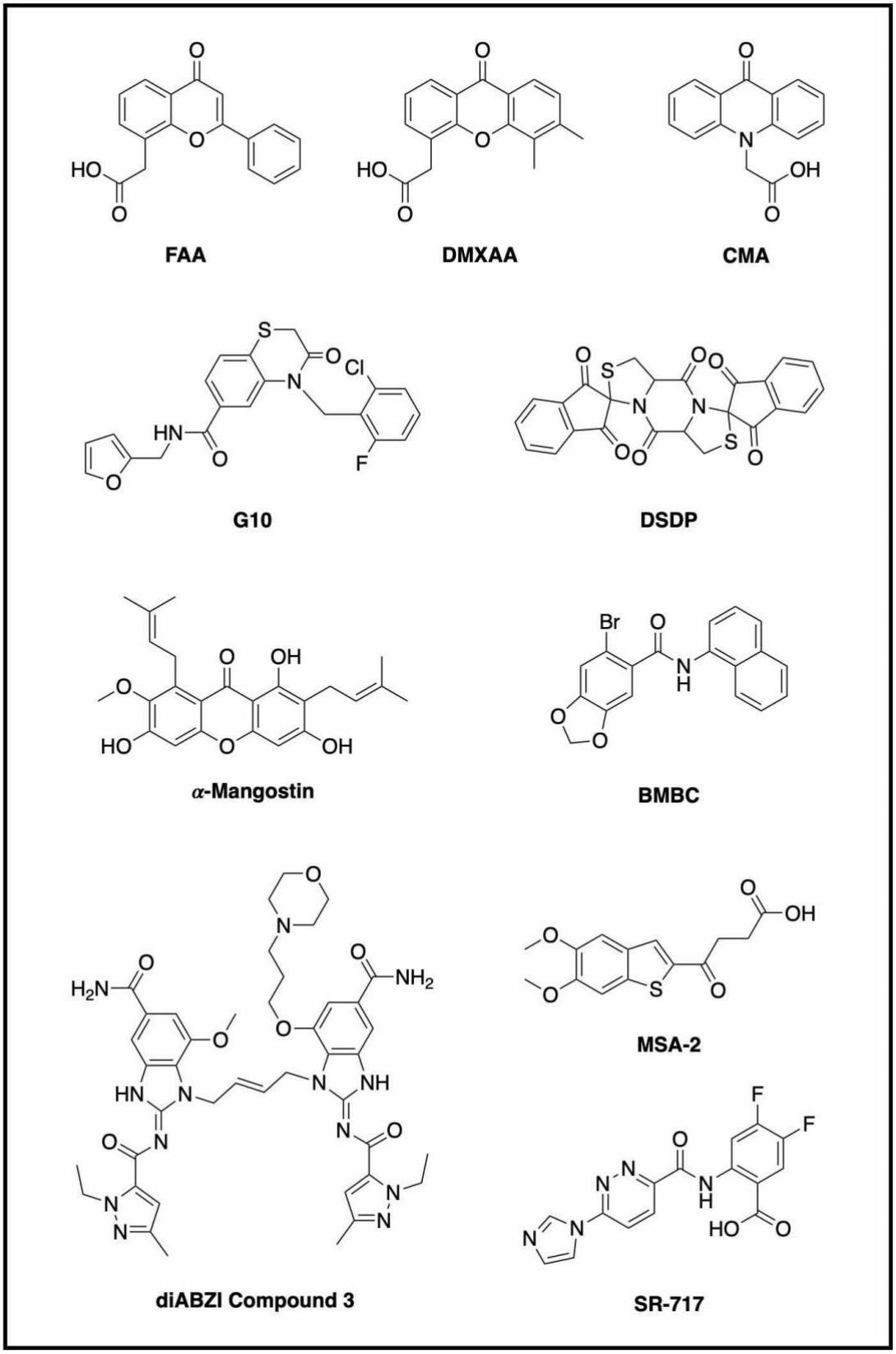

Figure 8: *Chemical structures of non-nucleotide, small molecule STING agonists*.

Liu *et al.* also developed a hSTING-specific agonist[331]. They identified a dispiro diketopiperzine compound, 2,7,2″,2″-dispiro[indene-1″-3″-dione]-tetrahydrodithiazolo[3,2-a:3′,2′-d]pyrazine-5,10(5aH,10aH)-dione (DSDP) through a high throughput screening assay. DSDP induced an IFN-dominant cytokine response in peripheral blood mononuclear cells (PBMCs) and human skin fibroblasts, suppressing the replication of yellow fever virus, dengue virus, and Zika virus. Although this agent shows promise for activating hSTING, it does not efficiently bind mSTING, which limits the use of mouse models for preclinical studies. Similar hSTING specificity was also observed with a well-known antiviral/anti-tumor agent, α-Mangostin, which induced type I IFN responses in 293T cells transfected with hSTING plasmids, but exhibited minimal activity in mSTING[332]. Although all of these molecules appear to hold great merit, a small-molecule candidate ideally would potently activate both hSTING and mSTING, allowing studies to span various tumor models in immunocompetent mice and enabling preclinical studies that can evaluate toxicity, pharmacodynamics, treatment regimen, and drug combinations.

Bicyclic benzamides and benzothiophene derivatives have also shown hSTING specific activity. Scientists at Curadev Pharma discovered that certain bicyclic benzamides could target the STING pathway as the compounds demonstrated the ability to induce the production of STING-associated cytokines (*e.g.* type I IFNs, CXCL10, and TNF-α) in human PBMCs[333-335]. Notably, a set of three intratumoral injections of their lead compounds administered every other day led to significant suppression of tumor growth in BALB/c mice with hSTING-expressing CT26 tumors. Investigators at Merck explored benzothiophene derivatives in the context of STING activation and identified compounds with micromolar potency in both direct STING binding and a cellular reporter assay for type I IFN production[336]. However, the multi-substituted benzothiophene lacked the ability to generate significantly higher type I IFN secretion compared to 2′3′-cGAMP. In mice with advanced MC38 tumors, the lead compounds were able to stimulate tumor regression when injected intratumorally every 3 to 7 days for up to 30 days, demonstrating their potential for clinical development[337].

One of the most promising non-nucleotide, small molecule STING agonists has recently been described by Ramanjulu and collaborators at GlaxoSmithKline. A series of amidobenzimidazole (ABZI) STING agonists was identified using a high throughput screening assay to monitor a library of small molecules competing with the binding of radio-labeled 2′3′-cGAMP to the CTD of hSTING[132]. The lead compound (*i.e.* Compound 1 – ***Figure 9A***), which emerged from the *in vitro* screen, could bind to one subunit of the hSTING homodimer with

significant, but relatively low potency (*e.g.* IC_{50} ~ 14 μM) compared to the natural STING ligand, 2′3′-cGAMP (*e.g.* IC_{50} ~ 200 nM). In order to improve potency, the researchers created a dimer of the lead compound, connecting two molecules together by replacing an N1-hydroxyphenethyl moiety with a four carbon linker. The resulting dimeric amidobenzimidazole ligand (diABZI) (*i.e.* Compound 2 – ***Figure 9B***) could target homodimeric STING and improved its competitive binding by 1000-fold (*e.g.* IC_{50} ~ 20 nM). All key contacts from the monomeric compound and STING were conserved and the linker was shown to have no interactions with the protein. Compound 2 induced dose-dependent secretion of IFN-β in human PBMCs at a level 18-fold higher than cGAMP. The molecule was further optimized through chemical substitution, yielding a diABZI (*i.e.* Compound 3 – ***Figure 9C***) with greatly improved *in vitro* activity. Compound 3 was 400-fold more potent than cGAMP in human PBMCs with wildtype STING and was also active in human PBMCs expressing other STING isoforms (*e.g.* HAQ/HAQ, R232H/R232H) as well as in murine PBMCs. Unlike cGAMP and DMXAA, this compound efficiently activated STING while maintaining an open STING confirmation, indicating that conformational change and lid interactions are not always necessary for STING activation (***Figure 9D***). Importantly, this diABZI compound was found to bind to both hSTING and mSTING, enabling preclinical analysis of pharmacological properties and antitumor efficacy in mouse tumor models. When administered intravenously using a three-dose regimen to mice with established CT26 colorectal tumors, diABZI resulted in a significant inhibition of tumor growth and an increased overall survival time with 8/10 mice in the study remaining tumor free. This finding was particularly notable as it represented the first published report of a non-nucleotide, small molecule STING agonist with both hSTING and mSTING binding capacity that could activate antitumor immunity and inhibit tumor growth when administered via an intravenous route.

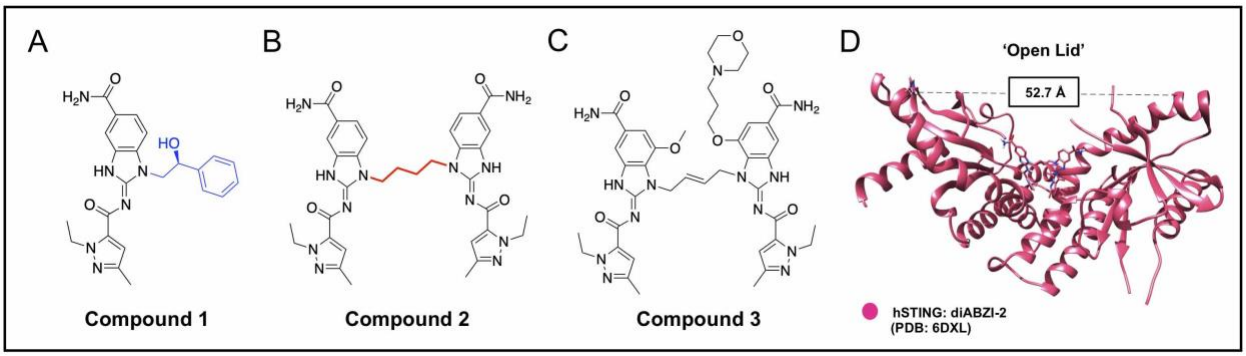

Figure 9: *Development of the dimeric amidobenzimidazole (diABZI) STING agonist*.

(A) Chemical structure of the monomeric ABZI STING agonist, Compound 1. **(B)** Chemical structure of the dimeric ABZI (diABZI) STING agonist, Compound 2. **(C)** Chemical structure of the

fully optimized diABZI STING agonist, Compound 3. **(D)** The 'Open Lid' configuration of a holo (*i.e.* ligand bound) hSTING dimer bound to diABZI Compound 2. Adapted with permissions from reference[145]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc. (PDB ID: 6DXL)[132]. Copyright © 2018 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc.

More recently, two reports have described non-nucleotide, small molecule STING agonists with potential to be delivered via multiple administration routes, including orally. Pan and co-workers at Merck identified MSA-2 (*i.e.* benzothiophene oxobutanoic acid – ***Figure 10A***) as a STING agonist using a high-throughput, cell-based phenotypic screen that measured IFN-β secretion from THP1 monocytes treated with a diverse library of ~ 2.4 million compounds[338]. MSA-2 was found to have high cell permeability, STING selectivity, and resulted in phosphorylation of both TBK1 and IRF3 in a dose-dependent manner. The mechanism through which MSA-2 activates STING is unique in that it forms a non-covalent dimer (***Figure 10B***) in solution and fills the CDN binding pocket with high affinity (*e.g.* K_D = 8 nM) and a slow off-rate (*e.g.* half-life of 1.3 hours). Interacting with each other through their aromatic cores, two molecules of MSA-2 predimerize in solution before binding to STING in the same site as 2′3′-cGAMP. The dimerized agonist creates a bridge across the STING homodimer, which noncovalently crosslinks the two STING subunits and allows for the stabilization of a closed lid conformation (***Figure 10C***), similar to that of 2′3′-cGAMP-bound STING. To further support this binding mechanism, a covalently linked monomer was synthesized by replacing both 5-methoxy groups with a propane linker, which also demonstrated potent STING binding activity. Interestingly, MSA-2 is currently the only small molecule reported to undergo such a reversible, noncovalent dimerization in solution to become a pharmacologically active ligand, highlighting the novelty of using MSA-2 rather than the covalent dimer. Computational modeling predicted that the hydrophobicity of MSA-2 (pKa ~ 4.7) increases in acidic conditions due to protonation of the carboxylic acid group with an attendant increase in the fraction of uncharged molecules, which exhibit higher membrane permeability. This suggests that the molecule may have higher cellular membrane permeability in the TME, which tends to be slightly acidic[339, 340]. Indeed, the investigators demonstrated higher MSA-2 concentrations in subcutaneous MC38 tumors than in plasma or in nontumor tissue (*e.g.* spleen, muscle), resulting in elevated IFN-β and proinflammatory cytokine production at the tumor site. As a result, treatment with MSA-2 resulted in complete tumor regression in 80−100% of mice bearing MC38 colon carcinoma tumors when delivered through IT, subcutaneous, and oral routes. Notably, MSA-2 administered orally in mice exhibited equal or better efficacy than a cGAMP analog dosed by intratumoral or subcutaneous routes. Treatment with MSA-2 also resulted in

long-term antitumor immunity and synergized with ICB (*i.e.* anti-PD-1) in MC38 colorectal, CT26 colorectal, B16-F10 melanoma, and LL-2 lung cancer models. This study highlights the promise of designing STING agonists that exploit biochemical signatures of the tumor microenvironment (*e.g.* pH, redox, *etc.*) to preferentially enrich STING activation at tumor sites with potential to reduce systemic inflammation and mitigate associated toxicities.

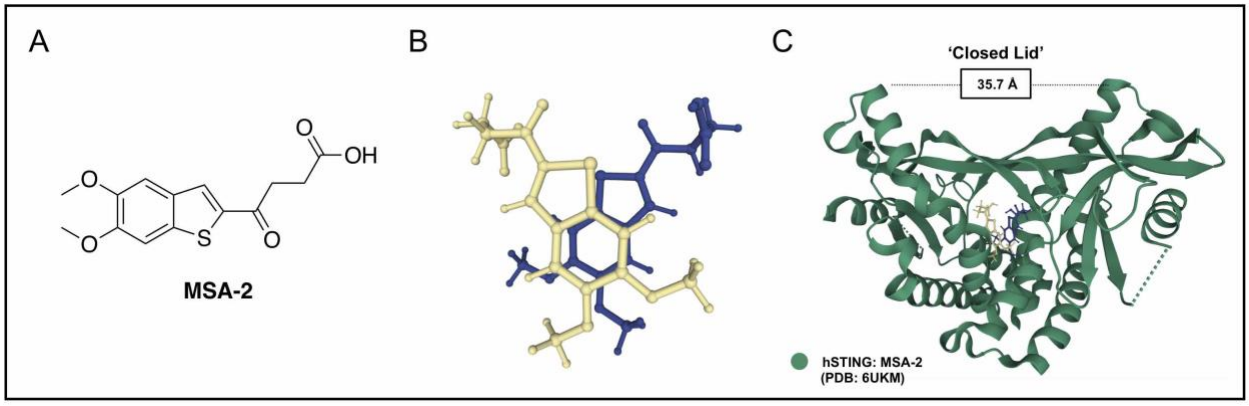

Figure 10: *Development of the MSA-2 (i.e. benzothiophene oxobutanoic acid) STING agonist*. **(A)** Chemical structure of MSA-2. **(B)** Self-dimerization of MSA-2 (PDB ID: 6UKM)[338]. **(C)** The 'Closed Lid' configuration of a holo (*i.e.* ligand bound) hSTING dimer bound to MSA-2 (PDB ID: 6UKM)[338]. Adapted with permission from reference[338]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc.

In a parallel effort, Chin *et al.* discovered other non-nucleotide, small molecule STING agonists (***Figure 11A***) with potential for systemic and oral administration through a series of cGAS/STING pathway–targeted cell-based phenotypic screens and subsequent structure-function analysis[145]. The investigators derived the non-nucleotide, small molecule, SR-717 from SR-001, which was one of the ~ 100,000 compounds in their starting library of commercially available compounds. The commercially acquired SR-001 exhibited a high level of activity in THP1 reporter cells for type I IFN (*e.g.* EC_{50} ~ 1.1 μM) and was found to increase the thermal stability of the soluble CDN-binding CTD of recombinant $hSTING^{REF}$ in a STING thermal shift binding assay. When the researchers chemically resynthesized SR-001 in-house, they were surprised to find that it was no longer active in the STING-binding assay, but was still consistently active in cell-based assays. Analytical characterization of the vendor-bought material revealed the presence of a small but significant amount of the de-esterified derivative, SR-012. This suggested that SR-001 was acting as a prodrug, with the ester being necessary for cell permeability and the active STING-binding species being that with the free carboxylic acid. In accord with this hypothesis, synthetic SR-012 could bind both mSTING and hSTING, but was

inactive in cell-based assays due to poor cytosolic delivery owing to its higher water solubility and low membrane permeability.

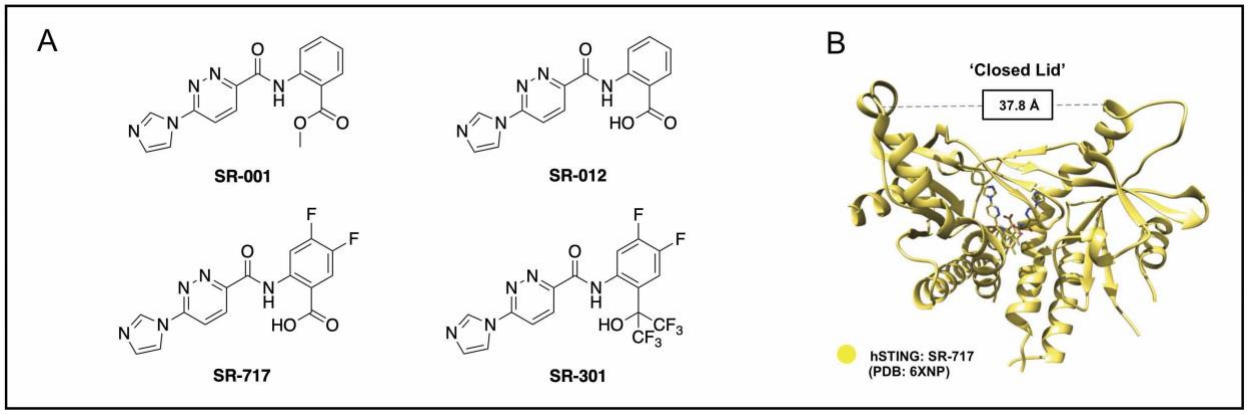

Figure 11: *Development of the SR-717 STING agonist*.

(A) Chemical structures of SR-001 (*i.e.* the prodrug screening hit), SR-012 (*i.e.* the elucidated STING agonist), SR-717 (*i.e.* the optimized STING agonist), and SR-301 (*i.e.* orally bioavailable analog of SR-717)[145]. **(B)** The 'Closed Lid' configuration of a holo (*i.e.* ligand bound) hSTING dimer bound to SR-717. Adapted with permission from reference[145] (PDB ID: 6XNP)[145]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc.

To address the low cell permeability of SR-012, Chin *et al.* synthesized SR-717 – another carboxylic acid–containing analog of SR-001[145], which includes a difluoro-substitution of the aniline ring system to increase membrane permeability. Crystallographic analysis demonstrated that two molecules of SR-717 bind at the base of the STING dimer intersubunit cleft, inducing a closed lid conformation (***Figure 11B***) that closely mimics the binding mode of 2′3′-cGAMP. The paired SR-717 molecules share similar contact residues as observed with 2′3′-cGAMP, which enable it to competitively bind to STING. Importantly, SR-717 was found to bind to all common human alleles of hSTING as well as mSTING, allowing *in vivo* analysis of pharmacological properties and efficacy in mouse tumor models. Therapeutic doses (*e.g.* 15 mg/kg or 30 mg/kg) of SR-717 administered intraperitoneally resulted in elevated plasma cytokine concentrations and STING-associated antitumor effects in syngeneic B16-F10 melanoma and MC38 colorectal adenocarcinoma mouse tumor models. intraperitoneal administration of SR-717 also inhibited the formation of pulmonary nodules following intravenous delivery of B16-F10 melanoma cells, suggesting its ability to combat metastasis. Surprisingly, unlike the study of Pan *et al.* that used MSA-2 with anti-PD-1 in MC38 colorectal, CT26 colorectal, B16-F10 melanoma, and LL-2 lung cancer tumors[338], Chin *et al.* did not observe a benefit of combining SR-717 with ICB (*i.e.* anti-

PD-1 or anti-PD-L1) in the B16-F10 tumor model[145]. Finally, using an analog of SR-717 with improved bioavailability, SR-301, they demonstrated a modest inhibition of tumor growth using an oral administration route.

4.3 cGAS Agonists

While the STING protein has appropriately garnered much interest as a druggable target for cancer immunotherapy, cGAS has been largely overlooked despite the potential of cGAS activation to more closely mimic the endogenous STING pathway[341]. It is possible that agonists of cGAS may offer more control over the level and kinetics of local STING signaling, which may be tailored to optimize antitumor immunity[79]. As mentioned previously, dsDNA in the cytosol can elicit tiered immune responses, the phenotype of which is determined by the physicochemical composition of the dsDNA[79]. The molecular weight of the cGAS-bound dsDNA (*i.e.* bp length) influences the prevalence and size of the resultant liquid-like droplets, which function as miniature bioreactors for the efficient production of 2′3′-cGAMP[58]. Accordingly, the localized production of 2′3′-cGAMP is tightly regulated, and the liquid-like droplets essentially act as *in situ* drug delivery depots that confer tunability over the degree of 2′3′-cGAMP production, which may be useful for promoting and controlling antitumor immunity.

The lack of development behind cGAS agonists might be attributable to the complexity of cGAS activation combined with the many challenges facing the therapeutic delivery of nucleic acids. Indeed, since freely administered dsDNA is rapidly cleared and degraded with minimal cellular uptake[342], dsDNA-based cGAS agonists will require molecular engineering approaches to protect dsDNA from degradation and promote cytosolic delivery of the dsDNA. In theory, small molecule cGAS agonists could circumvent the delivery issues that are associated with the negative charge, hydrophilicity, and relatively large molecular weight of dsDNA. While no small molecule cGAS agonists have been reported to date, Hall *et al.* have identified a potential small molecule binding site on the cGAS enzyme that may cause catalytic activation of cGAS and is certainly worth investigation[343]. However, like with most small molecule drugs, an effective small molecule activator of cGAS would need to demonstrate pathway specificity and not significantly affect other cellular processes via off-target activity. Furthermore, phase separation requires multivalent interactions for the assembly of macromolecular complexes[344], and therefore a small molecule activator of cGAS may not be able to induce the same liquid-like phase transition that is dependent on DNA-bridging. Indeed, a small molecule agonist would likely have to exhibit self-multimerization to achieve efficient 2′3′-cGAMP production via cGAS oligomerization, which adds

another level of complex requirements for the design and development of a small molecule cGAS agonist. Thus, most small molecules would seemingly be unlikely to generate the intracellular microreactors for 2′3′-cGAMP production without assistance from other molecules, and this is perhaps the primary reason that the development of small molecule cGAS agonists has not yet been reported. Notably, DNA-bridging is not required for cGAS *inactivation*, and there already exist several small molecule cGAS inhibitors that have been developed for applications outside of cancer (*e.g.* autoimmunity)[110, 341, 345, 346].

While not a canonical small molecule per se, the metal ion, manganese(2+) (Mn^{2+}) is worth discussing as it has recently been shown to be capable of independently activating monomeric cGAS in the absence of dsDNA without the need for oligomerization[65, 347]. Notably, Mn^{2+} can affect STING signaling in several unique ways, and this will be discussed in greater detail in ***Section 7*** along with other potentiators of the STING pathway. Interestingly, Mn^{2+} enhances the catalytic activity of cGAS and can also allosterically enhance the dsDNA binding activity of cGAS in conjunction with the ATP/GTP substrate pair, which sensitizes cGAS to oligomerization by lowering the threshold for cGAS activation in regard to both cytosolic dsDNA length and local dsDNA concentration in the cytosol[65]. Thus, in addition to oligomerization-free cGAS activation, Mn^{2+} may also trigger cGAS activation in cells by promoting the recognition of dsDNA already present in the cytosol at low concentrations and/or low molecular weights (*e.g.* short dsDNA lengths less than ~ 45 bp) that are ordinarily below the natural threshold for cGAS activation. Accordingly, the recently reported therapeutic efficacy of Mn^{2+} as a monotherapeutic STING pathway potentiator for cancer therapy[348] cannot be solely attributed to oligomerization-free cGAS activation. It remains to be determined whether any cGAS activator that does not somehow induce cGAS oligomerization and droplet formation could achieve therapeutically relevant STING activation and whether they would allow for control over the degree of STING signaling.

In addition to Mn^{2+}-encompassing therapies, DNA-based cGAS agonists that employ delivery technologies to achieve cytosolic accumulation of exogenous dsDNA can also facilitate the pharmacological activation of cGAS. Indeed, targeting cGAS activation via intracellular delivery of dsDNA has now been explored by several research teams including our group[349-353]. Notably, Garland *et al.* developed NanoISD, a DNA-based cGAS agonist designed for use as an intratumoral immunotherapy[353]. NanoISD is a nanoparticle formation that is assembled by complexing an exonuclease-resistant cGAS ligand (*i.e.* 95 bp phosphorothioate-capped dsDNA) with endosomolytic polymer micelles that can simultaneously enable cytosolic delivery of nucleic

acids and inhibit endonuclease degradation of loaded nucleic acids via steric interference. The resultant DNA/polymer nanoparticle complexes are ~ 60-90 nm in diameter and have a positive surface charge of +14.87 mV. It was demonstrated that NanoISD confers deoxyribonuclease resistance, enhances cellular uptake, and promotes endosomal escape of the 95 bp phosphorothioate-capped dsDNA into the cytosol of cells, resulting in potent activation of the STING pathway via cGAS. Furthermore, NanoISD relayed many of the same antitumor effects established for agonists of the STING protein; NanoISD was shown to induce proinflammatory cytokine production, prompt the maturation of antigen presenting cells, promote tumor infiltration of NK cells and $CD8^+$ T cells, reduce tumor burden, and enhance responses to ICB (*i.e.* anti-PD-1 and anti-CTLA-4). Moreover, when administered at the same dose and with the same treatment regimen in the B16-F10 tumor model, the therapeutic benefit of NanoISD was comparable to that of CpG DNA (*i.e.* ODN 1826), a well-established innate immune activator, analogues of which are currently being investigated in human clinical trials for the treatment of cancer[354]. While CpG DNA relies on the cellular expression of TLR9, which is mostly restricted to plasmacytoid dendritic cells and B cells in humans[355], cGAS and STING proteins are more ubiquitously expressed in mammalian cells[356-358]. Additionally, TLR9 signaling can only occur in cells that are directly exposed to CpG DNA, in contrast to STING signaling, which can be locally propagated from cell-to-cell through the transfer of endogenous 2′3′-cGAMP following DNA-induced cGAS activation. Therefore, cGAS agonists may represent a more accessible treatment for promoting antitumor immunity via DNA sensing. Regardless, novel agonists of cGAS increase the arsenal of potential cancer immunotherapies, providing additional opportunities for immune modulation. Accordingly, NanoISD is a promising nucleic acid therapy with clear indications for the treatment of immunologically cold cancers.

5. Drug Delivery Barriers and Pharmacological Challenges

5.1 Intracellular Delivery Barriers for Cyclic Dinucleotides

CDNs have advanced into clinical trials for intratumoral administration and have demonstrated a favorable safety profile and evidence of STING pathway activation. However, initial phase I data for ADU-S100 and MK-1454 demonstrated limited efficacy, though responses in several tumor types were observed when the STING agonists were administered in combination with anti-PD-1 monoclonal antibody treatment (spartalizumab and pembrolizumab, respectively)[326, 359]. Unfortunately, despite very promising pre-clinical data, the lack of impressive clinical responses has recently dampened enthusiasm for intratumoral administration of CDN STING agonists. Indeed, clinical trials of ADU-S100 and Merck's CDN STING agonist (MK-1454)

are no longer recruiting (***Table 1***). While a limited clinical response may be attributed to a multitude of factors (*e.g.* study design and outcomes, dose and regimen selection, patient cohort selection, use of combinations, *etc.*) that may be independent of the activity or immunostimulatory effects of the agent per se, these early clinical studies nonetheless motivate the importance of considering potential drug delivery and pharmacological barriers that may limit the therapeutic efficacy of CDNs (***Figure 4***).

STING is localized on the endoplasmic reticulum with the ligand binding domain facing cytosolically, and, hence, access of CDNs to the cytosol is thought to be a critical for their activity. However, intracellular delivery of CDNs via passive diffusive transport across the plasma membrane is limited by their negative charge and high aqueous solubility[360]. CDNs are also relatively large in size (*e.g.* ~ 700 Da) compared to traditional small molecule drugs typically designed to be less than 500 Da, further limiting their ability to diffuse passively through the membrane[195]. Current evidence suggests that the activity of free cGAMP can likely be attributed the aforementioned membrane transport processes (*e.g.* VRACs, connexin gap junctions, SLC19A1, *etc.*) or via cellular uptake through pinocytosis or endocytosis, but the efficiency of these processes appears limited[212] given the relatively high EC_{50} values (*e.g.* high micromolar) for free cGAMP typically measured in cell culture assays. Beyond its low cellular permeability, 2′3′-cGAMP activity is also hindered by the hydrolase, ENPP1, which cleaves phosphodiester bonds in the extracellular space[195]. However, as mentioned previously, nonhydrolyzable analogs can mitigate this effect and have shown improved potency with EC_{50} values ~ 10-fold higher than 2′3′-cGAMP as determined by measuring dose-dependent IFN-β production in a human THP1 monocyte cell line[195]. While a significant improvement, the potency of these analogs is still limited by their relatively poor cell membrane permeability. Thus, comparable to nucleic acid-based therapeutics (*e.g.* siRNA, mRNA, miRNA, *etc.*), the activity of CDNs is limited by intracellular delivery barriers, prompting the recent development of new chemical strategies for circumventing this challenge. In order to improve membrane permeability and stability against enzymatic degradation, some research groups have modified current CDNs to include fluorine substitutions for 2′-hydrogens or 2′-hydroxyl groups on the pentose rings[361-363] (***Figure 12***). Lioux *et al.* developed a non-canonical CDN, adenosine-inosine monophosphate (cAIMP) that includes both fluorine and thiophosphate substitutions (Dithio-2′-F-cAIMP – Compound 53) to address both of these delivery challenges[362]. Similarly, Pimková Polidarová *et al.* utilized phosphorothioate-linked 3′3′-ci-di(2′F,2′dAMP) to develop phosphoester CDN prodrugs that further enhance permeability by using biolabile protecting groups that mask the negatively charged phosphate groups and

release the free, parent drug under intracellular conditions (*e.g.* exposure to intracellular enzymes)[363]. Beyond chemically modifying CDNs, nanotechnology has also been developed to circumvent intracellular delivery challenges which we describe in detail in ***Section 6***.

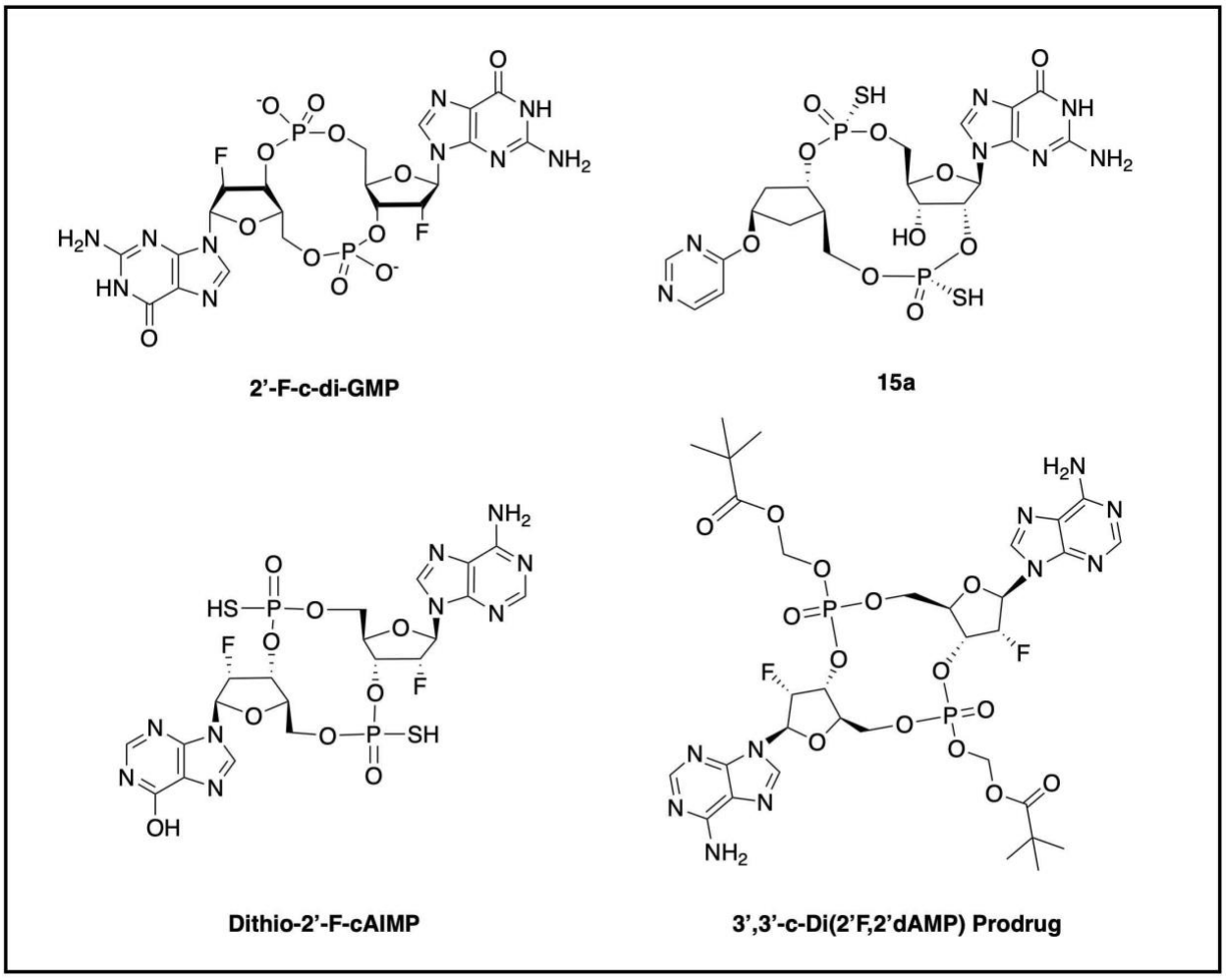

Figure 12: *Chemical structures of modified CDN STING agonists.*

Chemical structures of various CDN STING agonists that have been chemically modified for improved stability, activity, and cell permeability. The CDNs modified to include fluorine substitutions for 2′-hydrogens or 2′-hydroxyl groups on the pentose rings (*i.e.* 2′-F-c-di-GMP[361], Dithio-2′-F-cAIMP (Compound 53)[362], and the 3′3′-c-Di(2′F,2′dAMP) Prodrug[363]) exhibit improved membrane permeability as well as stability against enzymatic degradation. The carbocyclic STING agonist, 15a, which comprises carbocyclic nucleotides, cyclopentane instead of ribose, and the imidazole portion of adenine replaced with a pyrimidine ring, exhibits significantly improved STING binding, cellular activity, and membrane permeability[364].

Current CDNs, and all STING pathway agonists more generally, also lack cell and tissue specificity. STING is expressed in many cell types including immune, non-immune (*e.g.* endothelial cells), and cancer cells[365], that may respond differently to STING activation with potential to impact therapeutic outcomes. Notably, there is significant evidence that STING overstimulation can be toxic to lymphocytes. For example, T cells have been found to have high STING expression, but in general, exhibit low type I IFN responses[86]. Instead, the IFN-independent activities of STING can trigger cell death in T cells[86, 237, 366], which may prove problematic for cancer immunotherapy where a central goal is to enhance T-cell infiltration and function in immunogenically "cold" tumors. Interestingly, tumors have been shown induce STING-mediated T cell death as an immune evasion mechanism[237]. On the other hand, exploiting the sensitivity of T cells to STING-mediated cell death could be advantageous for the treatment of T cell lymphomas. Similarly, STING activation can trigger apoptosis in both normal and malignant murine B cells[273, 367, 368] and may also reduce responsiveness to B cell receptor (BCR) activation, resulting in reduced antibody responses[369], though this has not been fully resolved[367]. Notably, STING is also known to be poorly expressed and partly dysfunctional in resting human B cells[370].

Hence, an emerging and important area of research that could address both the challenge of intracellular delivery and cell-specificity is the development of targeting strategies to deliver CDNs to specific cell types based on differential expression of internalizing receptors. In particular, considering the toxicities involved with STING hyperactivation in lymphocytes, it may be particularly advantageous to target tumor-associated myeloid cells, tumor cells, or endothelial cells to improve immunotherapy responses to CDNs. To our knowledge, there are no reports of directly targeted CDNs, though the drug carrier technologies we discuss below are poised to enable more selective targeting to specific cell types with potential to enhance delivery efficiency and efficacy.

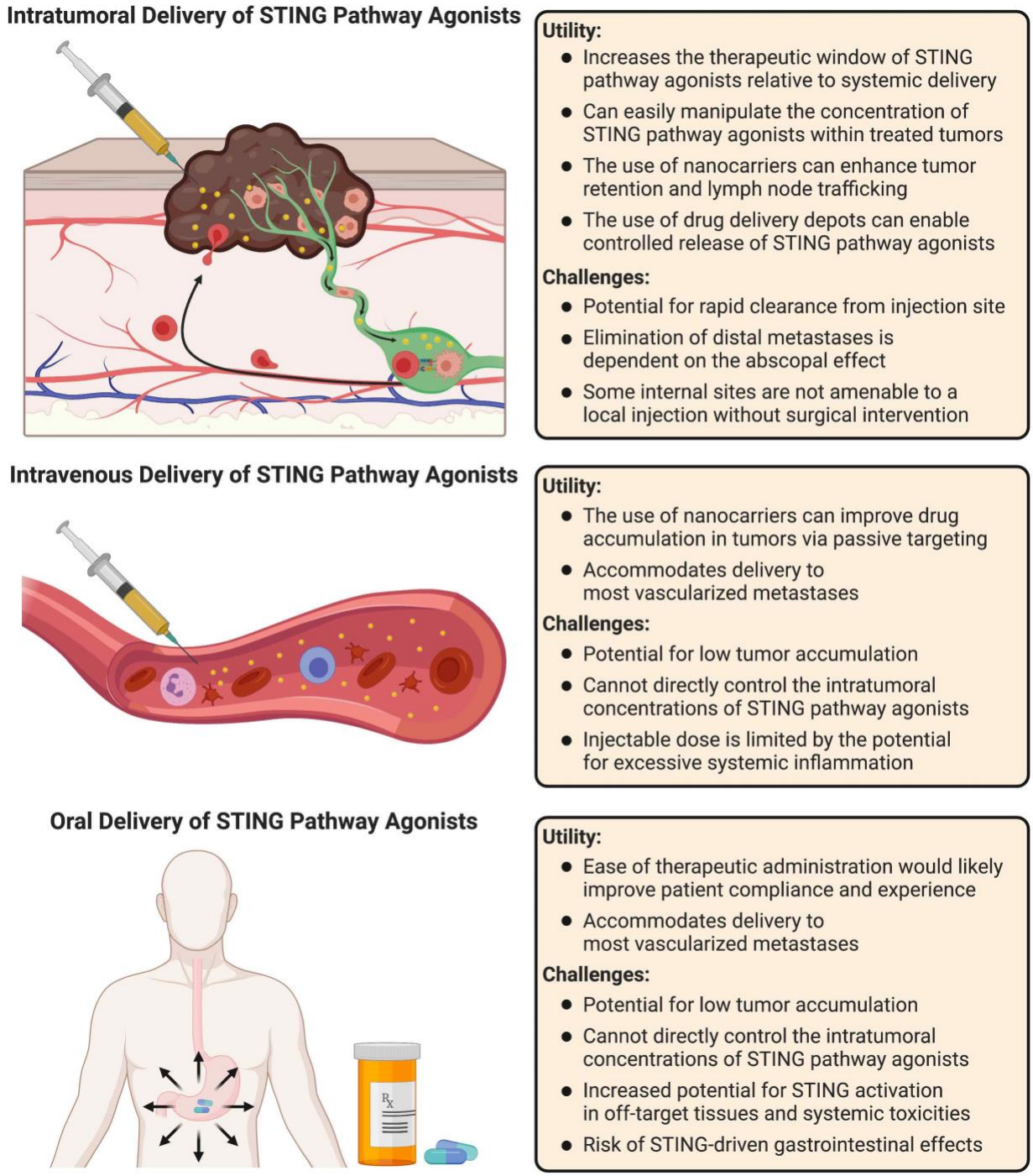

Figure 13: *Utility and challenges facing the administration of STING pathway agonists*.

The route of therapeutic administration can significantly impact the efficacy of STING pathway agonists. Administration can be local (*e.g.* intratumoral) or systemic (*e.g.* intravenous, oral), and each delivery route is associated with unique utility and challenges. Figure created with *biorender.com*.

5.2 *Utility and Challenges with Intratumoral Administration of STING Pathway Agonists*

Intratumoral injections offer the advantage of directly targeting a tumor site with relatively well-defined initial concentrations of a therapeutic, while reducing systemic drug exposure and associated risks of toxicity[371]. Indeed, the intratumoral administration of STING agonists has performed exceptionally well for murine transplantable tumor models, mediating the rejection of many locally-injected tumors and significantly enhancing the therapeutic benefit of ICB and radiotherapy[289, 372]. Accordingly, clinical evaluation of innate immune agonists, including CDNs, has primarily employed an intratumoral administration route. In addition to treating the injected tumor, such intralesional therapy can also act as an *in situ* cancer vaccine that primes and/or activates a peripheral tumor antigen-specific CTL response capable of eliminating distal disease (*i.e.* abscopal effect). Although STING activation via intratumoral administration of CDNs is still being explored clinically as a therapeutic strategy for treating solid tumors, there are several challenges that are inherent to this administration route (***Figure 13***), which may contribute to the underwhelming clinical responses observed in recent clinical trials.

The properties of solid tumors can vary widely between patients and cancer types and tumor injection techniques lack standardization, making it difficult to develop an overarching administration and dosing scheme. Parameters such as tumor size, morphology, anatomical location, degree of vascularization, interstitial pressure, mechanical properties, among other variables, can cause significant deviations in intratumoral drug concentration and/or distribution with potential to impact immune responses and therapeutic outcomes[373]. Moreover, cellular composition and level of STING expression can be variable between tumors, which may significantly affect a STING agonist's mechanism of action[373].

Due to their small size and high water solubility, CDNs rapidly diffuse from the injection site, and therefore, concentrations of CDNs in tumors following intratumoral administration can be transient and highly variable, potentially leading to different CDN dose-dependent effects[258, 373, 374]. Indeed, the immune and therapeutic effects elicited by intratumorally administered CDNs have been reported to have a bell-shaped dose-response relationship in mouse tumor models. In recent work by Sivick *et al*., high intratumoral doses of CDNs resulted in an ablative response, that efficiently inhibited growth of the injected tumor, but also resulted in systemic drug and cytokine exposure that resulted in immune cell death in the tdLNs, thereby impairing priming of anti-tumor adaptive immunity[232]. By contrast, lower doses of CDN were slightly less effective at inhibiting injected tumor growth, but resulted in a more immunogenic antitumor response

characterized by a stronger and more durable peripheral tumor antigen-specific CTL response. As a primary goal of intratumoral administration is to induce a systemic adaptive antitumor immunity, careful selection of local CDN dose appears critical to generating a robust T cell response via *in situ* vaccination. Therefore, an uneven dispersion of CDNs following injection may result in different responses within the same tumor (*i.e.* one section may receive an ablative dose while another receives an immunogenic dose), further complicating dose selection and potentially confounding interpretation of clinical outcomes. Indeed, it has recently been shown that variations in needle design (*i.e.* the use of a multi-side hole needle) can have a profound effect on intratumoral drug deposition and therapeutic efficacy in mouse tumor models[375].

The rapid clearance of CDNs from the injection site also limits the efficiency at which they accumulate and activate STING in the tdLNs, which are prime targets for immunotherapeutic intervention due to their important role in initiating T cell responses to tumor antigens[374, 376]. Since blood capillaries are permeable to molecules smaller than ~ 5−10 nm in diameter, CDNs rapidly clear from interstitial space and primarily partition into the blood stream instead of distributing into the lymphatics. This is supported by studies in mice by Sivick *et al.,* who demonstrated that intratumorally administered CDNs rapidly distribute systemically[232] as well as by plasma pharmacokinetic analysis in patients following local CDN administration[326]. By contrast, nanoparticles (NPs) between 10−100 nm in diameter preferentially drain into lymphatic vessels, resulting in increased accumulation in LNs and uptake by APCs.[377-379]. This has motivated the design of nanocarriers for CDNs that can exploit lymphatic transport to promote CDN delivery to LNs[194, 374, 380]. Indeed, this approach has been leveraged to enhance the vaccine adjuvant properties of CDNs[374, 380] and could similarly enhance immune responses to tumor antigens by stimulating STING activation in tdLNs, which may also be immunosuppressed. However, lymph vessels might also be dysfunctional in some tumors[381], potentially restricting lymphatic drainage of nanoscale STING pathway agonists.

Though there are many advantages to direct intratumoral injection, some tumors are not readily accessible without the aid of advanced image-guided administration techniques and, in certain cases, intratumoral injection may simply not be feasible. Furthermore, STING agonists normally require multiple, repeated intratumoral injections to achieve therapeutic efficacy in murine tumor models, but such dose regimens may not be possible and/or practical for many clinical cases[382]. For example, in an important recent study by Brody and colleagues, patients with indolent non-Hodgkin's B cell lymphoma were administered intratumoral injections of Flt-3

ligand (Flt3L) daily for nine days, followed by two days of radiation therapy, followed by eight injections of the immunostimulatory adjuvant poly-ICLC[383]. While the results of this study offer compelling evidence in support of *in situ* vaccination, such frequent dosing schedules may also limit patient compliance, which is known to be dependent on the complexity of treatment[384]. Notably, adherence rates for medication have been reported as low as 52% for patients in the United States with at least one chronic disease[385]. Additionally, repeated injections can cause problematic local inflammation and associated complications (*e.g.* vascular catastrophe)[373]. Indeed, one clinical trial combining ADU-S100 and spartalizumab ICB (*i.e.* anti-PD-1 monoclonal antibody therapy) required weekly intratumoral injections and resulted in several side effects, including injection site pain, diarrhea, fatigue, and pyrexia[326].

The timing, kinetics, and intensity of immunotherapeutic interventions can also significantly affect the generation and maintenance of antitumor immunity, and therefore suboptimal therapeutic dosing regimens can lead to poor therapeutic responses and/or resistance to immunotherapy[386, 387]. Accordingly, there is an opportunity for strategies that modulate the intratumoral delivery of STING pathway agonists to improve therapeutic responses by enabling more precise control over local drug concentrations and pharmacokinetic profiles. Future strategies involving intratumoral delivery of STING pathway agonists could also focus on improving and more tightly regulating tdLN accumulation to maximize antitumor T cell priming and activation. Notably, drug depot technologies, which will be discussed in detail in ***Section 6.2***, can address many of the challenges facing intratumoral administration of STING pathway agonists as they can be delivered locally and facilitate controlled and sustained release to precisely regulate local dose and STING signaling kinetics, while also potentially reducing the need for multiple injections.

5.3 *Utility and Challenges with Systemic Administration of STING Pathway Agonists*

Systemic (*e.g.* IV, oral) administration of STING pathway agonists has the potential to mitigate many of the aforementioned challenges of intratumoral administration. However, this route of administration still presents many key barriers that can limit the efficacy of STING-activating therapeutics (***Figure 13***). Due to their size and hydrophilicity, intravenously administered CDNs have short serum half-lives (*e.g.* ~ 2 minutes in mice), which, along with their poor drug-like properties, significantly limits their overall exposure, tissue distribution, and activity[326, 388, 389]. Accordingly, systemically administered CDNs have proven largely ineffective in limiting tumor growth in mouse tumor models[24, 194, 388]. To improve efficacy for intravenous

administration, Vyskocil *et al.* approached some of these limitations by developing a novel carbocyclic STING agonist (*i.e.* 15a), which comprises carbocyclic nucleotides, cyclopentane instead of ribose, and the imidazole portion of adenine replaced with a pyrimidine ring[364] (***Figure 12***)**.** 15a exhibited a half-life of ~ 23.4 minutes in BALB/c mice when administered intravenously at 1 mg/kg with systemic exposure (*i.e.* 3.9 μM in plasma collected 5 minutes after in injection) at or above cellular concentrations required for its activity. Furthermore, the unique structure of 15a resulted in significantly improved STING binding, cellular activity, and membrane permeability, leading to a robust antitumor effect in CT-26 tumor models. Although this agent seems promising, it is also a relatively new molecule, and therefore further work will be necessary to determine its efficacy across a larger range of tumor types and treatment regimes.

The barriers listed above, coupled with the preclinical efficacy of DMXAA, a molecule with more canonical drug-like properties, motivated the recent development of the non-nucleotide, small molecule STING agonists that are described in ***Section 4.1.2.*** Such agents have enhanced cellular permeability and would ideally also exhibit improved pharmacokinetic properties relative to CDNs, allowing for optimal exposure and STING activation. One significant recent example of a non-nucleotide, small molecule STING agonist is an amidobenzimidazole compound described by investigators at GlaxoSmithKline[132]. The lead compound (*i.e.* Compound 3) exhibited a half-life of 1.4 hours and an AUC of 3 μg h^{-1} ml^{-1} when administered intravenously at 3 mg/kg in healthy BALB/c mice, and concentrations in the blood exceeded the *in vitro* EC_{50} as determined in murine PBMCs (*e.g.* ~ 200 ng/mL)[132]. Notably, intravenous administration of Compound 3 resulted in significant tumor growth inhibition in BALB/c mice with subcutaneous CT26 colorectal tumors[132].

Two additional non-nucleotide, small molecule STING agonists that have recently been described, MSA-2 and SR-717, have been shown to exert therapeutic effects when administered orally, subcutaneously, or intraperitoneally. In MC38 tumor-bearing C57BL/6 mice, MSA-2 had a plasma half-life of ~ 14 minutes when delivered subcutaneously at 50 mg/kg and ~ 1.7 hours when given orally at 60 mg/kg (note that these half-lives are estimates from published pharmacokinetic data, where calculated half-lives were not described)[338], whereas in healthy C57BL/6 mice, SR-717 and its analog for oral delivery (*i.e.* SR-301) had half-lives of 6.37 hours when administered intraperitoneally at 3 mg/kg and 11.11 hours when dosed orally at 5 mg/kg, respectively[145].

However, unlike more conventional cancer therapeutics, relationships between pharmacokinetics, efficacy, and toxicity have not yet been fully defined for STING pathway agonists, and therefore, what the ideal pharmacokinetic properties should be is currently unknown. Given the complex relationships between the kinetics and magnitude of STING activation on immunity and toxicity, it will be important to better understand the underlying pharmacokinetic-pharmacodynamic relationships that govern the efficacy of STING pathway agonists.

In clinical trials of intratumorally administered ADU-S100, a maximum tolerated dose was not reached up to 800 μg in dose-escalation studies[326], consistent with the relatively low cellular uptake of CDNs. However, primary concerns underlying the systemic administration of STING pathway agonists are the lack of tumor or cellular specificity and the potential for inducing a toxic systemic inflammatory response. While STING activation in extratumoral cell populations (*e.g.* myeloid cell populations in secondary lymphoid organs) may exert antitumor effects, STING activation would ideally occur primarily within tumor sites to generate the local inflammatory context and chemokine gradient necessary for the recruitment of T-cells into the TME[373] while restricting STING activation in the blood stream and at other organ sites to minimize systemic inflammatory side effects. Currently, the therapeutic window for systemically administered STING agonists appears to be limited by non-specific systemic STING activation that can induce a cytokine storm, also known as cytokine release syndrome[390], similar to the response observed in sepsis[391]. Indeed, there is evidence linking overactivation of STING signaling in macrophages and monocytes to sepsis in mouse models[392, 393]. Symptoms from cytokine storm can range from mild fever, fatigue, headache to more serious physical outcomes such as hypotension, neurotoxicity, and multi-organ system failure[391]. STING induces a broad spectrum of cytokines, and the predominant mediators of efficacy and toxicity have not been fully resolved. However, defects in type I IFN signaling can result in excessive and unbalanced production of proinflammatory IL-6 and TNF-α, which has been linked to severe COVID-19 disease[394] and may also be involved in mediating inflammatory toxicities induced by STING agonists. Moreover, with the recent reports describing MSA-2 and SR-301 as orally available STING agonists, it will also be important to understand the potential implications of STING activation in the gastrointestinal tract, which has a complex role in mediating homeostasis in the gut with oral administration of STING agonists previously being reported to cause or exacerbate colitis[395, 396].

As results from clinical trials of other STING agonists emerge, particularly for non-nucleotide, small molecule agonists that are likely to activate STING indiscriminately and systemically, it will be critical to more fully understand the determinants of toxicity and efficacy to inform the design of strategies to widen the therapeutic window. One promising strategy may be to achieve more tumor selective STING activation using tumor-targeting agents (*e.g.* antibodies), prodrug strategies, and/or environmentally-responsive agonists. For example, MSA-2 bears a carboxylate with a pKa of 4.78 and, therefore, upon reaching an acidified TME (*e.g.* pH ~ 6.0−6.5) that occurs in some cancers[339, 340], the carboxylic acid protonates, reducing its negative charge and aqueous solubility and thereby increasing its cell membrane permeability[338]. To our knowledge, the MSA-2 small molecule agonist offers the only published report of an environmentally-responsive or targeted STING agonist, though antibody-targeted agonists of other pattern recognition receptors (*e.g.* TLR-7 agonists) have been described to preferentially trigger innate immunity at tumor sites[397]. An important and unresolved question is to which cell population(s) STING pathway agonists should be targeted either within tumors, in the circulation, and/or residing in secondary lymphoid tissue. For example, targeting of tumor-associated macrophages with STING pathway agonists has potential to promote an M1-like phenotype that would create an antitumor immune environment by promoting antigen presentation, secretion of proinflammatory cytokines, and infiltration of CTLs. Nanoparticle platforms for CDN delivery have recently emerged (discussed in ***Section 6***) and a large tool box of strategies exist for integrating targeting ligands onto nanocarriers, which offers promise for enhancing tumor targeting of STING pathway agonists.

It is also important to consider the possibility that STING activation can potentially cause immune tolerance and even the onset of autoimmune disease[12]. Notably, STING signaling can induce production of indoleamine 2,3 dioxygenase (IDO), which is both counter-regulatory to anti-tumor T cell function and has broad tolerogenic effects[398]. IDO activation induces downstream effects that drive immunosuppressive regulatory T cell (T_{REG}) differentiation and push macrophages and DCs towards an immunosuppressive phenotype, limiting immune response and promoting tumorigenesis[398]. Interestingly, intratumoral administration of STING agonists has resulted in more tolerogenic responses in a Lewis lung carcinoma mouse model[217]. There is also some evidence that suggests that certain factors linked to STING pathway activation, such as chromosomal instability, STING activation in mesenchymal stromal cells, and cGAMP transfer via the astrocyte gap-junctional network may contribute to metastasis, suggesting the need to further explore proper dosing regimens and targeting strategies that may avoid these adverse effects.[47,

[152, 399] Beyond tumorigenesis, dysregulation of STING can also contribute the development of inflammatory and autoimmune disease such as vascular and pulmonary syndrome, lupus-like syndromes, and STING-associated vasculopathy[400]. Researchers have even shown that cGAS/STING activation or increased expression can induce acute pancreatitis, colitis, and liver fibrosis[396, 401, 402]. However, it should be noted that most of these arise from sustained or chronic STING activation and, hence, may not be manifested in an immune-oncology setting. Nonetheless, they reflect the dichotomous role of STING signaling and the importance of carefully regulating the dose and kinetics of STING pathway agonists in promoting immunity instead of tolerance or chronic inflammation.

With a number of clinical trials for a diverse array of STING agonists underway or ongoing (***Table 1***), it will be interesting to learn in the coming months and years to what extent systemically administered agents have a therapeutic window and what acute and chronic side effects, if any, may manifest. Regardless, the potential for inflammatory side effects associated with systemic delivery of STING pathway agonists, and innate immune agonists more generally, motivates the need for tunable drug delivery technologies and/or novel agents that can help mitigate toxicity while still promoting the desired anti-tumor immune response. Such strategies potentially include nanocarriers that can improve the pharmacokinetic properties of STING pathway agonists such as half-life and AUC while exploiting dysfunctional tumor vasculature to enhance tumor accumulation, environmentally-responsive pro-drugs that activate selectively at tumor sites, and molecularly-targeted STING pathway agonist that enrich STING activation at tumor or immune priming sites and/or within specific cell populations. This is a nascent but important and rapidly expanding area of research with vast potential for expanding the therapeutic window of STING pathway agonists, and below we discuss emergent drug delivery strategies for improving the efficacy of STING pathway agonists for both systemic and local administration.

6 Delivery Technologies for STING Pathway Agonists

6.1 Nanotechnology for the Delivery of Cyclic Dinucleotides

Although CDNs exhibit great therapeutic promise for eliciting antitumor immunity, a variety of delivery challenges remain and restrain their potential. Much of this can be attributed to the anionic phosphate groups on CDNs that significantly limits their passive diffusion across the lipophilic plasma membrane and restricts their access to the cytosol for STING binding[193, 360, 403]. Thus far, clinical responses to intratumorally administered CDNs have proven modest due, in part, to the delivery barriers described above, including rapid clearance, lack of cytosolic delivery,

and nonuniform drug distribution[360, 374, 403, 404]. Similarly, systemic administration of CDNs is typically ineffective even in preclinical models due to a number of pharmacological shortcomings.

NP delivery platforms have great potential for improving the efficacy of STING pathway agonists as they offer numerous opportunities for enhancing CDN activity (***Figure 14***). First, NPs provide a strategy for modulating pharmacokinetic and biodistribution properties of drug cargo. A large and well-established tool box of chemical strategies exists for modulating the key physicochemical properties of NPs (*e.g.* size, shape, surface chemistry, stability, mechanical properties, permeability, *etc.*) and can be employed to modulate the pharmacokinetic and distribution behavior of STING pathway agonists to optimize immunotherapeutic benefit and minimize off-target inflammatory effects[405, 406]. For example, systemically administered NPs have a well-established capacity for passively targeting solid tumors, which provides a facile and promising approach for increasing drug accumulation in tumors. However, it should be noted that while it is clear that NPs can preferentially accumulate in human metastatic tumors[407-411], the primary mechanism of tumor accumulation is controversial[412-414]. The enhanced permeability and retention (EPR) effect, which was first proposed in 1986, describes how preferential tumor accumulation of NPs can be attributed to hypervascularity, defective vasculature, and poor lymphatic drainage in solid tumors[415, 416]. The EPR effect has been a guiding principle of NP delivery for over 30 years, but recent work has challenged the importance and relative contribution of the EPR effect. Indeed, Sindhwani *et al.* found that NPs enter tumors using an active process through endothelial cells[417], suggesting that physical gaps in tumor vasculature may not be as important as previously thought. Regardless, NP size and surface properties remain important design criteria that can be tuned to promote tumor accumulation.

Second, NPs can be designed with environmentally-responsive functionalities to enhance cytosolic CDN delivery and/or trigger drug release in response to a specific microenvironmental stimuli (*e.g.* pH, hypoxia, reactive oxygen species, *etc.*) for tumor-selective drug release or activation[418]. Third, NPs allow for co-packaging of multiple agents into a single particle at defined ratios, with potential to enable synergy between STING pathway agonists and other agents (*e.g.* chemotherapeutics, STING pathway potentiators) to widen the therapeutic window. Finally, NPs provide a versatile platform for introducing targeting ligands (*e.g.* antibodies, peptides, glycans) with potential to enhance tumor, lymphoid organ, and/or cell-specific delivery of STING pathway agonists. Below we describe recent advancements in NPs for the delivery of STING pathway agonists, which has primarily been directed at improving the delivery of CDNs.

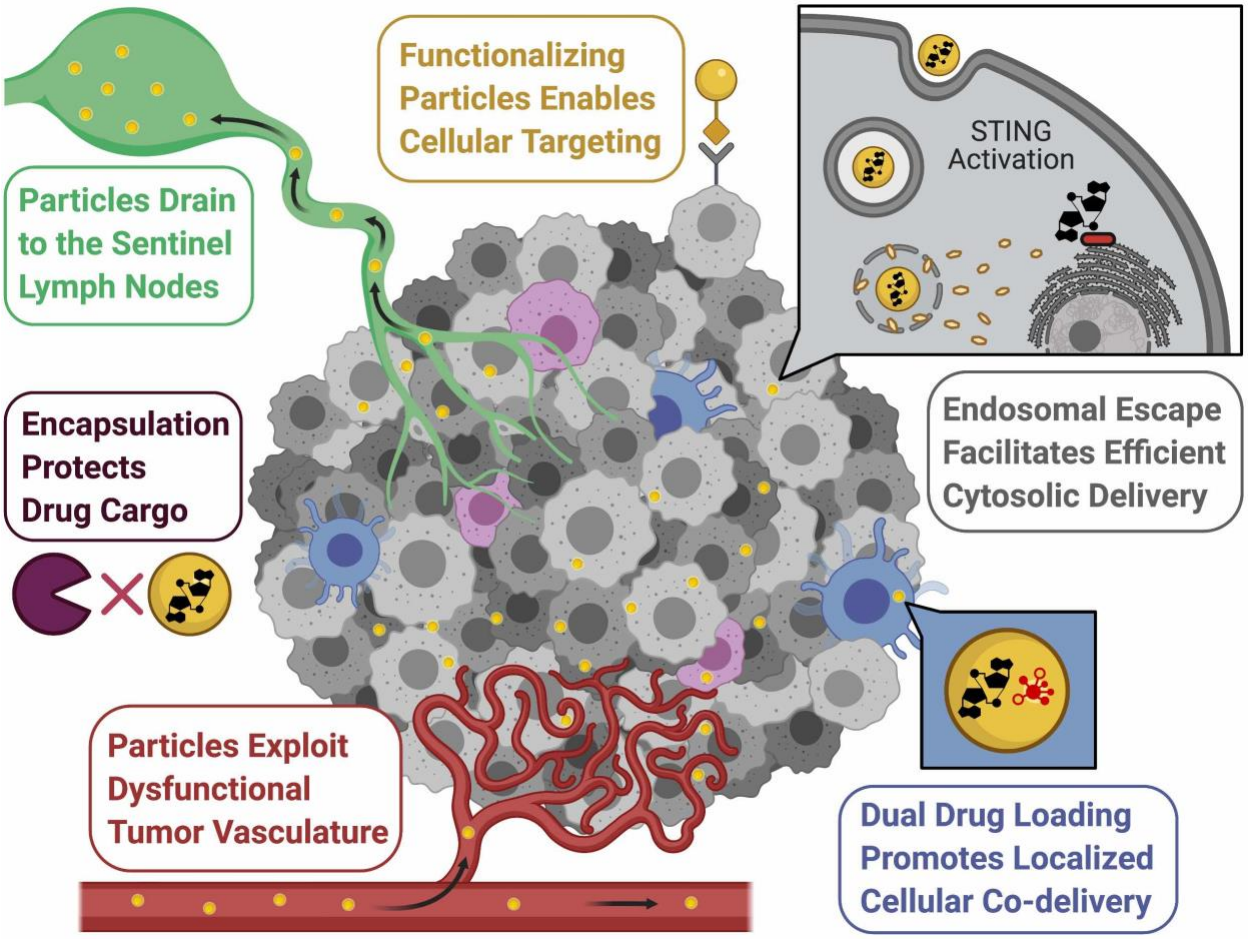

Figure 14: *Opportunities for nanotechnology in the delivery of STING pathway agonists*.
Nanotechnology can be employed to overcome many drug delivery challenges and therefore has potential to greatly improve the therapeutic efficacy of STING pathway agonists. Notably, nanotechnology can exploit dysfunctional tumor vasculature, protect drug cargo, promote lymphatic drainage, enable cellular targeting, facilitate cytosolic delivery, and allow for cellular co-delivery of various drugs. Figure created with *biorender.com*.

6.1.1 Lipid-based Delivery Systems

Many research groups have sought to address some of the barriers to CDN delivery through the development of liposomal NP formulations (***Figure 15***)[173, 252, 360, 374, 419-427]. Liposomes are logical candidates for CDN delivery since they have an aqueous core for loading of hydrophilic cargo[428-430]. Koshy *et al.* utilized a cationic liposome formulation (***Figure 15A***) consisting of varying amounts of 2 kDa polyethylene glycol (PEG) along with the cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and cholesterol, repurposing a formulation that has been used in gene delivery applications and has been evaluated in cancer patients[360]. While more

cytotoxic than their charge neutral counterparts, cationic liposomes can interact with the anionic cell membrane, allowing for enhanced internalization, and can also promote endosomal escape into the cytosol[431]. In these studies, the authors chose to load a phosphorothioated cGAMP analog into the liposomes, as that chemical modification confers resistance to degradation by ENPP1 and therefore can elicit a greater degree of STING signaling by avoiding rate-limiting CDN degradation[195]. A primary goal of the work was to evaluate the effect of surface PEGylation density on particle properties and attendant effects on antitumor efficacy. The liposome formulations used in the study contained 0, 5, and 10 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(PEG)-2000] (DSPE-PEG(2000)) with a 1:1 DOTAP:cholesterol ratio. Non-PEGylated liposomes resulted in an increased diameter compared to PEGylated particles, likely due to anionic serum protein binding on the cationic liposome surface causing liposome aggregation. While all NP formulations enhanced CDN uptake by BMDCs and increased STING-mediated proinflammatory cytokine expression relative to free cGAMP, PEGylation reduced the positive zeta-potential of the liposomes and thereby reduced CDN uptake by BMDCs and lowered STING activation relative to the non-PEGylated NPs, reflecting the familiar interplay between charge, uptake, toxicity, and activity that has also been described for nucleic acid therapeutics (*e.g.* siRNA)[432]. They also evaluated the effect of PEG surface density on inflammatory gene expression and inhibition of tumor growth using an intratumoral administration route in an orthotopic B16-F10 melanoma model. While there were relatively modest differences between the formulations on *Ifnb1* and *Cxcl9* expression, formulations lacking PEG (*e.g.* 0% PEG) were significantly less effective than both PEGylated liposomes as well as the free CDN, which led to complete regression of tumors in half of the mice. By evaluating the intratumoral distribution of a fluorescently-labeled CDN, this was attributed to the very poor tumor penetration of the CDN when loaded into the PEG-free liposome owing to aggregation at the injection site. Interestingly, though similar responses were observed in primary tumor regression between free cGAMP and the PEGylated liposomal formulations, the later appeared to confer greater protection from tumor rechallenge. Importantly, the authors also evaluated intravenous administration of the 5% PEGylated liposome in a lung metastatic B16-F10 model. Consistent with the capacity for DOTAP/cholesterol liposomes to passively target the lungs, they demonstrated increased inflammatory gene expression in tumor-bearing lung tissue, but a very modest effect on lung metastatic burden even when combined with ICB (*i.e.* anti-PD-1 and anti-CTLA-4). Hence, while further optimization of the delivery technology appears to be necessary for treatment of lung metastasis, this work highlights the importance of NP corona chemistry on tumor distribution and antitumor immunity.

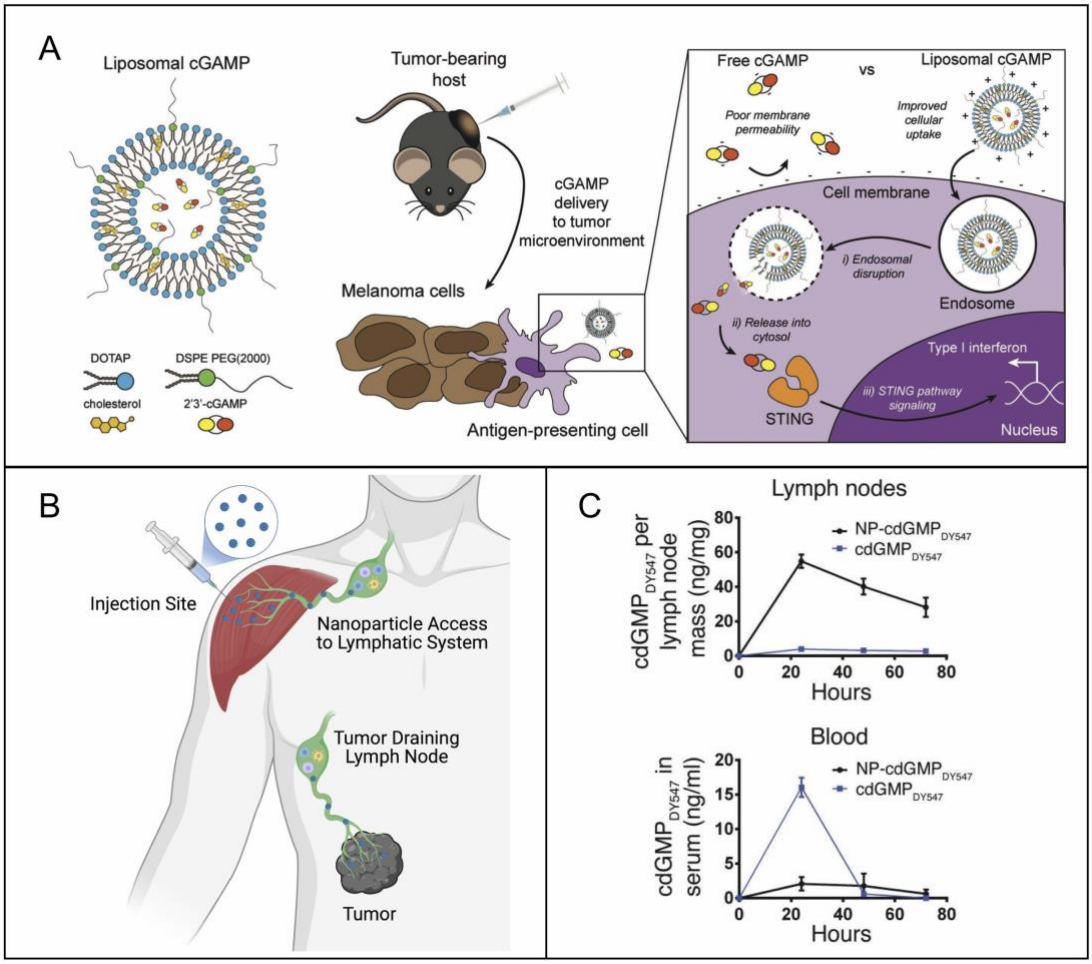

Figure 15: *Lipid-based CDN delivery systems*.

(A) Schematic of a cationic liposomal 2′3′-cGAMP formulation, which comprises DOTAP, cholesterol, and DSPE-PEG(2000), and the proposed mechanism of intracellular 2′3′-cGAMP delivery. Reproduced with permission from reference[360]. Copyright © 2017 John Wiley & Sons - Books; permission conveyed through Copyright Clearance Center, Inc. **(B)** Schematic demonstrating that nanoparticles can enhance drug delivery to draining lymph nodes via lymphatic transport. Figure created with *biorender.com*. **(C)** By exploiting the lymphatic transport of nanocarriers, CDN (*i.e.* cdGMP) concentration increases in the draining lymph nodes of the injection site and decreases in the blood stream when delivered with a lipid nanoparticle. Reproduced with permission from reference[374]. Copyright © 2015 American Society for Clinical Investigation; permission conveyed through Copyright Clearance Center, Inc.

Similarly, Cheng *et al.* recently developed a non-PEGylated liposomal NP using a mixture of hydrogenated (soy)L-α-phosphatidylcholine (Soy-PC) and DOTAP to deliver 3'3'-cGAMP (referred to as NP-cGAMP)[252], resulting in a formulation that could be delivered IV. Liposomes were made using a 100:1 ratio of Soy-PC to DOTAP to encapsulate cGAMP through thin-film hydration followed by membrane extrusion. This method resulted in particles that were ~ 85 nm

in diameter with a +15 mV zeta-potential and a cGAMP encapsulation efficiency of ~ 43%. They evaluated their formulation in basal-like triple negative breast cancer C3(1) breast cancer models that are resistant to anti-PD-L1 ICB. Of high translational significance, they also evaluated their approach in a genetically engineered mouse C3(1) Tag model that generates spontaneous primary and secondary tumors with diverse immunosuppressive microenvironments that more accurately reflects human breast cancer. Impressive antitumor effects were observed in both an orthotopic transplant and the genetically engineered mouse model, with NP-cGAMP outperforming free cGAMP in terms of controlling tumor growth and prolonging survival. Notably, the formulation was well tolerated as indicated by minimal mouse weight loss following intravenous administration, and a single dose of cGAMP-loaded liposomes was enough to suppress tumor growth. The antitumor effect of NP-cGAMP was shown to be dependent on both $CD4^+$ and $CD8^+$ T cells as well as macrophages and was correlated with reprogramming of protumorigenic M2-like macrophages towards a more M1-like phenotype that support T cell infiltration and antitumor effector function. Additionally, by inducing a type I IFN response, NP-cGAMP may also have induced tumor cell apoptosis and inhibited proliferation. While less striking results were observed in a B16-F10 model, potentially reflecting differences in immunogenicity, the high degree of efficacy and safety in the C3(1) Tag model afforded by this liposomal formulation offers translational promise for breast cancer immunotherapy.

An alternative cationic lipid to DOTAP was described by Miyabe *et al.* who developed a synthetic, pH-responsive lipid, YSK05, which has optimal membrane fusogenic activity at pH 6.4 and therefore high endosomal escape activity[419]. In their initial report, they evaluated several helper lipids and lipid compositions for enhancing the *in vitro* activity of the bacterial CDN, c-di-GMP, ultimately arriving at a liposomal formulation comprising YSK05:POPE:cholesterol:DMG-PEG at a 40:25:35:1 ratio. Upon demonstrating that the formulation increased expression of CD80, CD86 and MHC class I in murine macrophages *in vitro*, they immunized mice with a model antigen (*i.e.* ovalbumin) mixed with c-di-GMP/YSK05 liposomes and demonstrated an enhanced CTL response and protection against challenge with an ovalbumin-expressing E.G7 thymoma cancer cell line. As a follow up to this study, the group demonstrated that intravenous administration of c-di-GMP/YSK05 liposomes reduced lung tumor burden in a metastatic B16-F10 melanoma model[433] and found that the antitumor effect was primarily mediated by infiltrating NK cells that destroyed tumor cells due to their low levels of MHCI-I expression. While other reports have implicated T cells as the critical effectors in response to STING agonists, the findings of Miyabe *et al*. are consistent with those of recently described by Nicolai *et al*., who found

intratumoral CDN administration promoted NK cell activation and antitumor function[279]. This is significant as strategies to bolster NK-based tumor immunity may be critical for cancers that are adept at evading T cell recognition via MHC-I loss or downregulation.

Liposomal systems have also been developed for dual-delivery of both CDNs and monophosphoryl lipid A (MPLA), an agonist of TLR-4, another important pattern recognition receptor that can lead to IFN-I and proinflammatory responses, as a strategy to more effectively activate local APCs at tumor sites[420, 421]. Here, Karathanasis and co-workers utilized liposome formulations that lacked cationic lipids. Their first formulation consisted only of 1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and methoxy-PEG-2000 1,2 distearoyl-sn-glycero-3-phosphoethanolamine (mPEG2000-DSPE) at 48.5, 48.5, and 3 mole percent, respectively, and generated relatively small liposomal particles (*i.e.* 60 nm in diameter)[420]. By co-encapsulating c-di-GMP and MPLA they were able to ensure delivery of both agonists to the same cell, resulting in synergistic activation of both STING and TLR-4 signaling. Owing to a lack of cationic lipids, this formulation was particularly well suited for systemic delivery, which resulted in liposome accumulation in APC-rich perivascular regions of tumors, primarily stimulating IFN-I responses within tumor-infiltrating APCs rather than the cancer cells. Impressively, intravenous administration of liposomes loaded with both CDN and MPLA significantly reduced the growth of 4T1 mammary tumors and prevented lung metastasis. In a subsequent study, a similar liposomal formulation consisting of c-di-GMP and MPLA encapsulated in 77:20:3 DPPC, cholesterol, and mPEG2000-DSPE was used to systemically treat Panc20 pancreatic ductal adenocarcoma tumors[421]. An important aspect of this subsequent work was the finding that synergy between c-di-GMP and MPLA can be increased by systematically adjusting the MPLA/c-di-GMP ratio in the formulation. Specifically, the authors fabricated and tested an $MPLA^{hi}$ (300 μg MPLA per 42 μmol lipids) and $MPLA^{lo}$ (300 μg MPLA per 42 μmol lipids) with a constant amount of CDN loading. They found that the $MPLA^{hi}$ variant generated a 1.6-fold higher IFN-β response than the $MPLA^{lo}$ formulation and, importantly, that dual-encapsulated particles enhanced IFN-β production relative to dose-matched single-agent particles (c-di-GMP-NPs and MPLA-NPs) by 11- and 22-fold. Importantly, this also manifested *in vivo*, with the $MPLA^{hi}$ formulation tending to increase the number of IFN-β secreting immune cells, and impressively, also inhibited Panc02 tumor growth to a greater extent than free agonists or the $MPLA^{lo}$ formulation. This study nicely illustrates the potential to potentiate STING pathway activation via coordinated delivery of another agent at a defined ratio, a distinctive advantage of leveraging a nanocarrier platform.

While an important advantage of nanocarriers for CDNs has been their ability to enable intravenous administration, they can also be harnessed to improve efficacy via other administration routes. An excellent example of this is the work by Liu *et al.* who designed a very elegant liposomal cGAMP formulation to specifically target pulmonary APCs to enhance anticancer immunity against lung metastases, which are common in many cancer types[422]. Because phagocytes such as macrophages and DCs can recognize membrane exposed phosphatidylserine (PS) on apoptotic cells, they integrated PS into a liposomal cGAMP formulation to provide an "eat me" signal for APCs. Their formulation was fabricated through a two-step water-in-oil reverse microemulsion and incorporated calcium phosphate (CaP) to improve cGAMP encapsulation and provide a release and endosomal escape mechanism triggered by a decrease in endo/lysosomal pH. Using a 5:4:1 ratio of phosphatidylserine:1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC):cholesterol resulted in NP-cGAMP particles that were anionic (*i.e.* −40 mV) and ~ 120 nm in diameter, yet still achieved a 72% cGAMP encapsulation efficiency. Both layers of liposome membrane contained anionic PS, which allowed for both APC targeting and neutralization of excessive cationic Ca^{2+} of CaP not complexed with cGAMP in the core. Using an aerosolized NP-cGAMP formulation to achieve delivery to the deep lungs via inhalation, particles rapidly distributed throughout both lungs and were efficiently endocytosed by pulmonary APCs. Inhalation of NP-cGAMP alone led to a decrease in the number of metastatic foci in both lungs in a melanoma model of lung metastasis. Importantly, combining NP-cGAMP with radiotherapy further increased therapeutic efficacy in both melanoma and breast cancer metastasis models, even leading to complete regression of lung metastases in some mice. This synergistic effect was attributed to enrichment of STING activation in pulmonary APCs that therefore more efficiently cross-primed antitumor $CD8^+$ T cells and generated a proinflammatory TME that supported T cell infiltration and inhibited immunosuppressive T_{REG} cells .

While the use of targeted nanoparticles for cell- or tissue-specific delivery of STING activation is still in its infancy, this work highlights the potential merits of targeting specific cell populations within a specific tissue, which may also minimize undesired inflammatory side effects. For example, Li *et al*. have also leveraged the idea of APC targeting by incorporating a DSPE-PEG-mannose conjugate into a liposomal cGAMP formulation as a strategy to target dendritic cells[423]; however, this was only evaluated using an intratumoral delivery route and the therapeutic impact of mannose-targeting was not established.

In a comprehensive evaluation of STING agonist targeting using nanocarriers, Covarrubia *et al.* developed a NP platform for systemic c-di-GMP delivery that was designed to target APCs within the perivascular niche of the TME through both passive accumulation and direct ligand binding[427]. The researchers sought to characterize NP accumulation and cellular uptake in mouse models that mimicked different cancer landscapes, including primary tumors, early metastasis, and late metastasis. The differential uptake by specific immune cell subsets was also examined. The nanoparticles were ~ 60 nm in diameter and consisted of 48.5 mol% DOPC, 48.5 mol% DPPC, and 3 mol% mPEG2000-DSPE. The c-di-GMP was loaded into the particle core through film hydration. For the ligand-functionalized particles, 3 mol% mPEG2000-DSPE was replaced with 3 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(PEG)-2000] (DSPE-PEG2000 amine) to allow for sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) coupling between amines on the particle surface and thiol groups on the targeting peptides. Three peptides were explored as targeting ligands for their ability to bind fibronectin (CREKA), P-selectin (CDAEWVDVS), and $\alpha_v\beta_3$ integrin (c(RGDfC)) to preferentially direct the particles to the TME and certain immune cells. Fibronectin is overexpressed on the perivascular extracellular matrix, while P-selectin is overexpressed by endothelial cells in the remodeled tumor vasculature. Additionally, $\alpha_v\beta_3$ integrin is typically expressed by dendritic cells and macrophages. Although this design offers potential for next-generation, targeted STING technologies, the untargeted NPs demonstrated the highest immune cell uptake and tumor accumulation with the exception of early stage metastasis of 4T1 tumors to the lung and liver, where integrin-targeting NPs had the highest uptake by lung DCs and liver macrophages. These findings are consistent with the increased circulation time of non-targeted nanoparticles and the ability of nanoparticles to exploit dysfunctional vasculature of established tumors for passive targeting. These results also demonstrate the potential impact of molecular targeting in enhancing CDN accumulation at sites of early metastasis, where the vascular endothelium might be less permeable. Notably, in a neoadjuvant therapy model using the 4T1 murine breast cancer and co-administered ICB (*i.e.* anti-PD-1), the untargeted NPs demonstrated significant therapeutic efficacy and outperformed the integrin-targeted NPs. Though not examined in this report, the authors mention that in future work, they will explore whether targeted immunostimulatory NPs are more effective in a purely metastatic setting. Collectively, this comprehensive study nicely demonstrates the potential of leveraging targeted STING activation for specific clinical scenarios and also highlights the importance of identifying immune and cancer-specific biomarkers towards enabling enhanced delivery of STING agonists and improved therapeutic results.

In another recent example of a targeted NP for CDN delivery, Gou *et al.* discovered that STING agonists significantly stimulated type I IFN secretion in Clec9a$^+$ dendritic cells and therefore designed a peptide-expressed biomimetic cancer cell membrane (EPBM)-coated nanovaccine platform to preferentially deliver STING agonists and tumor antigens to this particular cell population[434]. Clec9a is a C-type lectin endocytosis receptor that is responsible for antigen uptake and cross-presentation. Notably, Clec9a has been utilized for targeted tumor vaccines, which motivated this group to develop a 12-mer Clec9a binding peptide (CBP-12) that could be incorporated into various delivery technologies. A retroviral vector encoding CBP-12 was constructed and used to incorporate this peptide into cancer cell membranes, which were then extracted and fused with a PLGA NP core containing 2′3′-cGAMP to form PLGA/STING@EPBM particles, which were ~ 160 nm in diameter. This resulted in enhanced CDN uptake in DCs, expression of ISGs, antigen cross-presentation, and T cell proliferation in the B16-OVA tumor model, which collectively inhibited tumor growth and prolonged survival. This platform also inhibited both tumor growth and lung metastasis in an anti-PD-1-resistant 4T1 tumor model. Additionally, this treatment strategy was further improved by co-administering radiotherapy, which was shown to increase the amount of Clec9a$^+$ DCs within the tumor microenvironment.

While not evaluated in the context of cancer immunotherapy, Wang *et al.* developed a novel liposomal cGAMP formulation for intranasal delivery and evaluated this as an adjuvant for vaccines to prevent respiratory viral infections, such as influenza[173]. CD8$^+$ tissue resident memory T-cells (T_{RM}) are essential immune effectors in viral infections, specifically those located within the lung[435-437]. Therefore, to generate a T_{RM} response, the investigators aimed to design a delivery platform that would not only target pulmonary APCs, but also alveolar epithelial cells without breaching the integrity of the pulmonary surfactant layer. To accomplish this, they designed pulmonary surfactant-biomimetic liposomes (PS-GAMP) to deliver cGAMP along with vaccine antigen (*e.g.* inactivated influenza virus) into the lung via intranasal immunization. The liposomes were considered PS-mimetic because they were based on PS constituents and ultimately an anionic formulation comprising DPPC:1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG):cholesterol:1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(PEG)-2000] (DPPE-PEG2000) at a mass ratio of 10:1:1:1 was found to most closely mimic the PS and demonstrated the strongest adjuvant properties of the formulations tested. Importantly, the authors also investigated non-PEGylated and cationic variants, which showed less adjuvancy and significantly more toxicity, implicating the negative charge and surface PEGylation as important to the activity and function of their liposomal cGAMP formulation. When used to adjuvant an H1N1

influenza vaccine, PS-GAMP generated a robust CTL response within the respiratory tract, which remained for 6 months post-vaccination, and also elicited cross-protection against other influenza strains. A particularly important result of this work of potential relevance to cancer immunotherapy was their discovery that PS-cGAMP uptake by alveolar macrophages required the surfactant proteins A and D and that cGAMP released into the cytosol of alveolar macrophages was transferred to alveolar epithelial cells through gap junctions, activating STING in both cell types. As cGAMP is known to spread via gap junctions, the design of delivery systems capable of exploiting such transfer mechanisms may prove valuable for improving tumor penetration of CDNs with potential to improve immunotherapeutic efficacy.

LNs act as command centers for orchestrating adaptive immune responses and are therefore another important target tissue for the delivery of STING pathway agonists in addition to tumor sites. Specifically, tdLNs are important sites for priming and expansion of antitumor T cells, yet, like tumor sites, may also be highly immunosuppressed and therefore contribute to T cell dysfunction[438, 439]. Additionally, cancer vaccines are a promising and re-emerging class of cancer immunotherapy, and owing to the critical role of STING activation in generating antitumor immunity, CDNs hold great potential as adjuvants for cancer vaccines[380, 440]. However, due to the rapid clearance of CDNs, their delivery to LNs is inefficient, a challenge that has partly motivated the development of strategies for targeting STING agonists to LNs. NPs of ~ 20−100 nm in diameter can passively target cargo to local draining LNs through drainage from interstitial space into the lymphatic system (***Figure 15B***)[441, 442]. In a seminal paper, Hanson *et al.* sought to exploit this transport phenomenon by encapsulating c-di-GMP into phosphatidylcholine liposomes with 5% PEGylation (NP-cdGMP)[374], electing to incorporate PEG, since it had been shown to enhance LN trafficking of liposomes in previous studies[443, 444]. Primarily due to its low molecular weight, they reported that subcutaneously administered free c-di-GMP inefficiently trafficked to the LN and instead distributed to the blood, resulting in minimal cellular uptake by APCs in the draining LN. By contrast, NP-cdGMP preferentially accessed the LN and improved CDN uptake by APCs (***Figure 15C***). Using NP-cdGMP as an adjuvant and ovalbumin (OVA) as a model subunit antigen, they found that liposomal CDN delivery elicited an ~ 3-fold greater $CD4^+$ and $CD8^+$ T cell response relative to a soluble mixture of OVA and c-di-GMP. Accordingly, immunization with NP-cdGMP as an adjuvant reduced tumor growth and prolonged survival in mice inoculated with an OVA-expressing EG.7 tumor. They also evaluated responses to the poorly immunogenic antigen MERP from HIV gp41 and further demonstrated the importance of LN targeting in optimizing CDN adjuvancy. Use of NP-cdGMP as an adjuvant significantly increased the expansion of helper T

lymphocytes, induced germinal center formation, and generated a robust and durable humoral response, while mitigating systemic cytokine production.

Finally, a unique approach to enhancing STING activation is to express activated STING itself. Tse *et al.* formulated lipid NP (LNP) mRNA vaccines to deliver the mRNA transcript of STING with a dominant gain-of-function mutation (V155M) that renders STING constitutively active even in the absence of cGAMP or other STING ligands[424]. The mRNA was synthesized *in vitro* by T7 RNA polymerase-mediated transcription with N1-methylpseudouridine in place of uridine. mRNA-loaded LNPs were formed by combining an ionizable lipid:DSPC:cholesterol:PEG-lipid at a ratio of 50:10:38.5:1.5 in ethanol with mRNA in aqueous buffer through synchronized syringe pumps at a 1:2 ratio. After filtration, the particles were 80–100 nm in diameter with greater than 80% mRNA encapsulation. Mice inoculated with TC-1 tumors transformed with HPV16 E6 and E7 oncoproteins were vaccinated with mRNA encoding antigen co-formulated with STING(V155M) mRNA in LNPs. This elicited E7-specific CTLs which suppressed tumor growth and prolonged survival. Similar results were found in a murine lung metastasis model using luciferase-expressing TC-1 cells. This innovative strategy has potential advantages over liposomal delivery of STING ligands, particularly for local administration, including efficient mRNA loading into LNPs, an established safety profile for mRNA-based vaccines, and the possibility of modulating STING activation kinetics through control of intracellular mRNA stability and degradation rate, which may be employed to maximize efficacy and mitigate undesired inflammation.

6.1.2 Polymeric Delivery Systems

While the use of liposomal and lipid-based carriers may provide translational advantages owing to the approval of other lipid-based drug formulations (*e.g.* Doxil, Patisiran), polymeric carriers afford a greater degree of synthetic control over key physicochemical properties and can confer integration of unique functionalities and, hence, polymers also have great potential for enhancing CDN delivery (***Figure 16***). In one of the first examples of a polymeric CDN delivery system, Lee *et al.* developed submicron-sized hydrogel particles loaded with both 3′,3′-cGAMP and 2′,3′-cGAMP (***Figure 16A***). The particles were assembled using thiol-modified linear polyethyleneimine (LPEI-SH) that was mixed with a solution of hyaluronic acid (HA) and cGAMP to form electrostatic complexes that were then added to an organic solvent and emulsified with surfactants via water-in-oil emulsion[445]. To introduce thiol groups and, therefore enable crosslinking, the secondary amines on LPEI were reacted with propylene sulfide to achieve ~ 5%

thiol backbone modification. Although cGAMP would typically be too small to be stably encapsulated into such porous hydrogels, the intermolecular disulfide linkages and ionic interactions between LPEI and HA and LPEI and cGAMP allowed for a 47% entrapment efficiency. This formulation strategy yielded spherical particles designed to be relatively large in size (*i.e.* diameter ~ 455 nm) with a positive zeta-potential of +49 mV to more specifically target phagocytotic macrophages and dendritic cells. Upon internalization, intracellular reduction of the disulfide bonds allowed for triggered cGAMP release. Hydrogel particles displayed higher cytocompatibility than LPEI or LPEI/HA complexes and, importantly, enhanced STING activation as measured by induction of IFN-β and IL-6 secretion relative to empty particles, free cGAMP, or LPEI complexed with cGAMP. The authors tested the efficacy of this system as a vaccine platform with OVA injected intramuscularly into C57BL/6 mice and demonstrated that the particle significantly elevated levels of anti-OVA total IgG in serum. While promising as a vaccine platform, further studies to test efficacy as a cancer immunotherapeutic are necessary.

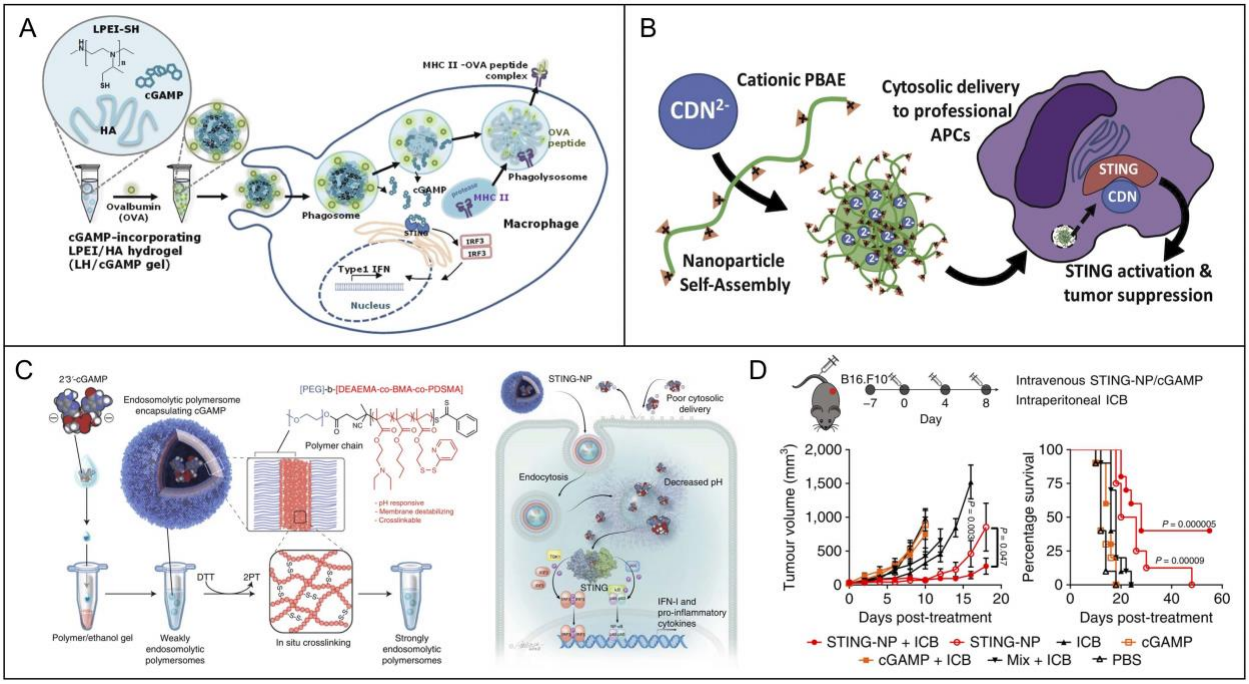

Figure 16: *Polymeric CDN delivery systems*.

(A) Formulation of cGAMP and ovalbumin (OVA) into linear polyethyleneimine / hyaluronic acid (LPEI/HA) hydrogels for enhanced STING activation and antigen presentation. Adapted with permission from reference[445]. Copyright © 2015 Elsevier Science & Technology Journals; permission conveyed through Copyright Clearance Center, Inc. **(B)** Nanoparticle assembly and cytosolic delivery of CDNs using cationic poly(beta-amino ester) (PBAE) nanoparticles to induce STING activation. Reproduced with permission from reference[446]. Copyright © 2017 Elsevier Science & Technology Journals; permission conveyed through Copyright Clearance Center, Inc.

(C) Chemical structure, formulation strategy, and intracellular delivery mechanism for STING-NPs (*i.e.* endosomolytic polymersomes for cytosolic delivery of 2′3′-cGAMP). Reproduced with permission from reference[194]. Copyright © 2019 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc. **(D)** Antitumor effect and prolonged survival of mice with B16-F10 melanoma treated with intravenous administration of STING-NPs alone and in combination with ICB (*i.e.* anti-PD-1 and anti-CTLA-4). Reproduced with permission from reference[194]. Copyright © 2019 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc.

Another early example of a polymeric CDN delivery system was described by Junkins *et al.* who employed an acid-sensitive microparticle platform for intracellular delivery of 3′3′-cGAMP[447] as a strategy to improve its activity as a vaccine adjuvant. To formulate particles, the researchers used acetalated dextran (Ace-DEX), which can be solubilized and electrosprayed to fabricate microparticles with high CDN encapsulation efficiency and excellent stability via an industrially scalable process. Notably, organic soluble Ace-DEX can be synthesized through one-step synthesis that converts the pendant hydroxyl groups of FDA-approved, water-soluble, 70 kDa dextran homopolysaccharide into acetal groups, which enhances their organic solubility and enables formulation into polymeric microparticles. Microparticles, having a diameter of ~ 1.54 ± 0.47 μm and zeta-potential of −32.0 ± 0.7, were formed through a coaxial electrohydrodynamic spraying method with Ace-DEX in an ethyl acetate/butanol/ethanol co-solvent mixed with cGAMP in molecular-grade water, achieving 90% cGAMP encapsulation efficiency. Acetylated microparticles provide a mechanism of cargo release that is triggered by a decrease in pH within the endo-lysosome owing to pH-dependent hydrolysis of acetals that results in regeneration of hydroxyl groups, aqueous chain solubility, dissolution of particles, and drug release[448]. The particles displayed an initial burst release followed by a controlled release profile of CDNs for 28 days. Importantly, these particles were of optimal size to be endocytosed by APCs, and the acid sensitivity allowed for intracellular release of CDNs within the phagolysosome. How the CDNs escaped the endosome into the cytosol to access STING was not described, though it is reasonable to suspect that a high concentration of CDN is achieved in the phagolysosome, which could drive diffusion of CDN across the phagosomal membrane and/or the use of membrane transporters. They found that loading of CDNs into Ace-DEX microparticles resulted in significantly enhanced proinflammatory cytokines and type I IFN responses, providing 1000-fold dose-sparing compared to soluble cGAMP for pro-inflammatory cytokines and type I IFN responses in primary APCs. The microparticles also significantly enhanced local type I IFN and IL-6 responses and preferentially trafficked to the LN while mitigating systemic inflammation when injected intramuscularly. CDN-loaded Ac-DEX particles were then evaluated as an adjuvant for

an influenza vaccine and were found to enhance the proinflammatory cytokine response while significantly reducing the required dose of CDN needed to stimulate anti-influenza immunity. This vaccine platform also increased anti-hemagglutinin antibody titers, specifically Th1 IgG response, resulting in complete protection against H1N1 influenza challenge.

In the first application of a polymeric CDN carrier for a cancer application, Wilson *et al.* employed biodegradable NPs composed of a poly(beta-amino ester) (PBAE) to improve cytosolic delivery of the phosphodiesterase resistant CDNs, ML-RR-CDA and RR-CDG (***Figure 16B***)[446]. PBAEs, which have been widely employed for delivery of nucleic acids (*e.g.* DNA, mRNA), was selected due to its biodegradability, structural diversity, ease of synthesis, ability to electrostatically bind nucleic acids, and endosomolytic activity. The PBAEs were synthesized at a molar ratio of 1.1:1 from monomers 1,4-butanediol diacrylate and 4-amino-1-butanol and then end-capped with a 0.2 M solution of 1-(3-aminopropyl)-4-methylpiperazine. A 500:1 polymer to CDN ratio was used for formulation, resulting in particles that had a diameter of ~ 100 nm with a positive zeta-potential of +10 mV. It is presumed that the CDNs were loaded via electrostatic interactions between phosphate groups on CDNs and cationic amino groups on PBAE, but the overall stability of this loading method is unclear as mono- or divalent electrostatic interactions may be insufficient to confer stability in physiological media. Nonetheless, this strategy increased CDN endocytosis by THP1 human monocytes and RAW 264.7 murine macrophages and was able to enhance activation of IRF3 *in vitro* relative to free CDN. When combined with ICB (*i.e.* anti-PD-1), Intratumoral administration of CDNs loaded into PBAEs strongly inhibited tumor growth in a B16-F1 melanoma model relative to dose matched free CDN combined with ICB, and also reduced the dose of CDN required exert comparable therapeutic activity by 10-fold. Of translational significance, it was demonstrated that the formulation could be lyophilized and stored for over 9 months without a significant loss of biological activity. This was an important aspect of this work as translational considerations such as stability and scalability have typically been ignored in the development of nanocarriers for STING pathway agonists.

While an abundance of cationic polymers (*e.g.* PEI, PBAE, *etc.*) have been developed for the delivery of macromolecular nucleic acids (*e.g.* siRNA, mRNA, *etc.*), CDNs bear only two negative charges and therefore cannot exploit the binding capacity afforded by multivalent interactions with polycationic carriers. Accordingly, our group has postulated that polymeric carriers for CDNs have distinctive design requirements and therefore require new drug delivery approaches[194, 380, 388, 449, 450]. Indeed, Shae *et al.* recently designed polymeric vesicles (*i.e.*

polymersomes) that have an aqueous core for efficient CDN loading and a vesicle membrane comprising amphiphilic diblock copolymer chains with pH-responsive endosomal membrane-destabilizing activity (***Figure 16C***)[194]. Critical to the structure and function of these polymersomes are well-defined $mPEG_{2kDa}$-*block*-[(2-diethylaminoethyl methacrylate)-*co*-(butyl methacrylate)-*co*-(pyridyl disulfide ethyl methacrylate)]$_{5kDa}$ (PEG-DBP) copolymers synthesized using reversible addition-fragmentation transfer (RAFT) polymerization. The carrier was designed such that at physiologic pH, the membrane-destabilizing DEAMA-*co*-BMA polymer segments are sequestered in the polymersome bilayer, shielded by a 2 kDa PEG corona to confer colloidal stability and increase circulation half-life. In response to endosomal acidification, the polymersomes disassemble to release CDNs and reveal membrane lytic domains that mediate endosomal escape of CDNs to the cytosol. An important aspect of the design is the copolymerization of thiol-reactive PDSMA groups into the second block for post-assembly crosslinking of the vesicle membrane. This increases chain molecular weight, and consequently endosomolytic activity, while also yielding less toxic, low molecular weight unimers upon reduction of disulfide crosslinks in the cytosol. Polymersomes were formulated via a modified direct hydration method, resulting in ~ 40% cGAMP encapsulation efficiency and yielding surface charge-neutral NPs with a median hydrodynamic diameter of ~ 100 nm, comparable properties to approved liposomal drug formulations, but with potent endosomolytic activity. As a result, these STING-activating NPs (STING-NPs) dramatically increased the immunostimulatory potency of cGAMP in monocyte, macrophage, dendritic cell, and melanoma cell lines, as well as human metastatic melanoma tissue. Although increased CDN cellular uptake contributed to increased activity, this was mostly attributed to potent endosomal escape as analogs with less endosomolytic activity were less efficient at STING activation. Consequently, in an aggressive and poorly immunogenic B16-F10 murine melanoma model, intratumoral administration of STING-NPs converted the TME to an inflamed and tumoricidal microenvironment, with significant upregulation of IFN-I and ISGs, pro-inflammatory cytokines, leukocyte-recruiting chemokines, pro-apoptotic mediators, and markers of DC maturation and T cell activation. In mice bearing two tumors, intratumoral treatment of one tumor (*i.e.* primary) resulted in a significant decrease in tumor growth rate for both primary and distal tumors, indicative of an abscopal effect. Intratumoral administration of STING-NPs was also applied for the treatment of neuroblastoma and was shown to trigger immunogenic cell death and inflame the TME to inhibit primary and distal tumor growth and improve response to ICB (*i.e.* anti-PD-L1)[449]. This study was also the first to evaluate STING activation as a therapeutic target in neuroblastoma, a pediatric cancer that is poorly responsive to immunotherapy and for which new treatment options are urgently needed.

Significantly, STING-NPs opened a therapeutic window for systemic, intravenous delivery of cGAMP, inhibiting tumor growth and increasing mean survival time in mouse models of melanoma (B16-F10 and YUMM1.7) and breast cancer (E0771)[194, 388]. Strikingly, a 40% complete response rate was observed in mice given STING-NPs in combination with ICB (*i.e.* anti-PD-1 and anti-CTLA-4), whereas neither free cGAMP nor ICB alone demonstrated a therapeutic benefit. It was further found that intravenously administered STING-NPs enhance the half-life of cGAMP by 40-fold from ~ 2 minutes to 90 minutes, resulting in increased tumor accumulation of cGAMP, elevated expression of IFN-I and proinflammatory cytokines in the TME, and a dramatic increase in the number of $CD8^+$ and $CD4^+$ T cells that infiltrated poorly immunogenic B16-F10 melanoma tumors[388]. Unsurprisingly, and consistent with the behavior of most nanoparticle delivery systems, STING-NPs also accumulated in the liver and spleen, resulting in activation of STING in these tissues and a transient elevation of serum cytokines that peaked around 4 hours and rapidly subsided 8−24 hours later. Importantly, intravenous administration of STING-NPs was safe and well-tolerated, resulting in only mild and transient weight loss, insignificant effects on blood chemistry, including markers of liver (*e.g.* ALT, AST) and kidney (*e.g.* creatinine) damage, and no signs of organ damage via histopathology. Nonetheless, splenic and hepatic toxicities are likely to be dose limiting for many nanoparticle-based STING pathway agonists, and therefore, strategies to minimize STING activation in the liver and spleen may allow for improved efficacy and safety.

A distinctive advantage of using nanocarriers for the delivery of STING pathway agonists is the opportunity to co-package additional cargo, which our group has exploited to achieve dual-delivery of CDNs and tumor peptide antigens to enhance response to cancer vaccines. Shae and Baljon *et al.* demonstrated that endosomolytic polymersomes could be co-loaded with cGAMP and several different peptide antigens, including murine tumor neoantigens[380]. Through co-loading of both tumor antigen and cGAMP in a common particle, this design mimics the natural immunological cues that underlie spontaneous antitumor immunity. Indeed, the vaccine platform helped ensure synchronous delivery of cGAMP and antigens to APCs in draining LNs, resulting in increased antigen-specific $CD8^+$ T cell responses that inhibited tumor growth and increased survival with combined with ICB in mouse models of B16-F10 melanoma and MC38 colon cancer.

Immunogenic cell death (ICD) is an inflammatory form of cell death that has been implicated in the generation of antitumor adaptive immunity in response to various cancer therapies[451]. Direct

STING activation can trigger ICD in some, but not all, cancer types, and some chemotherapy agents can induce ICD[452], whereas others do not. In order to mimic the sequence of events in the process of STING-mediated ICD, Chattopadhyay *et al*. aimed to devise a platform to deliver CDN STING agonists prior to treatment with cytotoxic chemotherapy agents. This process stimulates STING activation immediately prior to chemotherapy-induced cell death, effectively adjuvating tumor cell debris to stimulate antitumor immunity[453]. To achieve this, they utilized poly(lactic-co-glycolic) acid (PLGA) nanoshells (NS), which allow for encapsulation of cGAMP (NS-cGAMP) in a large aqueous core and pH-responsive release following internalization[454]. Although most other CDN carrier systems have been designed to passively target APCs, the authors used this technology to deliver a STING agonist directly to cancer cells followed by subsequent treatment with a cytotoxic agent, a process they refer to as “synthetic ICD” with the “immunogenic” component conferred via exogenous CDN delivery. These particles were formulated through a double emulsion process with low-viscosity, carboxyl-terminated PLGA (50:50 ratio, 0.15−0.25 dL/g), resulting in particles that were ~ 100 nm in diameter with 10 nm thick shells that were able to encapsulate cGAMP at 42% efficiency and enhance STING activation in both immune and cancer cells relative to free CDN. The investigators found that while Intratumoral administration of NS-cGAMP had a modest tumor inhibitory effect in multiple mouse models, therapeutic responses were improved when combined with chemotherapy agents (*i.e.* irinotecan, doxorubicin, cisplatin). This work offers evidence that coordination of tumor cell death and liberation of tumor antigen (here, via chemotherapy) and activation of innate immunity via the STING pathway can act as an *in situ* vaccine that enhances priming and activation of tumor antigen-specific T cells. However, how to optimally sequence and/or coordinate chemotherapy with administration of STING agonists to maximize antitumor immunity has not been widely explored and will likely depend on the tumor type and type of chemotherapy agent.

Towards answering this question, Liang and Wang *et al.* developed a polymeric carrier for dual-delivery of the chemotherapeutic agent SN38 and the murine STING agonist DMXAA[455]. To achieve this, they synthesized a triblock copolymer with a 5 kDa PEG first block, a second block comprising a redox-responsive prodrug monomer of SN38, and a third block of diethylamino-ethyl methacrylate (DEAMA), which enabled electrostatic interactions with the carboxylic acid group of DMXAA. Through control of block molecular weight, the group established a formulation that assembled into particles that were ~ 30 nm in diameter with greater than 80% loading of DMXAA. Using an intravenous administration route in multiple tumor models, it was demonstrated that NPs containing both SN38 and DMXAA were most effective in inhibiting tumor growth and, notably,

also more effective than a formulation comprising a mixture of SN38-loaded NPs and free DMXAA. This improved response was attributed to coordinated tumor cell killing and increased infiltration and activation of APCs in the TME capable of processing and presenting liberated tumor antigen for cross-priming of $CD8^+$ T cells. While alternative sequencing regimens were not considered, these studies suggest that dual-loading of chemotherapy and STING pathway agonists may improve therapeutic responses and they also motivate the design of carrier systems that are optimized to maximize synergy between cytotoxic agents and STING pathway agonists.

To avoid use of chemotherapy while still aiming to enhance tumor antigen uptake by APCs, Lu *et al.* also leveraged a polymeric carrier for dual-delivery of cGAMP and an siRNA to inhibit expression of the phagocytosis checkpoint, signal regulatory protein α (SIRPα)[456]. SIRPα is expressed by APCs and binds to the "don't eat me signal," CD47 expressed by cancer cells as an immune evasion mechanism to prevent phagocytosis. The group co-loaded cGAMP and siRNA into particles, which had a diameter of ~ 100 nm and were comprised of PEG-*block*-PLGA copolymers and the cationic lipid DOTAP. The researchers demonstrated the capacity of the system to both enhance cGAMP activity as well as knock down SIRPα in dendritic cells. Using an intravenous administration route in a B16-F10-OVA melanoma model, some enhancement in tumor growth inhibition was demonstrated when both cGAMP and SIRPα siRNA were co-delivered relative to formulations containing just cGAMP or just siRNA. The improved therapeutic response was attributed to enhanced priming and infiltration of antigen-specific T cells. Similar to the work by Liang and Wang *et al.*, this study adds further support for the concept of coordinating STING activation with strategies to increase the amount of tumor antigen available for uptake, processing, and cross-presentation by activated APCs in tumors and tdLNs.

6.1.3 Inorganic Delivery Systems

Inorganic materials have also been utilized for CDN delivery and STING activation, and offer potential advantages such as access to particles of very small size, low polydispersity, and the potential to leverage imaging modalities to evaluate tumor accumulation. An *et al.* was among the first to leverage an inorganic carrier, using cationic, amine-modified silica NPs (CSiNPs) that allowed for the electrostatic interaction with CDNs while also increasing endocytosis via interactions with anionic cell membranes[457]. Notably, due to their cationic nature, CSiNPs have intrinsic cytotoxicity, which the investigators postulated could promote the generation of tumor-associated antigens from necrotic tumor cells to improve response to *in situ* vaccination (*i.e.* intratumoral immunotherapy). Using silica NPs with a diameter of 30 nm and a 240:1 NP to CDN

mass ratio, they achieved 65% CDN encapsulation efficiency, yielding particles that were 35 nm in diameter and had a positive +18 mV zeta-potential. In a B16-F10 melanoma model, intratumoral injection of CSi-c-di-GMP-NPs resulted in an increase in necrotic tumor cells while also prolonging CDN release and achieving a more uniform distribution compared to free CDN. The combination of CSiNPs and CDN, but not individually, activated local APCs and triggered expansion of antigen-specific CTLs, resulting in reduction in tumor growth and prolonged survival. Significantly, an ~ 40% complete response rate was observed and surviving mice were fully protected against tumor rechallenge.

Similarly, Park *et al.* developed biodegradable mesoporous silica NPs (bMSNs) to enhance the cellular uptake and cytosolic delivery of cyclic-di-adenosine monophosphate (c-di-AMP)[458]. By reducing the density of the Si-O-Si matrix to increase the pore size and accelerate biodegradation rate compared to conventional MSNs, and also surface-modifying bMSNs with amino groups to allow for electrostatic loading of CDNs, the authors were able to achieve particles that were ~ 80 nm in diameter with over 90% CDN loading efficiency via simple mixing. At a physiological pH of 7.4, CDNs were released from the particle very quickly (*i.e.* within an hour), but were released slowly over the course of several hours at pH 6.0, mimicking the acidic conditions of some tumors. Despite such rapid release of CDN, bMSNs increased cellular uptake of CDNs by bone marrow-derived dendritic cells (BMDCs), resulting in increased CD40 and CD86 expression and release of cytokines and chemokines including IL-6, IL-12p40, IFN-β, CXCL10, CCL2, CCL3, and CCL5. In a B16-F10-OVA model, a single intratumoral injection of CDN loaded bMSNs completely inhibited tumor growth, whereas only 50% of mice survived following injection of free CDN.

A similar approach was pursued by Bielecki *et al*. who used mesoporous silica NPs (MSNs) as a nanocarrier for c-di-GMP to boost antitumor immunity in glioblastoma multiforme (GBM)[459]. The group generated high-surface area, mesoporous structures from tetraethylorthosilicate particle nucleation with cetyltrimethylammonium bromide and functionalized MSNs with N^1-(3-trimethoxysilylpropyl) diethylenetriamine to generate amino groups for electrostatic loading of c-di-GMP as well as reactive handles for surface PEGylation. This process yielded PEGylated NPs with a diameter of ~ 60 nm that transitioned from cationic (*i.e.* +59.1 mV) to nearly charge neutral zeta-potential following loading of c-di-GMP, which was highly efficient (*i.e.* 99.2% loading efficiency). In contrast to Park *et al.*, CDN release was slow at neutral pH, but accelerated at pH 5.5 due to deprotonation of surface amino groups and reduced

electrostatic interactions, allowing for release of CDNs under acidic conditions. The investigators evaluated their platform in an orthotopic GL261 GBM model using an intravenous administration route. They found that MSNs were able to cross the compromised blood–brain barrier in the GBM model to directly access APC rich near-perivascular regions of the brain tumor where they were efficiently taken up by APCs. This triggered a reprogramming of the immunosuppressive GBM TME, resulting in the recruitment of inflammatory macrophages and DCs and to tumor sites, while sparing healthy brain tissue, and, importantly, inhibiting tumor growth.

Chen *et al.* also employed a similar MSN system to deliver c-di-GMP[460], using N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride to generate cationic particles for electrostatic loading of c-di-GMP; Rhodamine B isothiocyanate (RITC) was integrated to allow for fluorescent imaging. Consistent with other reports, cationic MSNs efficiently loaded c-di-GMP (*i.e.* greater than 95% encapsulation efficiency), resulting in slightly smaller particles (*i.e.* ~ 47 nm in diameter) that enhanced CDN uptake and activity. Efficacy was evaluated via intratumoral administration in a 4T1 breast cancer model where MSN-mediated c-di-GMP delivery increased tumor infiltration of macrophages, dendritic cells, and T cells and inhibited tumor growth to a greater degree than free cGAMP. Although not investigated in this series of studies using MSNs, the tunable pore diameter and a large internal surface area of this platform offers the possibility of co-loading different therapeutics, such as chemotherapies or ICB to further enhance efficacy.

6.1.4 Biologically-Derived Carriers

Biologically-derived drug carriers, including cells[440], bacteria[461], virus-like particles[462], extracellular vesicles[463-466], and proteins[467, 468], have also been explored to enhance STING activation in cancer immunotherapy (***Figure 17***). In one of the first uses of a CDN for a cancer immunotherapy application, Fu *et al.* developed STINGVAX, a cell-based cancer vaccine system that combined the synthetically-derived, phosphodiesterase-resistant, (R_P,R_P) dithio CDA diastereomer (RR-S2 CDA) and irradiated GM-CSF-secreting tumor cells as a source of tumor antigens and evaluated vaccine efficacy in SCCFVII, TRAMP, Panc02, CT26, and B16 tumor models[440]. Vaccines were prepared by pre-incubating the CDN with lethally irradiated GM-CSF-secreting cancer cell lines prior to subcutaneous injection. Whether or not this resulted in CDN uptake by cancer cells was not directly evaluated, but it is conceivable that cells acted as a CDN carrier in these studies and served to co-localize STING agonist with tumor-derived antigens. The investigators found that this cell-based vaccine platform activated DCs in the tdLNs, induced naïve $CD8^+$ T cell priming, significantly reduced tumor growth, and increased survival in multiple

mouse tumor models. Notably, when combined with ICB (*i.e.* anti-PD-1), STINGVAX treatment not only led to cancer regression in both B16 and CT26 tumor models, but also long-term tumor-specific memory as demonstrated in CT26 rechallenge studies. Due to the proven safety profile of both GM-CSF-secreting whole cell vaccines and locally administered CDNs, this cancer vaccine platform appears well positioned for being translated into clinical trials.

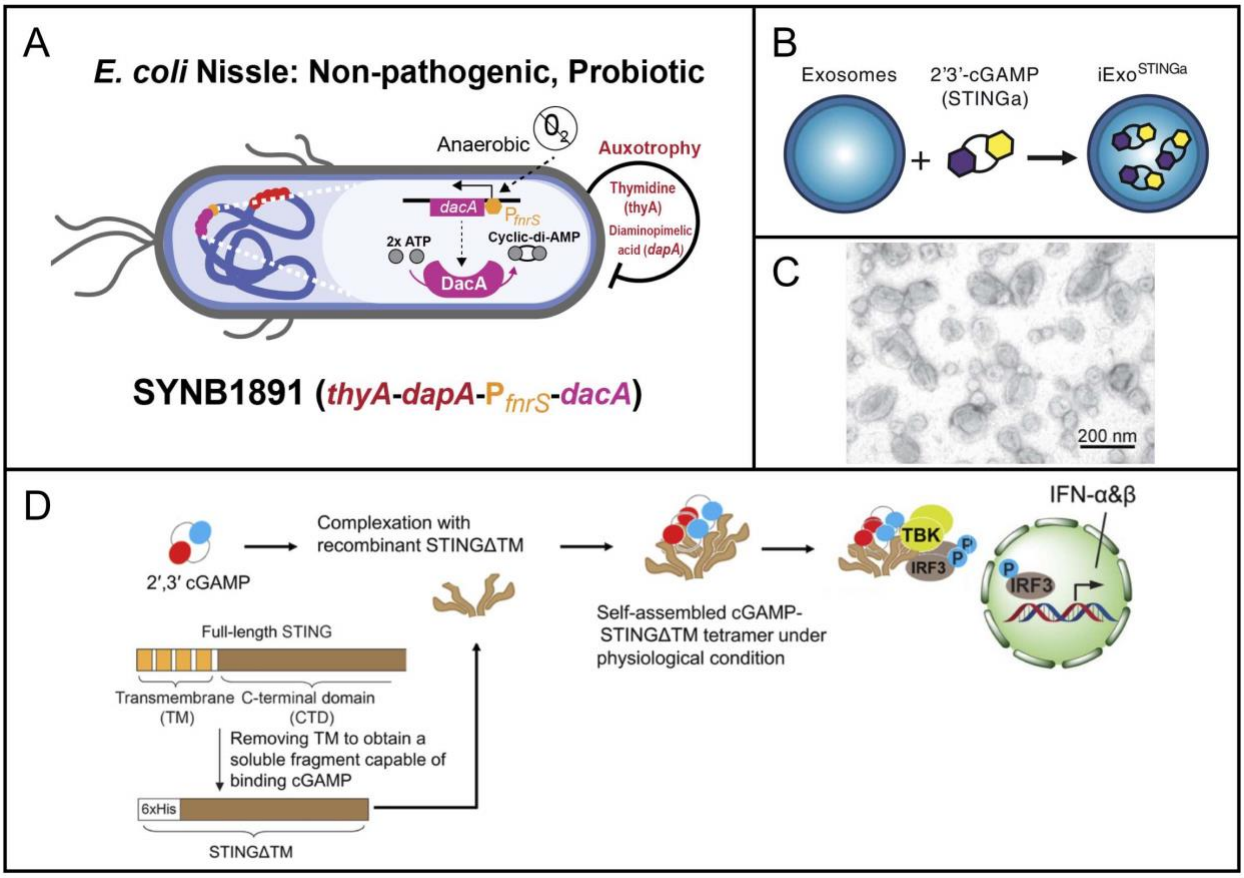

Figure 17: *Biologically-derived CDN carriers*.

(A) Schematic of the SYNB1891 bacteria strain, which has been engineered to localize in the hypoxic tumor environment, activate STING in tumor APCs through enzymatic production of c-di-AMP, and trigger complementary proinflammatory pathways through additional PRR activation. Reproduced with permission from reference[461]. Copyright © 2020 Springer Nature. Distributed under a CC BY 4.0 license http://creativecommons.org/licenses/by/4.0/. **(B)** Schematic of iExo^{STINGa} exosomes, which have been engineered to deliver 2′3′-cGAMP. Reproduced with permission from reference[464]. Copyright © 2021 The Authors. Published by Elsevier Inc. on behalf of American Society for Biochemistry and Molecular Biology. Distributed under a CC BY 4.0 license http://creativecommons.org/licenses/by/4.0/. **(C)** Representative TEM image of extracellular vesicles used for CDN delivery. Reproduced with permission from reference[466]. Copyright © 2021 Codiak BioSciences, Inc. Distributed under a CC BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/. **(D)** Schematic illustrating the delivery of 2′3′-

cGAMP using recombinant, transmembrane-deficient STING to induce type I interferon responses. Reproduced with permission from reference[467]. Copyright © 2020 The Authors, some rights reserved; exclusive licensee AAAS. Distributed under a CC BY-NC 4.0 license http://creativecommons.org/licenses/by-nc/4.0/.

Engineered bacteria have also recently been explored as vectors for CDN delivery, and offer some unique advantages over synthetic systems, since bacteria can endogenously produce CDNs, target APCs via active phagocytosis, trigger complementary pattern recognition receptors, and selectively colonize in tumors[461]. Leventhal *et al*. engineered bacteria capable of tumor-selective production of CDNs (***Figure 17A***), selecting *E. coli* Nissle 1917 as the bacterial vector since it has increased serum sensitivity, has a well-defined genome, and is susceptible to a broad range of antibiotics, making it a good candidate for engineering of gene circuits to regulate CDN production. Diadenylate cyclase is an enzyme that produces high amounts c-di-AMP (CDA) and was selected as the CDA-producing enzyme. To construct a gene circuit to allow for tumor-selective CDA production, they incorporated a hypoxia-inducible promoter, which bypasses the need for the delivery of exogenous inducers and allows for site-specific activation due to the hypoxic nature of the TME. By introducing 4-hydroxy-tetrahydropicolinate synthase gene and thymidylate synthase gene deletions as a method of biocontainment, they could prevent intratumoral and extratumoral bacterial proliferation, increasing safety to enable possible translation of this living therapeutic. The removal of antibiotic resistance genes resulted in the finalized strain, SYNB1891, which was STING-inducing, tumor-specific, safe, and compliant with manufacturing regulatory guidelines. Type I IFNs were produced in a phagocytosis-dependent manner in both mouse and human APCs. SYNB1891 also activated parallel innate immune signaling pathways, such as TLR-4, resulting in the expression of complementary proinflammatory cytokines to improve immune response. SYNB1891 was delivered to B16-F10 tumor-bearing mice through three intratumoral injections during the span of a week, which delayed tumor growth in a dose-dependent manner and lead to complete tumor rejection in 30−40% of mice. Significant tumor rejection was also seen in A20 B cell lymphoma tumors, further illustrating the therapeutic efficacy and versatility of this system. Overall, this work offers an elegant example of how rationally-designed microorganisms can potentially be leveraged for conditional activation of STING signaling at tumor sites as well as the promise of synthetic biology approaches for regulating cGAS/STING signaling to maximize efficacy and safety.

Extracellular vesicles (EVs) are endogenously generated, lipid-bound nano- and microparticles that are secreted by cells as a natural mechanism for shuttling of diverse cargo, including nucleic acids, proteins, and metabolites, between cells[469]. Accordingly, there has been considerable recent interest in the production and engineering of EVs as therapeutics and/or drug carriers due to their versatility and high degree of tropism for specific cell and tissue targets[470, 471]. Moreover, EVs have also been identified as a mechanism of transfer of DNA and/or CDNs between cells[104, 182, 300, 472], offering a bioinspired strategy for delivery of cGAS/STING agonists that has recently been pursued by several groups in the context of vaccines and cancer immunotherapy (***Figures 17B and 17C***)[463-466]. This is exemplified by the work of Jang and colleagues at Codiak BioSciences[465] who have recently developed exoSTING, an EV that is exogenously loaded with a synthetic CDN (cAIM(PS)$_2$ Difluor (R_P,S_P)), which is InvivoGen's cyclic adenine monophosphate-inosine monophosphate bisphosphorothioate with a fluorine atom at the 2' position of each nucleoside. EVs isolated from suspension culture-adapted HEK293 cells were loaded with CDNs via simple incubation with sufficiently high concentrations of CDN to allow for passive loading into or onto EVs, followed by washing away of unloaded CDN. It was found that exoSTING was ~ 100−200-fold more potent than free CDN in human PBMC, resulting in significant improvements in antitumor activity when administered intratumorally in a B16-F10 melanoma model. Robust therapeutic effects were observed using very low CDN doses (0.01−0.1 μg), which could be attributed to improved preferential uptake and intracellular delivery in APCs as well as prolonged CDN retention within the TME, with exoSTING increasing CDN half-life by ~ 5-fold and reducing clearance rate by ~ 10-fold. Importantly, it was also reported that exoSTING circumvented the immune ablative effects associated with intratumoral delivery of free CDNs, likely due to the preferential uptake and STING activation in APCs with reduced STING activation in T cells. They also evaluated intravenous administration of exoSTING in a mouse model of hepatocellular carcinoma and demonstrated a significant improvement in antitumor efficacy relative to free CDN, including a ~ 38% complete response rate. Based on these preclinical studies, Codiak has recently initiated a phase 1/2 clinical trial (*i.e.* NCT04592484) to evaluate intratumorally administered exoSTING in patients with injectable solid tumors.

Finally, the STING protein itself has been used as a CDN carrier[467, 468]. As discussed in ***Section 3.1***, STING is commonly epigenetically silenced in cancer cells and, therefore, CDNs and other STING pathway agonists may not result in STING activation in the tumor cell compartment[473]. Previous studies have shown that the transmembrane (TM) domain of STING is essential for its translocation, oligomerization, and signaling[474], and, therefore, TM-deficient

STING would not be expected respond to CDNs or other agonists. However, He *et al*. discovered that the titration of cGAMP into TM-deficient STING (STINGΔTM) protein under physiological conditions triggered the formulation of self-assembled tetrameric structures that were ~ 30 nm in diameter and comprised of cGAMP and STINGΔTM (***Figure 17D***)[467]. When delivered *in vitro* with commercial transfection reagents, the cGAMP-STINGΔTM complex triggered STING signaling and cytokine production even in STING-deficient cell lines. Therefore, the ribonucleoprotein complex is not only a strong affinity carrier for cGAMP (*e.g.* K_D ~ 73 nM), but it is also able to initiate oligomerization and provide a scaffold for TBK1 recruitment and downstream signaling when cytosolically delivered regardless of STING expression or haplotype. Importantly, the cGAMP-STINGΔTM complexes coupled with commercial transfection reagents were able to activate STING signaling *in vivo*, both when used as a vaccine adjuvant that enhanced antigen-specific humoral and T-cell responses to a model antigen (*i.e.* OVA) as well as when administered IT, which inhibited tumor growth in a CT26 colon cancer model. The researchers subsequently furthered the therapeutic development and translatability of STINGΔTM by incorporating a peptide-based cell-penetrating moiety via genetic fusion with a known cell-penetrating domain to bypass the need for any synthetic delivery material[468].

6.2 Delivery Platforms for Controlled and Sustained Release of Cyclic Dinucleotides

While direct intratumoral injection of CDN STING agonists has demonstrated an excellent safety profile in patients with evidence of on-target STING activation, results emerging from these clinical trials has been largely underwhelming so far. While there a multitude of factors that likely contribute to these disappointing outcomes, this can be at least partially attributed to the delivery and pharmacological challenges discussed in ***Section 5***, including rapid clearance from the injection site, inconsistent tumor penetration, and lack of control over the magnitude and kinetics of local STING activation. These challenges have inspired the development of injectable or implantable controlled release depot technologies that can enable spatiotemporal control over the delivery of STING pathway agonists with potential to improve the efficacy, safety, and clinical feasibility of intratumoral immunotherapy (***Figure 18***).

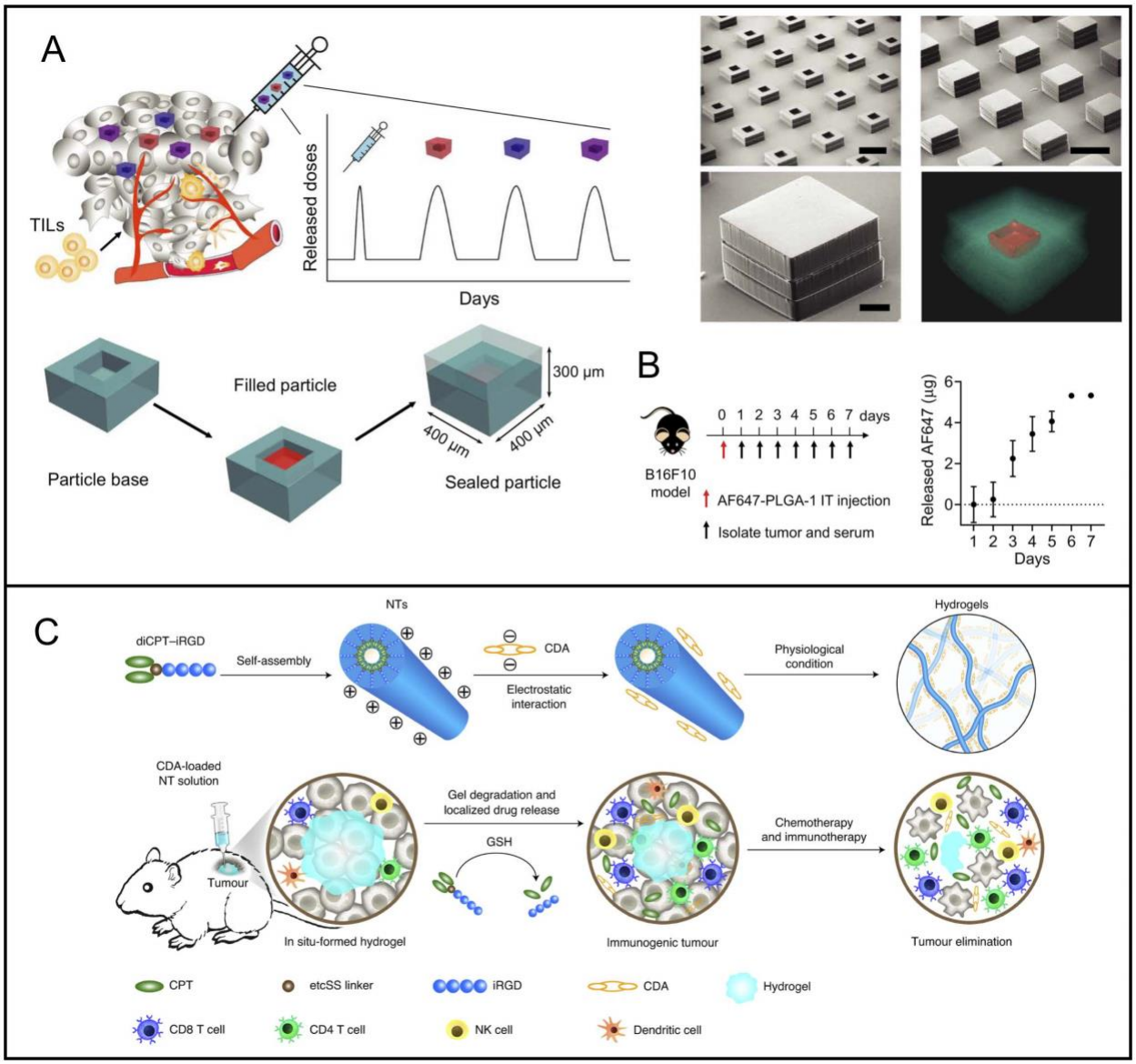

Figure 18: *Controlled-release delivery systems for local delivery of STING pathway agonists*.

(A) Schematic and fabrication of PLGA microparticles for temporally programable pulsatile cargo release. Reproduced with permission from reference[475]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc. **(B)** Cumulative *in vivo* release of AF647 from microparticles in the B16-F10 tumor model. Reproduced with permission from reference[475]. Copyright © 2020 American Association for the Advancement of Science; permission conveyed through Copyright Clearance Center, Inc. **(C)** A nanotube hydrogel for TME regulation and chemoimmunotherapy tumor sensitization. A peptide-drug conjugate was created by linking the hydrophilic tumor-penetrating peptide, iRGD to the hydrophobic anti-cancer drug, camptothecin (CPT). The diCPT-iRGD conjugates self-assembled into cationic supramolecular nanotubes, which electrostatically bound anionic c-di-AMP (*i.e.* CDA) and enabled localized and sustained drug release within the tumor microenvironment for a combination of cancer immunotherapy and chemotherapy. Reproduced with permission from

reference[476]. Copyright © 2020 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc.

An excellent and early example of this was the work of Leach *et al.* who developed STINGel – a peptide hydrogel-based delivery platform for intratumoral administration of CDNs[477]. They utilized a positively charged, MultiDomain Peptide (MDP) that self-assembles to form a hydrogel that effectively mimics the extracellular matrix, is biodegradable, and can be strategically functionalized[478]. The group used the MDP hydrogel denoted, $K_2(SL)_6K_2$, for intratumoral delivery of the CDN, ML-RR-S2 CDA. The cationic lysine groups of $K_2(SL)_6K_2$ allowed for electrostatic interactions between the hydrogel carrier CDN as well as with surrounding cells and tissue following injection. Importantly, MDPs demonstrate a time and stress-dependent viscosity, allowing them to be delivered through a syringe and localized at the injection site *in vivo*. Under buffered conditions *in vitro*, they found that MDP hydrogels sustained the release of CDN for ~ 15 hours, whereas CDN was released from a collagen gel within 5 hours, suggesting that MDPs could allow for higher sustained doses of CDN at the injection site. Utilizing a murine MOC2-E6E7 oral cancer model, they found that a single injection of STINGel significantly reduced tumor growth relative to free CDN as well as CDN administered using a collagen hydrogel, resulting in a 60% complete response rate and induction of immunity that protected from tumor rechallenge. The improved efficacy observed with the MDP hydrogel relative to collagen is notable as this suggests that hydrogel properties and/or CDN release rate are important variables that may be further optimized to maximize antitumor effects. Overall, the use of the MDP hydrogel improves the localized delivery of CDNs and has potential to allow for fewer intratumoral injections.

Although surgical resection is a common and effective treatment for operable tumors, it can also remove neoantigens and effector immune cells that are necessary for proper immunosurveillance and antitumor mechanisms; additionally, the wound healing process can result in local immunosuppression that may inhibit antitumor immunity[479]. In order to improve the immunogenicity of the tumor resection site, Park *et al.* developed an intraoperative scaffold for sustained and localized release of the CDN, 2′3′-c-di-AM(PS)2 (R_P,R_P) (STING-RR), R848 (*i.e.* agonist of TLR-7 and TLR-8), and other immunomodulators[480]. They selected hydraulic acid (HA) as their hydrogel platform due to its biocompatibility and biodegradability, and leveraged a thiol-modified HA that could be crosslinked via Michael addition using a PEG diacrylate crosslinker. They evaluated over 20 formulations to establish an optimal crosslink density that allowed for fabrication of a sufficiently stiff scaffold for surgical implantation, while still allowing for

biodegradation. In mice, scaffold degradation began 5 weeks after implantation, with complete resorption by week 12. After resecting primary 4T1 breast tumors in mice, they locally administered the therapeutic hydrogel, which stimulated a type I IFN response and promoted the infiltration of NK cells, dendritic cells, and T-cells in the TME. This treatment was superior to intratumoral injection of either free STING-RR or R848, and was able to eradicate metastatic tumors that had already developed within the lung prior to surgery. Intraoperative placement of the immunotherapeutic hydrogel was required for therapeutic benefit as systemically administered STING-RR or STING-RR injected locally along with an empty hydrogel had no effect, highlighting the importance of implanting the biomaterial delivery system during this critical intraoperative window.

Adoptive T cell and CAR-T cell therapy is an emerging treatment for many solid tumors; however, their efficacy has been limited due to inefficient lymphocyte trafficking from the circulation into tumors and inhibition of T-cell expansion and function in immunosuppressive “cold” TMEs[481, 482]. To circumvent these barriers, Stephan *et al.* developed a biodegradable, microporous scaffold for efficient local delivery of tumor-targeting T-cells and immune activators[483] and subsequently applied this technology to also deliver CDNs[484]. The scaffold matrix consisted of polymerized alginate that incorporated GFOGER peptides through carbodiimide chemistry with embedded mesoporous silica microparticles loaded with CDN (*i.e.* c-di-GMP) and displaying anti-CD3, anti-CD28 and anti-CD137 antibodies. GFOGER peptides mimicking collagen, which lymphocytes naturally utilize for migration, were strategically integrated to allow for binding and migration of lymphocytes in the scaffold while antibodies displayed on microparticles provided immunostimulatory cues for T cells, which could be loaded into the scaffold by hydrating it in the presence of T cells. When implanted into a surgical cavity, T cells robustly expanded and migrated out of the scaffold into the tumor resection site and to tdLNs, eliminating residual tumor and preventing relapse. By also incorporating c-di-GMP into the microparticles, the biomaterial implant also acted as a “self” vaccine site, with STING activation providing a local immunostimulatory milieu that supported T cell priming and activation, including *de novo* priming of endogenous T cells capable of eliminating tumor cells not recognized by the CAR T cells. As a result, scaffold co-delivery of c-di-GMP along with tumor-specific CAR T cells stimulated strong antitumor responses in both melanoma and pancreatic tumor models, and elicited long-term immunity against tumor rechallenge.

As discussed previously, most current intralesional STING agonist–based therapies require frequent injections, which can lead to complications and low patient compliance. Recently, a PLGA microparticle platform was developed to allow for long-term, pulsatile release of cGAMP within the tumor site following local injection[475]. Lu *et al.* used soft lithography techniques to fabricate arrays of cubic (400 x 400 x 300 μm) PLGA microparticles that can be filled with an aqueous solution and then sealed with a lid to form a closed cavity, resulting in 100% drug encapsulation, high loading capacity, and minimal leakage (***Figure 18A***). By fabricating particles using PLGA with different properties (*e.g.* molecular weight, lactide/glycolide ratio), the biodegradation rate of the walls could be tuned, allowing for a time-dependent burst release of capsule contents (***Figure 18B***). By mixing populations of particles designed with different release rates, a pulsatile release profile of cGAMP could be finely tuned to mimic dosing regimens commonly used for repeated intratumoral injection (*e.g.* four injections with 3−4 days between). Particles could be administered using a syringe and aggregated at the tumor injection site of B16-F10 melanoma and orthotopic 4T1 breast tumor models, where they were able to release cargo in a programmed, pulsatile manner; particles could be directly injected into a tumor or into a surgical bed following tumor resection. A single injection of cGAMP-loaded microparticles almost exactly mirrored the therapeutic effect of four injections of free cGAMP, highlighting the capacity of the approach to reduce or minimize the need for multiple intratumoral injections. This was associated with a conversion to a more immunogenic TME, characterized by the infiltration of CTLs, NK cells, DCs, memory T cells, and macrophages with a shift from an M2 to an M1 macrophage phenotype. They further demonstrated that their approach could reduce lung metastasis, enhance responses to ICB, and generate protection against tumor rechallenge. As an example of the potential clinical utility of their approach, they also evaluated the depots in an orthotopic pancreatic tumor model, which is not readily accessible for repeated injection, and demonstrated reduced tumor burden with a single injection of cGAMP-loaded microparticles.

Another recently described depot technology was specifically focused on harnessing synergy between STING agonists and chemotherapy since some chemotherapeutic drugs trigger cell death by inducing DNA damage and activating cGAS/STING signaling.[476] Wang *et al.* developed a drug-loaded supramolecular hydrogel system composed of a self-formulating peptide–drug conjugate, di-camptothecin–iRGD, which comprised a neuropilin-1-binding, tumor-penetrating iRGD peptide and the chemotherapeutic, camptothecin (***Figure 18C***). By linking the hydrophobic camptothecin to the water soluble peptide through a disulfanyl-ethyl carbonate linkage, which is susceptible to reduction via glutathione, a drug amphiphile was synthesized.

Peptide-drug conjugates spontaneously assembled into supramolecular nanotubes *in situ* with a positively charged surface that enabled electrostatic complexation of the CDN, c-di-AMP. After intratumoral injection, a hydrogel forms immediately and acts as a depot that enables release both camptothecin and c-di-AMP over time. The hydrogel degraded with a near linear profile and sustained local release of CDN over at least two weeks following injection. Local intratumoral administration induced STING-dependent activation of type I IFNs and CXCL10, resulting in infiltration of NK, DCs, and CTLs. In multiple tumor models (CT26 colon cancer, 4T1 breast cancer, GL-261 glioma), a single injection of the hydrogel strongly inhibited tumor growth and increased survival compared to a soluble mixture of CDN and camptothecin, the hydrogel lacking CDN, or a similar hydrogel lacking camptothecin. This offered a compelling demonstration of both the utility and improved efficacy achieved using a sustained CDN release depot as well as the potential to leverage biomaterial design to harness synergy between chemotherapy and STING activation.

6.3 Biomaterials with Intrinsic STING Activity

In addition to CDN delivery vehicles, researchers have also developed and/or used biomaterials that can intrinsically activate the cGAS/STING pathway, either directly or indirectly (***Figure 19***). Some of the first evidence that biomaterials could stimulate inflammation via cGAS/STING came from Carrol *et al*., who demonstrated that the cationic polysaccharide, chitosan exerted vaccine adjuvant properties via the cGAS/STING pathway[485]. Chitosan has been known for its promising adjuvant capabilities (*e.g.* promotion of DC activation, enhancement of adaptive immunity, *etc.*), though its mechanisms of action have remained undefined[486, 487]. Carrol *et al*. utilized a *Mycobacterium tuberculosis* vaccine construct to demonstrate the ability of chitosan, comprising randomly distributed D-glucosamine and N-acetyl glucosamine, to enhance surface expression of CD40 and CD86 by DCs and elicit highly polarized Th1 and IgG2c antibody responses. However, these responses, as well as the secretion of CXCL10, were mitigated in the absence of IFNAR, showing that type I IFN signaling was essential for this immune activation. They also demonstrated that these responses were specifically dependent on the cGAS/STING pathway due to reduction of IFN-β and CXCL10 production in cGAS/STING deficient mice. Interestingly, they found that chitosan induced the production of mitochondrial-specific ROS, suggesting that cGAS/STING activation occurred in response to the release of mitochondrial DNA into the cytosol. In total, this study defined chitosan as an effective adjuvant able to bolster adaptive immune response through the cGAS/STING pathway.

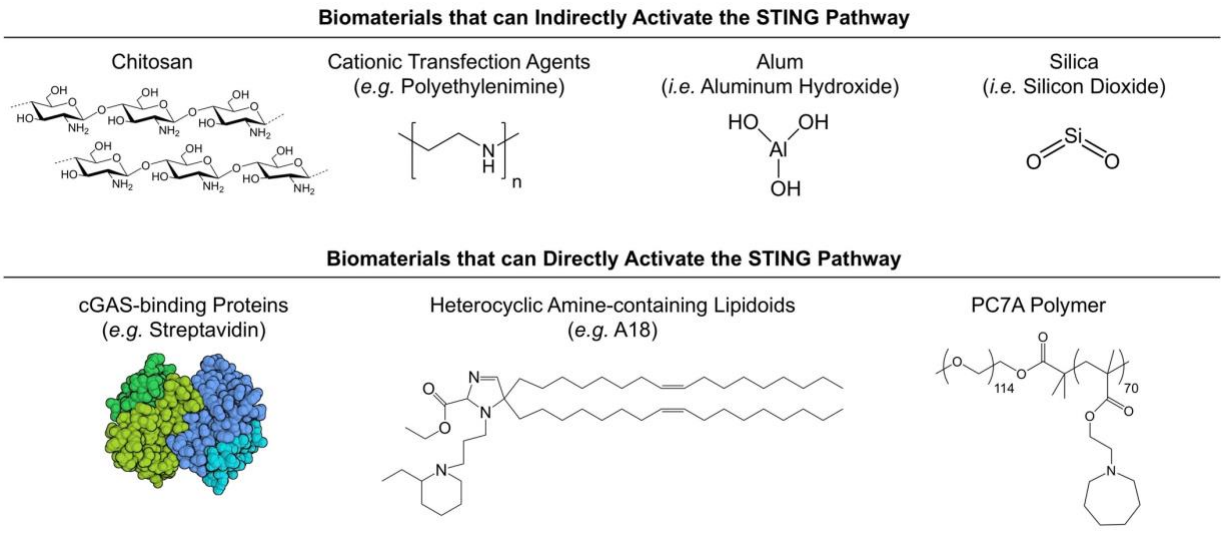

Figure 19: *Biomaterials that can intrinsically activate the STING pathway*.

There is a growing list of biomaterials known to activate the STING pathway, either directly or indirectly. Direct activation of the STING pathway involves molecules that can bind to and functionally activate either cGAS or STING proteins. Indirect activation of the STING pathway most commonly involves endogenous cGAS activation and is typically achieved by inducing the cytosolic relocation of DNA from mitochondria and/or nuclei. Figure created with *biorender.com*.

More recently, Turley *et al.* have identified optimal characteristics of chitin-derived polymers for the activation of DCs and the induction of antigen-specific cellular immune responses[488]. The researchers found that degree of chitin deacetylation, acetylation pattern, and its regulation of mitochondrial ROS are the key determinants of its immune enhancing effects. Notably, only chitin-derived polymers with a high degree of deacetylation enhanced the generation of mitochondrial ROS and thereby the STING-mediated induction of type I IFN. It was determined that chitin-derived polymers with a degree of deacetylation less than 80% are poor adjuvants, while a fully deacetylated polyglucosamine polymer is most effective as a vaccine adjuvant. Furthermore, for the chitin-derived polymers that are not fully deacetylated, a heterogenous acetylation pattern (*i.e.* clustering of the remaining acetyl groups) was favorable to a homogenous acetylation pattern (*i.e.* even distribution of the remaining acetyl groups), which was likely due to charge distribution and its effect on mitochondrial stress. Indeed, when packed closely together, positively charged species, such as the free amines generated by deacetylation, might more efficiently promote mitochondrial association and mitochondrial membrane disruption[489-491].

Since the initial characterization of chitosan as a cGAS/STING pathway activator, Qiutong *et al*. has applied chitosan in a nano-complex with anti-PD-L1 antibodies to achieve pulmonary delivery of ICB through inhalation in lung metastasis[492]. Direct pulmonary delivery of therapeutics allows for a reduction in systemic distribution and high, localized drug concentration[493]; however, antibody delivery is normally limited by alveolar mucosal barriers that inhibit the permeation of large, hydrophilic, and anionic biomacromolecules[494]. The direct complexation of positively-charged chitosan to antibodies allows for interactions with anionic sialic acid of mucins to enhance permeability and delivery of macromolecular therapeutics directly into the lung. The innate adjuvant activity of chitosan also allows for complimentary STING activation to synergize and improve ICB therapy. The nanocomplex was prepared in one step by mixing positively-charged chitosan and negatively-charged antibodies, allowing for polyelectrolyte complexation. At a 1:1 ratio, this nanocomplex formed particles with a diameter of ~ 60 nm and surface charge of +24 mV that could stick to the negatively-charged mucus lung epithelium to prolong retention and reversibly open tight junctions of lung mucosa to increase the penetration of ICB. Repeated inhalation of this complex promoted the infiltration of immune cells, especially CTLs, at and around tumor lesions of B16-F10 melanoma lung metastases. The enhanced retention and penetration of ICB and additional STING-activating adjuvant effects allowed for prolonged survival over 60 days.

There have been several other positively charged molecules that have been identified as indirect activators of the cGAS/STING pathway via mitochondrial DNA release. Positively charged molecules are attracted to the negative membrane potential of mitochondria (*i.e.* ~ –170 mV)[490, 491] and are therefore more likely to associate with mitochondrial membranes and cause membrane disruption. Notably, cationic nanocarriers (*e.g.* transfection agents) can activate the cGAS/STING pathway via mitochondrial damage and the subsequent release of mitochondrial DNA into the cytosol[495, 496]. Furthermore, the most prevalent adjuvant in licensed human vaccines, aluminum hydroxide (*i.e.* alum)[497] also exhibits a positive surface charge at physiological pH[498] and can similarly activate the cGAS/STING pathway via the release of self dsDNA[499-501]. However, STING-driven gene expression appears to be heavily restricted and often undetectable in many cell types in response to alum-induced activation[502], which suggests that alum is a relatively poor STING pathway agonist and also highlights how indirect activation of the cGAS/STING pathway can be quite multifaceted.

There is some evidence that certain non-positively charged biomaterials are also capable of indirectly activating cGAS/STING via the release of self dsDNA. Indeed, Benmerzoug *et al.* observed that silicosis patients with a history of intense silica (*i.e.* silicon dioxide) exposure to the lungs exhibit increased circulating dsDNA in their plasma and increased expression of CXCL10 in their sputum[50]. The researchers then identified the mechanisms behind silica-induced lung inflammation, determining that silica can intrinsically induce self-dsDNA release and a subsequent STING-mediated IFN-I response. Notably, the mechanism of silica-induced self-dsDNA release was not determined. As silica tends to be charge-neutral to slightly negatively charged[503], it is unlikely that silica triggers mitochondrial dsDNA release in the same manner as the positively charged species that indirectly activate cGAS. Alternatively, the researchers proposed that mitochondrial DNA may accumulate in the cytosol in response to silica exposure through the initiation of apoptosis in a BAX and BCL-2-dependent manner[504, 505], activation of mitochondrial permeability transition pore[506, 507], or deficient control by transcription factor A mitochondria (TFAM)[505]. Interestingly, *in vitro* studies suggested that the mechanism of STING activation varied by cell type. After silica exposure, mitochondrial dsDNA in dendritic cells was released into the cytosol and activated the cGAS/STING signaling axis in a conventional manner, while macrophages exhibited an alternative and less common form of STING activation. The activation of STING in macrophages following silica exposure was cGAS-independent and instead required the accumulation of extracellular dsDNA, which was then internalized and processed through DDX41 and IFI204 (*i.e.* the murine ortholog of IFI16) DNA sensors. Nonetheless, STING pathway activation was identified as essential for the onset of silica-induced lung inflammation, and DNase I was proposed as a potential therapeutic treatment to clear extracellular dsDNA and thereby attenuate STING signaling.

While biomaterials that indirectly activate cGAS/STING signaling can be quite useful, they primarily depend on the induction of cell-stress mechanisms, which may not be suitable for certain applications. Indeed, many of the biomaterials that indirectly activate the cGAS/STING pathway also stimulate other immunomodulatory pathways. For example, chitosan also triggers the cellular production of IL-1β and IL-18 via activation of the NLRP3 inflammasome[508]. Furthermore, the biomaterials that trigger the intracellular release of mitochondrial DNA can indirectly activate several different DNA sensors (*e.g.* cGAS, AIM2, TLR9, *etc.*)[509, 510] due to the relatively large size (*i.e.* ~ 16.5 kb in length) and nucleotide composition of mitochondrial DNA[511]. Accordingly, the downstream signaling cascades induced by such biomaterials are heavily context-dependent.

Immunomodulatory biomaterials with pathway specificity do not involve pathway cross-talk and can therefore offer enhanced control over therapeutically programming immune responses. Investigators have recently sought to develop synthetic biomaterials that can directly activate STING via direct binding interactions with the STING protein (***Figure 20***). This approach was first elegantly demonstrated by the group of Jinming Gao, who synthesized a library of ultra-pH sensitive NPs comprised of a PEG first block and a second, pH-responsive block containing tertiary amines with linear or cyclic side chains[512]. Having demonstrated that all polymers could efficiently load the model antigen OVA, they screened the ability of carriers to elicit an OVA-specific CTL response and identified a lead polymer, PC7A, that contained a cyclic amine side chain and was selected due to its ability to induce strong CTL responses without the use of any exogenous adjuvants. Using OVA, they demonstrated that OVA-PC7A NP was capable of eliciting a 20-fold higher CTL response compared to commonly used adjuvants including alum, LPS, and CpG. Th1 and Th2 responses assessed via measurement of IgG1 and IgG2c titers were also shown to be higher or comparable to these common adjuvants. Following subcutaneous injection, OVA-PC7A NPs accumulated in the peripheral LNs and significantly increased OVA-positive $CD8\alpha^{+}$ DC cells. Interestingly, these effects were reduced in $cGAS^{-/-}$ mice, demonstrating a dependence on cGAS/STING signaling to exert adjuvant effects, and additional pull-down methods using the CTD of STING elucidated that PC7A could bind to STING directly. Collectively, these findings suggested that PC7A was acting as a direct STING agonist. The therapeutic efficacy of the vaccine was observed in B16-F10, MC38, and HPV E6/7 TC-1 tumor models. The vaccine also demonstrated synergy with anti-PD-1 antibody therapy in B16-OVA and TC-1 models. PC7A-NPs were also used to deliver cGAMP in models of HIV-1 infection, which can inhibit activation of the STING pathway[513]. Through IFN-I signaling, this NP system for cGAMP delivery was able to elicit strong, long-acting antiretroviral responses to HIV-1.

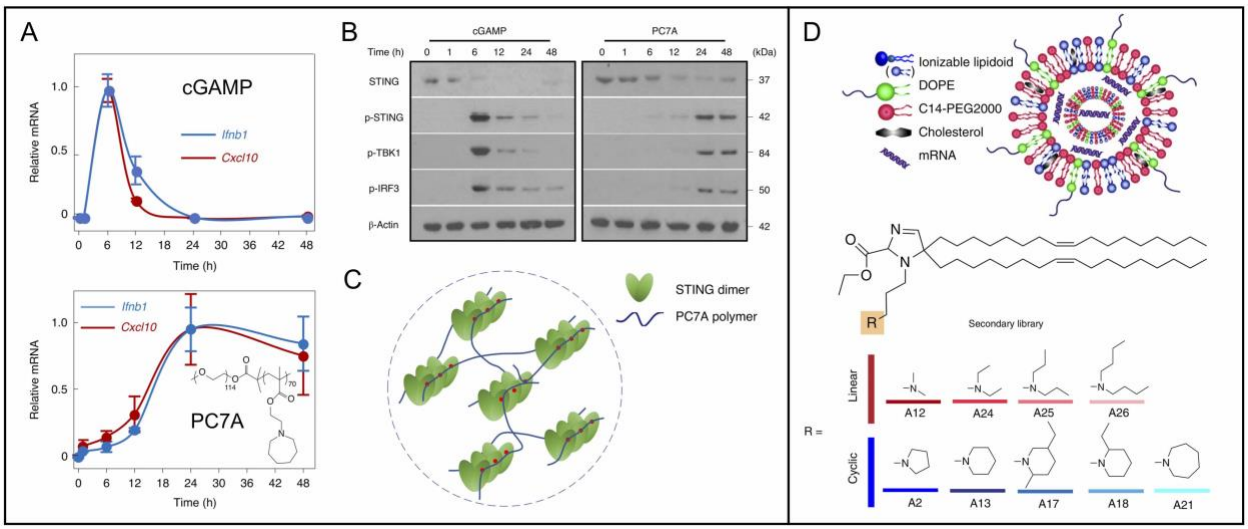

Figure 20: *Biomaterials that can directly bind and activate STING*.

(A) Relative *IFNB1* and *CXCL10* mRNA levels over time in THP1 cells treated with 2′3′-cGAMP or the synthetic diblock copolymer, PC7A, along with the chemical structure of PC7A. Reproduced with permission from reference[164]. Copyright © 2021 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc. **(B)** PC7A led to sustained TBK1/IRF3 phosphorylation and slower STING degradation compared to 2′3′-cGAMP in THP1 cells. Reproduced with permission from reference[164]. Copyright © 2021 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc. **(C)** Schematic of STING oligomerization and the uncharacteristic immunostimulatory condensation (*i.e.* unlike that of the natural STING phase-separator, which negatively regulates STING-driven gene expression[146]) induced by PC7A. Reproduced with permission from reference[164]. Copyright © 2021 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc. **(D)** Schematic of mRNA-encapsulating LNPs incorporating STING-activating ionizable lipidoids. A18 was selected as the lead cyclic lipid candidate. Reproduced with permission from reference[514]. Copyright © 2019 Springer Nature BV; permission conveyed through Copyright Clearance Center, Inc.

In an important follow-up study, the researchers identified an alternative STING binding site for PC7A, indicating that PC7A does not compete with 2′3′-cGAMP in STING binding[164]. Hence, PC7A could be combined with 2′3′-cGAMP for synergistic STING activation, which is particularly useful for STING variants that exhibit reduced activity to 2′3′-cGAMP stimulation (*e.g.* the REF (R232H) STING isoform, present in ~ 14% of humans)[160]. Indeed, synergy between co-delivered 2′3′-cGAMP and PC7A NPs was demonstrated in MC38 and TC-1 tumors. Notably, they found that PC7A deterred lysosomal degradation of STING by buffering endosomal pH and induced a unique liquid-liquid phase condensation of STING, both of which led to enhanced and sustained downstream signaling as compared to 2′3′-cGAMP treatment (***Figures 20A-C***). The formation of the PC7A-induced STING condensates was found to correlate with activity, unlike the inhibitory STING phase-separator that negatively regulates the pathway upon substantial

pathway activation[146]. Though not addressed in their work, PC7A may inhibit the STING phase-separator in addition to promoting its own immunostimulatory biocondensates. Thus, the prolonged activity of PC7A-induced STING signaling could potentially be attributed to PC7A-mediated inhibition of the STING phase-separator in addition to the established ability of the polymer to prevent lysosomal degradation of STING.

Taking a similar approach, Miao *et al.* synthesized a combinatorial library of over 1,000 ionizable lipid-like materials and screened the ability of lipid nanoparticle formulations to stimulate potent immune responses to mRNA-based vaccines[514]. Although mRNA vaccines are advantageous due to their ability to intracellularly express whole protein antigens, their efficacy is challenged by mRNA hydrolysis and inadequate antigen loading and APC maturation[515]. After synthesizing and optimizing functional lipid libraries through a three-dimensional multi-component reaction system, they found that lipids containing cyclic-amino headgroups, unsaturated lipid tail, and a dihydroimidazole linker (***Figure 20D***) were not only highly efficient at delivering mRNA, but also stimulated type I IFNs and ISGs in a STING-dependent manner as evidenced by reduced innate and antigen-specific adaptive immune responses in STING KO cells and mice. Using a lipid pulldown assay, they also demonstrated that lipids with cyclic-amino headgroups, in contrast to those with linear structures, could directly associate with STING, which was further supported by dynamic molecular docking simulations that estimated a K_D of ~ 50 μM for A18, the lead cyclic lipid candidate from the library. By formulating antigen-encoding mRNA into LNPs comprising A18, the fusogenic helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), C14-PEG, and cholesterol at an undisclosed molar ratio, the investigators generated a potent mRNA cancer vaccine capable of stimulating strong CTL and Th1 $CD4^+$ T cell responses. Using mRNA encoding the tumor antigen tyrosine-related protein 2 (mTRP2), the A18 mRNA LNP vaccine inhibited tumor growth and increased survival in mice with B16-F10 tumors to a greater degree than an analogous LNP formulated using a lipid with a linear headgroup (*i.e.* A25), or using the established MC3 lipid, demonstrating the importance of head group design and resulting STING activation in therapeutic vaccine efficacy. To further define the efficacy of the A18 mRNA vaccine, it was formulated with mRNA encoding the human papillomavirus E7 protein and co-administered with anti-PD-1 in a TC-1 tumor model, resulting in robust cures in these mice, further demonstrating its efficacy as an mRNA delivery vehicle and intrinsic STING stimulator.

7. Therapeutic Potentiators of the STING Pathway

There is a growing list of known STING pathway potentiators (***Figure 21***), which currently includes certain metal ions (*e.g.* Mn^{2+} and Mg^{2+}), cGAS-binding proteins, inhibitors of DNA methyltransferases, various inhibitors of NF-κB signaling, as well as ENPP1 inhibitors for therapies that utilize endogenous 2′3′-cGAMP (*e.g.* radiotherapy). These therapeutic agents have potential to improve the efficacy and/or safety of STING pathway activation for cancer immunotherapy and could be utilized in combination with STING pathway agonists either through local co-administration or by rational co-incorporation into drug delivery platforms.

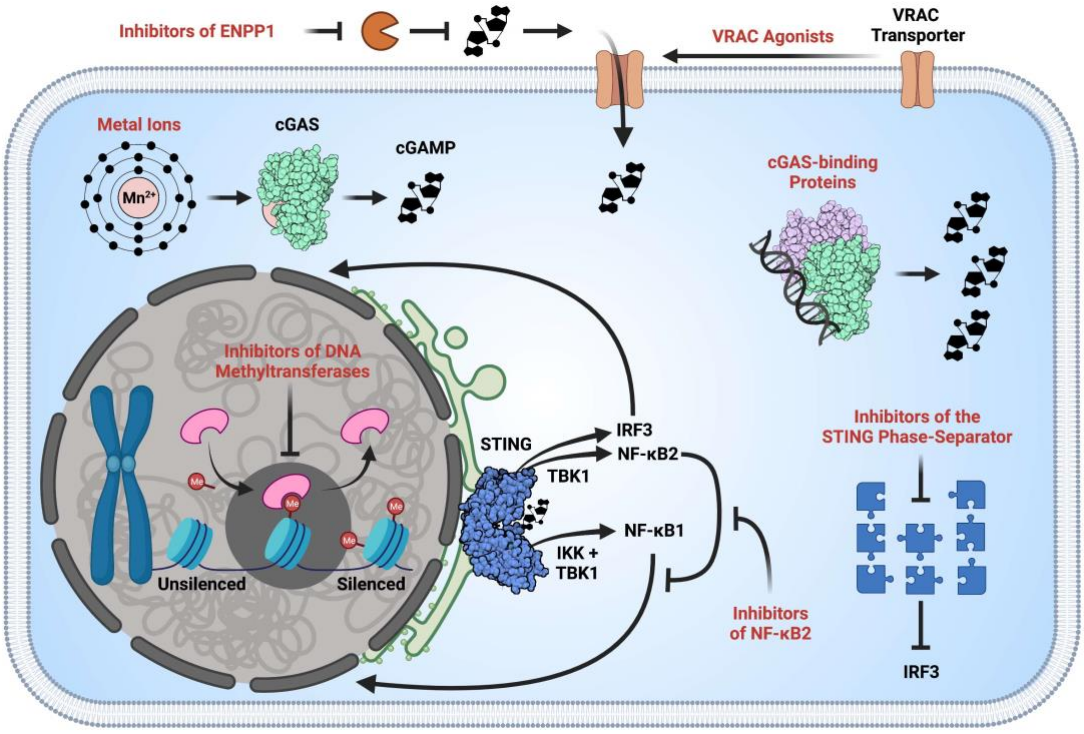

Figure 21: *Strategies for potentiating STING signaling*.

The magnitude of STING-driven gene expression and/or profile of the resultant immune response can be modulated by many different biochemical agents (*i.e.* potentiators). Depicted in this figure are some notable potentiators of the cGAS/STING pathway. These potentiators include: certain metal ions (*e.g.* Mn^{2+} and Mg^{2+}), which can amplify STING signaling through a variety of mechanisms; cGAS-binding proteins, which can augment 2′3′-cGAMP production by sensitizing cGAS to dsDNA; inhibitors of DNA methyltransferases, which can restore the activity of epigenetically silenced cGAS and STING proteins; various inhibitors of NF-κB signaling, which can influence downstream gene expression; inhibitors of the STING phase-separator, which have potential to prevent the inhibition of STING signaling induced by the liquid-liquid phase condensation of STING; VRAC agonists, which may enable enhanced transmission of CDN STING agonists; and inhibitors of ENPP1, which can be used to increase local 2′3′-cGAMP concentrations by deterring the degradation of 2′3′-cGAMP. Figure created with *biorender.com*.

It is well established that transition metals can regulate the function of enzymes, as nearly half of all enzymes utilize metal cofactors[516, 517]. Manganese, which is one of the most abundant metals within mammals, was recently identified as a natural, triggerable potentiator of the STING pathway in 2018[518]. Indeed, the majority of manganese(2+) (Mn^{2+}) within cells is confined to membrane-enclosed organelles (*e.g.* Golgi and mitochondria) at cellular steady-state and therefore avoids innate immune sensors in the cytosol. However, upon viral infection, Mn^{2+} is released into the cytosol, where it can promote STING pathway activation[518].

Over the past several years, cytosolic Mn^{2+} has been reported to potentiate STING signaling in several unique ways: 1) Mn^{2+} can independently activate monomeric cGAS in the absence of dsDNA without the need for cGAS oligomerization[65, 347]; 2) In conjunction with the ATP/GTP substrate pair, Mn^{2+} allosterically enhances the dsDNA binding capacity of cGAS. Conversely, dsDNA enhances the Mn^{2+} binding capacity of cGAS, which is also amplified by larger molecular weight dsDNA. Accordingly, coupling Mn^{2+} with cytosolic dsDNA can lower the threshold for STING pathway activation by several orders of magnitude[518] as they both act in a concerted manner for maximal cGAS-substrate recognition[65]; 3) Mn^{2+} accelerates the overall catalytic activity of dsDNA-bound cGAS resulting in much greater production of cGAMP[65, 518]; 4) Mn^{2+} may increase the binding affinity of cGAMP to STING. Some reports suggest that Mn^{2+} can augment cGAMP-STING binding affinity[146, 518], though a more recent publication found that Mn^{2+} does not affect the binding affinity between STING and STING agonists[519]. Thus, this particular point remains to be clarified; 5) Mn^{2+} induces the phosphorylation of both TBK1 and p65 in a STING-independent manner and when in the presence of STING agonists, Mn^{2+} enhances the assembly of the enhanceosome, resulting in greatly increased production of IFN-β[519].

Collectively, all these attributes make cytosolic Mn^{2+} an exceptionally potent and noteworthy STING pathway potentiator. Additionally, it has also recently been determined that Mn^{2+} is essential in the innate immune sensing of tumors and that combining Mn^{2+} with ICB can synergistically boost antitumor immunity[348]. Furthermore, a phase 1 clinical trial investigating the combination of Mn^{2+} and anti-PD-1 antibody yielded promising efficacy in patients with advanced metastatic solid tumors[348] (NCT03991559).

Several research groups have already begun to develop nanotechnology and/or depots for Mn^{2+} delivery to promote enhanced pharmacological STING pathway activation for cancer

immunotherapy[352, 520-524]. Wang *et al.* reported a biomaterial-based delivery approach that coupled the divalent cation chelator, alginate with Mn^{2+} in the context of radiotherapy[520]. The researchers found that intratumoral injections of Mn^{2+} by itself could indeed enhance the antitumor immune response following RT, but that the timing of administration was critical for efficacy. Free Mn^{2+} was metabolized out from tumors within minutes and DNA did not accumulate in the cytosol of cells until ~ 24 hours post RT treatment. Accordingly, Mn^{2+} injected intratumorally immediately after RT was unable to enhance the therapy, while intratumoral injection 24 hours after RT did demonstrate efficacy. They subsequently employed alginate to act as a depot to control the release of Mn^{2+} for up to 72 hours. Administration of the alginate-manganese complexes 24 hour after RT lead to 90% tumor inhibition rate and a significantly extended average survival time.

Hou *et al.* created a multifaceted NP for STING pathway activation in tumors[521]. Doxorubicin (DOX) was encapsulated within amorphous porous manganese phosphate (APMP) NPs, which were then coated them with phospholipids (PL) for improved stability in systemic circulation and triggerable phospholipase-mediated degradation within tumor cells. When administered IV, the resultant PL/APMP-DOX NPs navigated to tumors, released DOX to induce DNA damage and subsequent cGAS activation, and released Mn^{2+} to augment cGAS/STING activity. The PL/APMP-DOX NP treatment boosted DC maturation and increased tumor infiltration of both cytotoxic T cells and NK cells in the 4T1 murine breast cancer model.

Zhou *et al.* also developed a multifunctional NP platform, which likely operates in a similar manner to the PL/APMP-DOX NPs (*i.e.* delivering DOX and potentiating the STING pathway with manganese)[352]. Their NP platform was prepared by co-assembling dsDNA-gold conjugates and DOX onto Mn_3O_4 nanoflowers. 59 bp poly(dA):poly(dT) was chosen as the dsDNA to activate the STING pathway. The poly(dT) single-stranded DNA was pre-conjugated onto AuNP through an Au-S bond and then annealed with complementary strand. This was then loaded onto Mn_3O_4 nanoflowers via a noncovalent attachment method. Finally, DOX was loaded onto the complex, resulting in a final particle diameter of ~ 354 nm and a surface charge of −7.7 mV. Following intravenous administration, manganese and gold from the nanoflower NPs were detected in B16-F10 tumors, suggesting some level of passive targeting. It was reported that the dsDNA stimulated the immune response by activating the STING pathway via cGAS, while the DOX exerted its chemotherapeutic antitumor activity. Though not addressed by the authors, it is likely that the DOX also contributed indirectly to the STING pathway activation via its DNA-damaging capacity and that the Mn^{2+} degradation product of nanoflower enhanced the STING signaling

within the tumors. The combination particles significantly inhibited tumor growth and prolonged survival in the 4T1 tumor model and successfully demonstrated potential for synergy between a STING-pathway agonist and a chemotherapy.

Chen *et al.* reported a thiolated and Mn^{2+} coordinated CDN nanovaccine (termed Mn-cGAMP NVs) that facilitates the cytosolic co-delivery of 2′3′-cGAMP and Mn^{2+} to potentiate an antitumor immune response against B16-F10 murine melanoma following intratumoral administration[522]. They utilized polymerized guanidine-containing disulfides to assemble with 2′3′-cGAMP and then coordinate with Mn^{2+} ions, forming particles that were ~ 176 nm in diameter. The Mn-cGAMP NVs attenuated primary tumor growth, inhibited distal tumor growth, and improved responses when administered in combination with anti-PD-L1 monoclonal antibody treatment.

Yang *et al.* engineered a biomimetic nanoplatform using cancer cell membranes extracted from B16-F10 cells to co-encapsulate manganese dioxide (MnO_2) NPs and the established photothermal therapy sensitizer, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR)[523]. Interestingly, manganese was the sole adjuvant in their system and it was used to induce STING signaling via cGAS activation. The resultant vesicles had a diameter of ~ 125 nm and displayed a negative surface charge of −19 mV. Notably, the researchers found that slightly acidic conditions (*e.g.* pH ~ 6.8) with high concentrations of hydrogen peroxide (*e.g.* 2.5 mM H_2O_2) triggered the release of Mn^{2+} from the vesicles and that the vesicles promoted the tumor accumulation of both Mn^{2+} and DiR following intravenous injection. The systemic administration of their construct coupled with targeted photothermal therapy enabled partial tumor regression in primary tumors, multinodular tumors, metastatic tumors, and recurrent tumors. Additionally, transcriptomic analysis of the tumors following treatment demonstrated the upregulation of STING-driven genes, supporting on-target STING activation *in vivo*.

Gao *et al.* described the development and characterization of PEGylated manganese phosphate (MnP-PEG) nanoclusters for cancer immunotherapy[524]. The particles were fabricated by mixing Mn^{2+} and PO_4^{3-} ions in solution followed by the addition of a phosphate-functionalized 5 kDa PEG polymer. The MnP-PEG nanoclusters were ~ 150 nm in diameter with a negative surface charge of −11 mV. It was determined that the nanoparticles could mediate endocytosis, acid-triggered Mn^{2+} release, and STING signaling. Furthermore, intratumoral administration of the MnP-PEG nanoclusters in the B16-F10 tumor model enhanced the tumor infiltration of DCs and

macrophages as well as activated (*i.e.* $CD69^+$) tumor-infiltrating $CD8^+$ and $CD4^+$ T cells and NK cells. The treatment also resulted in antitumor efficacy as a monotherapy and improved responses to ICB (*i.e.* anti-PD-1 therapy).

Sun *et al.* developed a new cancer immunotherapeutic that co-delivers Mn^{2+} and CDA in coordination nanoparticles[519]. After screening various metal ions for potential synergy with STING agonists, they identified Mn^{2+} as a noteworthy potentiator of the STING pathway. Indeed, Mn^{2+}-mediated potentiation of the STING pathway was found to be independent of STING variants and STING agonist structures, and the intratumoral treatment of mice bearing CT26 tumors with a soluble mixture of CDA and Mn^{2+} resulted in a significant increase in antigen-specific T cells and an attendant inhibition of tumor growth compared to either monotherapy. Interestingly, it was subsequently determined that Mn^{2+} can self-assemble with CDA in methanol to form coordination polymers with diameters ranging from nanometers to micrometers; however, these complexes were shown to be unstable under physiological conditions. In light of these physicochemical properties, the researchers developed a nanoparticle system that could stabilize the CDA–Mn^{2+} coordination polymers. Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-$(histidine)_{11}$ was added as an additional coordination ligand to promote the formation of a stabilizing hydrophobic core, which was then coated with an outer PEG-lipid layer by resuspending in a solution of DOPC:cholesterol:DSPE-PEG5000 (4:1:1 molar ratio), thereby allowing for aqueous suspension of the resultant particles. The final platform, denoted CMP_{CDA}, comprised particles that were ~ 118 nm in diameter with a neutral charge, and the loading efficiencies of CDA and Mn^{2+} were 39.6% and 25.3%, respectively. Notably, this new immunotherapeutic could be delivered intravenously, where a significant increase in serum IFNβ, TNFα, CXCL-9 and CXCL-10 was observed in mice with CT26 tumors compared to those treated with a soluble mixture of CDA and Mn^{2+}. Systemic treatment with CMP_{CDA} significantly decreased CT26 tumor growth, eliminated established tumors in 50% of mice, and conferred resistance to tumor rechallenge, whereas treatment with soluble agents had no response. This platform was also validated in tobacco carcinogen-associated syngeneic squamous cell carcinoma and B16-F10 tumor models, where it was found to outperform the highly potent diABZI small molecule agonist, demonstrating the significant potential of metalloimmunotherapy in nano-based cancer therapeutics.

There exist several known cGAS-binding proteins that also bind DNA and thereby promote cGAS activity[525-528]. By providing additional binding sites for cytosolic DNA, these cGAS-binding proteins enhance the recognition of DNA by cGAS, which augments 2′3′-cGAMP production and

STING signaling. Polyglutamine binding protein 1 (PQBP1) has been described as a proximal innate sensor of a human immunodeficiency virus type 1 (HIV-1) infection, as it was found to enhance the IRF3-dependent innate response in primary human monocyte-derived DCs (MDDCs) by directly binding reverse-transcribed HIV-1 DNA and cGAS[525]. The CCHC-type zinc-finger (ZF) protein, ZCCHC3 was similarly reported as a co-sensor of cGAS, capable of improving the innate immune response to cytosolic dsDNA and the DNA viruses, herpes simplex virus 1 (HSV-1) and vaccinia virus[526]. GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) was identified as another positive regulator of cGAS activity with the inhibition of G3BP1 partially rescuing cGAS-mediated autoinflammation in a *Trex1*$^{-/-}$ mouse model[527]. Lastly, the secreted bacterial protein, streptavidin was recently reported to bind both DNA and cGAS to promote cGAS-dependent immune responses against the DNA virus, HSV-1[528]. Notably, streptavidin exhibits exceptionally strong noncovalent interactions with biotin and has accordingly been extensively used for many biotechnological applications, such as molecular purification, molecular detection, and drug delivery. Therefore, the unique interaction of streptavidin with cGAS and DNA, which can lead to immunostimulation, complicates the clinical and biotechnological usage of streptavidin. Indeed, careful consideration should be given when choosing to use streptavidin in certain applications. However, since enhanced STING signaling is beneficial for many cancer types, these cGAS-binding proteins have potential for therapeutic use in combination with cGAS-activating cancer therapies, though molecular engineering or nanotechnology would likely need to be employed for *in vivo* delivery of these molecules.

As briefly mentioned in ***Section 3.4***, inhibitors of DNA methyltransferases are approved for the treatment of certain cancers and are also capable of improving intratumoral STING signaling and tumor immunogenicity[297, 299]. Indeed, Falahat *et al.* recently determined that promoter hypermethylation of cGAS and STING genes mediates transcriptional silencing and impairs STING signaling function in melanoma, which disrupts tumor antigen presentation and the accumulation of tumor infiltrating lymphocytes[299]. By inhibiting DNA methylation with a clinically available DNA methyltransferase inhibitor (*i.e.* 5-aza-2'-deoxycytidine), the researchers were able to restore the activity of cGAS and STING and thereby improve antigenicity through the augmentation of MHC class I surface expression and antigen presentation. This ultimately resulted in enhanced T cell recognition of melanoma. Therefore, inhibitors of DNA methyltransferases could possibly be used along with STING pathway agonists to improve antitumor immune responses in cancers where STING is epigenetically silenced.

Recent studies conducted by Hou *et al.* have demonstrated that irradiation-induced STING signaling activates both canonical NF-κB (*i.e.* NF-κB1) and noncanonical NF-κB (*i.e.* NF-κB2) in tumor-localized DCs[238]. Interestingly, the researchers also found that the NF-κB2 pathway negatively regulates NF-κB1–mediated gene transcription and that they could enhance the antitumor effect of irradiation in murine models by inhibiting downstream signaling of the noncanonical pathway with intratumoral injections of a specific NF-κB2 inhibitor (*i.e.* SN52). Thus, targeted inhibition of NF-κB2 represents another possible strategy for potentiating the therapeutic effects of STING signaling in cancer. Tuning the downstream signaling that follows STING activation holds tremendous promise, because it may yield outcomes where beneficial effects of STING signaling (*e.g.* antitumor immunity) are maximized and negative effects (*e.g.* toxicity, immune regulation, *etc.*) are minimized. Interestingly, an inhibitor of downstream NF-κB1 signaling (*i.e.* SN50) has recently been characterized in combination with vaccine adjuvants and was described as an immune potentiator capable of decreasing markers associated with poor tolerability and improving the protective response of vaccination[529], which suggests that therapeutic context is certainly important as well.

Carozza *et al.* found that many cancer cells continuously export endogenous 2′3′-cGAMP and that 2′3′-cGAMP is rapidly degraded by ENPP1 in the extracellular space[166]. They also determined that depletion of extracellular 2′3′-cGAMP by intratumoral injection of wildtype STING decreased the tumor infiltration of immune cells and eliminated the curative effects of tumor irradiation. Moreover, intratumoral administration of ENPP1 inhibitors elevated extracellular 2′3′-cGAMP concentrations and promoted improved responses to radiation therapy as demonstrated by delayed tumor growth. Notably, ENPP1 inhibitors would also limit levels of immunosuppressive adenosine in addition to elevate the levels of 2′3′-cGAMP[198]. Accordingly, ENPP1 inhibitors are currently being explored in preclinically with cGAS-activating therapies[530], as they are likely to synergize with therapies that involve endogenous 2′3′-cGAMP.

In addition to the established potentiators of the STING pathway, there are still many other possible agents that might also propagate STING signaling, such as inhibitors of the STING phase-separator and VRAC agonists, both of which could synergize with cGAS-activating therapies by enhancing the production and spread of 2′3′-cGAMP. Though such agents have not yet been directly explored in the context of STING signaling and cancer immunotherapy, future investigation is certainly warranted.

8. Summary, Perspectives, and Future Directions

Since elucidating its critical role as a central link between innate and adaptive immunity in cancer immune surveillance, the cGAS/STING pathway has emerged as one of the most exciting and promising targets in immuno-oncology. Indeed, as indicated by the rate of publications, academic interest is increasing exponentially (***Figure 22***), and many pharmaceutical companies are developing STING pathway agonists and racing to translate them into the clinic. This fervent research-and-development activity is motivated by the clear and growing need for new immunotherapeutic strategies to increase immune recognition and eradication of tumors, particularly those that do not, or only poorly, respond to currently FDA-approved ICB antibodies. While an expanding number of therapeutic candidates are being developed to address this challenge (*e.g.* alternative checkpoint inhibitors, cytokine therapeutics, cell-based therapies, *etc.*), the multimodal activity of the STING pathway to "jump-start" and propagate the cancer immunity cycle offers compelling rationale for its enormous potential as an immunotherapy target. Indeed, preclinical studies of an increasing number of STING agonists have demonstrated remarkable results in many tumor models, sometimes resulting in complete and durable therapeutic responses in a majority of treated mice, even in models of highly immunosuppressive tumors.

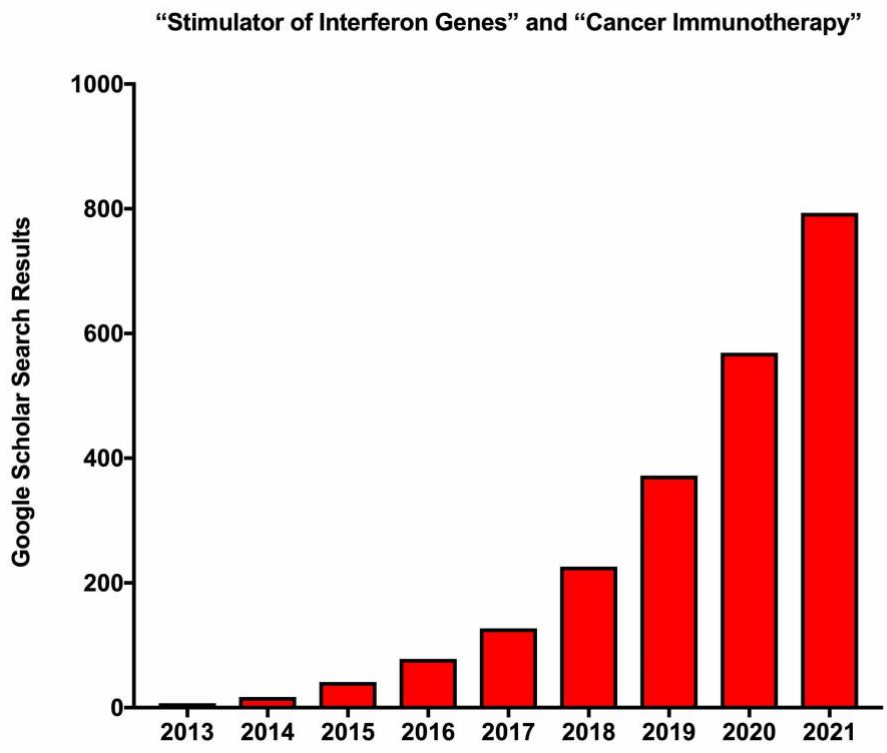

Figure 22: *Rate of Publications.*
Google Scholar search results for: "stimulator of interferon genes" "cancer immunotherapy".

Unfortunately, early clinical studies evaluating STING agonists in cancer patients have been less successful and arguably disappointing relative to initial, though perhaps unrealistic, expectations. Notably, data from the Aduro/Novartis (ADU-S100)[326, 531] and Merck (MK-1454)[359] clinical trials of intratumorally administered CDNs (***Table 1***) were underwhelming with low response rates observed in treated patients (*e.g.* 2.1% overall response for MIW815 (ADU-S100) monotherapy, 0% overall response for MK-1454 monotherapy, and 24% overall response for MK-1454 in combination with pembrolizumab (*i.e.* anti-PD-1 monoclonal antibody therapy)). Additionally, neither study demonstrated consistent abscopal effects (*i.e.* shrinkage of non-injected distal tumors), which is a primary goal of an intralesional therapy (*i.e. in situ* vaccination). While not uncommon in drug development, such a gap between the remarkable preclinical efficacy, which was observed across many investigators, types of STING agonists, and tumor models, and these initial clinical outcomes motivate the need to better understand both the biological and pharmacological mechanisms that are restraining efficacy and to develop new agents, delivery systems, and/or drug combinations to more fully realize the immunotherapeutic potential of the STING pathway in patients. Below we offer additional perspective into emerging chemical and materials-based strategies for addressing known and putative barriers to the efficacy of STING pathway agonists, many of which appear poised for future clinical evaluation.

In considering the clinical trial data from the recent Aduro/Novartis and Merck studies, it is important to recognize that these studies enrolled patients with a range of solid tumor types, and many patients also had advanced disease that had progressed following other treatments. Additionally, the trials did not involve focused biomarker screening (*i.e.* testing for specific gene expression and/or protein signatures that are known to be more conducive to a given therapy), which can help to identify patients that are more likely to respond to treatment. Moreover, clinical observation of an abscopal response following intratumoral administration currently remains the exception rather than the norm[371], and therefore it is perhaps unrealistic to expect robust therapeutic responses from a single intratumorally injected agent. This is noteworthy, as 11/15 of the ongoing trials (***Table 1***) appear to be focused on intratumoral administration of STING agonists. Thus, care should be taken when interpreting the results of these trials, as multiple factors have likely contributed to the unfavorable clinical outcomes observed thus far, a number of which may not be attributed to STING as a drug target or even the agents themselves.

As described in ***Section 5.2***, while intratumoral administration clearly has potential, especially for some cancer types and clinical scenarios, free CDNs (*e.g.* ADU-S100) rapidly clear

from intratumoral injection sites and display poor pharmacokinetic properties with a short half-life (*e.g.* 10–20 minutes). Considering the complex temporal relationships among innate immunity, antigen processing, and T cell priming[382, 387], such transient drug exposure, and the resultant burst of local STING activation at the tumor site, is likely suboptimal for *in situ* vaccination to generate systemic T cell responses that are capable of mediating abscopal responses. While this challenge could perhaps be addressed with repeated administration, the short half-life of CDNs (and most STING agonists described to date), coupled with the intrinsically transient nature of the resulting inflammatory response, may require a frequency of local administration that would not be feasible, particularly in clinical scenarios that require image-guided injection of tumors. This motivates the need for drug delivery technologies that allow for improved and tunable control over the retention and/or distribution of STING agonists following intratumoral administration as well as a deeper understanding of the interplay among STING agonist pharmacokinetics, pharmacodynamics, and antitumor immunity that can inform the design of local delivery strategies to improve clinical responses. Notably, delivery vehicles have been employed to improve the therapeutic efficacy of two other leading innate immune agonists, CpG ODN[354] (*i.e.* agonist of TLR-9) and poly-IC[532] (*i.e.* agonist of TLR-3 and MDA-5), which has led to ongoing clinical trials for intratumoral administration; CMP-001 is a CpG ODN encapsulated into a virus-like particle (NCT04695977), and poly-ICLC is an electrostatic formulation comprising poly-IC stabilized with poly-L-lysine and carboxymethylcellulose (NCT03789097).

Considering the significant recent advancements in nanoparticles for CDN delivery (***Section 6.1***) that can improve cellular uptake, promote more efficient cytosolic delivery, harness lymphatic drainage to reprogram tdLNs, and/or increase local retention of CDNs, clinical investigation of these technologies for intratumoral administration should be a priority, as they offer a relatively simple approach for addressing the pharmacological shortcomings of locally administered CDNs. Indeed, the results of the clinical trials by Codiak Biosciences using an exosome-based delivery system for CDN delivery and by Synlogic with a bacterial-based delivery system (***Table 1***) are much anticipated as they may help in assessing the extent to which the delivery barriers facing freely administered CDNs have impeded clinical efficacy.

Comparable to local oncolytic virus therapy (*e.g.* T-VEC[533]), nanoparticle-based STING agonists appear to be ideal for direct injection into solid tumor sites. However, in post-surgical settings, implantable and/or injectable biomaterial scaffolds may be better suited for administration into resection cavities to control the release of STING agonists to boost antitumor

immunity. In addition to allowing for sustained and tunable drug release that may reduce the necessary number of local injections for efficacy, biomaterial scaffolds and depots also afford important opportunities for protecting drug cargo from clearance and/or degradation, promoting cell-specific interactions via targeted (*e.g.* chemokine-induced) cellular infiltration, and programming the coordinated (*e.g.* combinatorial, pulsatile) release of multiple chemically-distinct agents. As the cancer immunity cycle requires the cooperation and coordination of multiple cell types, the use of scaffolds or gels for cell-specific orchestration has potential to improve therapeutic responses for STING pathway agonists. Notably, DCs, and conceivably other cell types, can be directed into gels for targeted, *in situ* manipulation as demonstrated by Mooney and co-workers, who have reported the development of injectable cryogels loaded with a chemoattractant (*i.e.* GM-CSF) to enhance the local accumulation of specialized antigen-presenting cells (*e.g.* DCs)[534]. Additionally, maximizing antitumor responses via intratumoral delivery (and immunotherapy more generally) is likely to require use of multiple agents that are properly sequenced. This is nicely illustrated in the work by Brody *et al.* described above (***Section 5.2***) where image-guided injection of Flt-3 ligand for nine days, two days of radiotherapy, and eight injections of poly-ICLC were employed to generate an abscopal response in lymphoma patients[383]. Likewise, the seminal studies by Wittrup and co-workers demonstrated the importance of sequencing a tumor-targeted IgG and IL-2 prior to IFNα in maximizing antitumor immunity[234].

While much work remains to be done to understand how to best combine STING pathway agonists with other therapeutics, both for local and systemic administration, major advancements in designer biomaterials for local drug delivery will enable the continued development of technologies for intratumoral immunotherapy that allow for optimal control over the release and/or activation of multiple agents in a spatiotemporally programmable manner. Such opportunities are nicely exemplified by the recent work described above in ***Section 6.2*** from the groups of Honggang Cui[476], Matthias Stephan[484], and the team of Robert Langer, Daniel Anderson, and Ana Jaklenc at MIT[475]. In particular, the work by Lu *et al.* from the MIT team is very promising as it uniquely allows for pulsatile, programmed release of not only STING agonists but also a diverse range of other molecules[475]. This presents an important opportunity to coordinate STING agonist delivery with other therapeutics agents (*e.g.* chemotherapy, ICB, *etc.*) to optimize antitumor immunity using fewer injections, and perhaps only a single administration, which would dramatically improve clinical utility and expand the number of patients that would be eligible for intratumoral administration.

Nonetheless, the translational challenges and the initial low clinically efficacy of intratumoral administration has motivated recent advancements in drug delivery systems for CDNs as well as other STING agonists that exert therapeutic effects when administered systemically, including both modified CDNs and non-nucleotide, small molecules. These medicinal chemistry developments address some, but not all, of the intracellular delivery and pharmacological limitations of natural CDNs (*e.g.* 2′3′-cGAMP) and first-generation synthetic CDNs (*e.g.* ADU-S100). While efficacy data for many of these agents has not been published in the academic literature, compounds such as MSA-2, SR-717, diABZIs, and 15a appear promising in preclinical studies, and a number of these have recently entered the clinical pipeline (***Table 1***), with data expected in the next several years. The outcome of these trials will be important for informing the continued development of STING pathway agonists and for identifying immunopharmacological barriers that limit their safety and efficacy.

The critical question for systemically administered STING agonists, regardless of type of STING agonist or formulation method, will be the width of the therapeutic window. Systemic administration of STING agonists can result in a transient systemic inflammatory response[132, 145, 338, 388] that can resemble a cytokine storm similar to that of other innate immune activators (*e.g.* PEG-Intron[535], CMP-001[354], *etc.*), which have been known to cause patients to temporarily experience flu-like symptoms. Indeed, systemic inflammation could very well prove to be dose limiting in patients receiving an intravenous or oral administration of STING agonists. Accordingly, as systemically administered STING agonists move forward, an important consideration will be how to expand their therapeutic window by minimizing inflammatory side effects. In addition to a growing clinical arsenal of approaches to combat cytokine storm and other deleterious systemic inflammation (*e.g.* anti-IL-6 monoclonal antibody therapy[536-538]), a multitude of exciting chemical, biomolecular, and pharmaceutical engineering strategies can be envisioned to address this critical challenge, including some already in development. For example, the non-nucleotide STING agonist MSA-2 described in ***Section 5.3*** leverages a protonizable carboxylic acid group that increases cell membrane permeability in the acidic microenvironment associated with some tumors[338]. Nonetheless, systemic administration of MSA-2 still induces STING activation in other tissues, and therefore, the degree to which exploiting the acidic TME drug widens the therapeutic window relative to other STING agonists remains to be seen in human clinical studies. Furthermore, it will also be important to consider that the pH of tumors (as well as other microenvironmental factors) can vary significantly between cancer types, patients, and tumor sites[339]. Thus, it will be important to continue to develop STING agonists and/or drug carriers

capable of selectively targeting tumor sites via other environmentally-responsive mechanisms (*e.g.* redox, protease expression levels, *etc.*). Fortunately, an expansive tool box of environmentally-responsive drug carriers and conditionally-cleavable chemical linkers have already been developed, primarily for chemotherapeutics, to enhance drug accumulation at tumor sites[539]. Leveraging such chemical strategies to exploit microenvironmental signatures to enrich STING activation at tumor sites has not been widely explored, but holds much potential for improving the efficacy and safety of systemically administered STING agonists.

Likewise, harnessing molecular targeting strategies such as antibodies, peptides, and glycans for STING agonists also holds significant promise for achieving more tumor selective activation of innate immunity and minimizing inflammatory side effects, provided an appropriate selective target can be identified. While no published reports are available, antibody drug conjugates (ADCs) for targeted STING agonist delivery are being developed by several companies, including Mersana (*i.e.* XMT-2056)[540, 541], Takeda (*i.e.* TAK-500)[208], and Curadev (*i.e.* CRD5500)[542]. Based on recent reports leveraging ADC technology for the delivery of other innate immune agonists[397], such targeting strategies appear well poised to be a major advancement in the field. However, this also raises the important and unknown question as to which cell type(s) in the tumor should STING agonists be targeted. Mechanistic preclinical studies, which have almost exclusively utilized intratumoral administration of CDNs, have implicated a number of different cell populations (*e.g.* cancer cells, endothelial cells, macrophages, dendritic cells) as being important contributors to STING agonist activity and therapeutic efficacy. This is perhaps not surprising given the relatively ubiquitous expression profile STING across cell populations as well as the multifaceted paracrine effects exerted by downstream innate effectors (*e.g.* type I IFNs and other proinflammatory cytokines) on many cell types within the TME. Which cell type(s) to target may also depend on the cancer type and/or the stromal composition of TME, as STING activation can trigger distinctive effects in different cell populations. A still unresolved question is the extent to which cancer cell–specific STING activation is important for therapeutic efficacy; that is, are there advantages (or potentially disadvantages) to activating STING signaling in cancer cells or is targeting stromal populations (*e.g.* macrophages, dendritic cells) sufficient or superior, with cancer cells primarily acting as bystanders during the initial phase of the innate immune response. This will be an important question to resolve, as STING signaling is often suppressed and dysfunctional in cancer cells[473]. Accordingly, in cases where cancer cell–intrinsic STING activation is critical to efficacy, careful patient selection and/or adjunctive therapies to enhance STING expression in cancer cells (*e.g.* treatment with DNA methyl transferase inhibitors[299]) will

be required. Clearly, more detailed knowledge of how specific cell types contribute to the therapeutic efficacy of STING agonists, and how this may also vary between cancer types, will be critical to the future design of molecularly-targeted STING agonists. Notably, in addition to their potential therapeutic utility, ADCs may also be of use as a research tool for addressing these outstanding questions. Indeed, Cetinbas *et al.* are pursuing such a strategy to dissect the importance of cell-specific STING pathway activation in tumors and have identified distinct differences in resultant immune profiles generated by non-targeted STING agonists and cancer cell–targeted STING agonists, which suggested that cancer cells can positively contribute to antitumor immunity in certain cases[543].

Another concern facing systemic administration of STING agonists, particularly non-nucleotide, small molecules, is the potential for inducing toxicity in T cells, which are important for therapeutic efficacy[210]. Indeed, T cells express high levels of the STING protein and appear to be highly susceptible to STING-induced apoptosis[86, 237, 366]. Many non-nucleotide, small molecule STING agonists directly access the cytosol via passive diffusion across cellular plasma membranes, resulting in indiscriminate STING activation in T cells with potentially deleterious effects on antitumor adaptive immunity. The use of nanoparticle-based drug carriers, including many described above, offer the possibility of minimizing such effects, since T cells have a relatively low capacity for endocytosis of nanoparticles[544]. Indeed, in our studies using STING-NPs, we have observed negligible uptake of nanoparticles (*i.e.* CDNs) by T cells in the TME, whereas we and others have demonstrated that nanocarriers can enhance uptake by cancer cells and myeloid cells in the tumor[194, 545].

While this represents an important advantage of using NP-based STING agonists, this is counterbalanced by the likelihood that a large fraction of administered nanoparticles, typically the vast majority regardless of nanoparticle properties, will accumulate in the liver with potential for hepatic STING activation. In our recent analysis of CDN biodistribution following intravenous delivery with a therapeutic dose of STING-NPs (*i.e.* polymersomes), we unsurprisingly observed a high degree of CDN accumulation in the liver, but a disproportionally low degree of hepatic STING activation as measured by inflammatory gene expression. We postulate that Kupffer cells, which have a well-established role in clearing nanoparticles from the circulation, were the primary contributors to STING activation in the liver as hepatocytes have been reported to have low levels of STING expression[546]. Hence, clearance of nanoparticles by the liver may not impose as significant of a barrier to intravenously administered nanoparticulate STING agonists as might

otherwise be anticipated. Nonetheless, evidence is also emerging that chronic STING activation in the liver can promote nonalcoholic fatty liver disease (NAFLD)[547]. Therefore, STING-induced liver damage is a valid safety consideration for any nanoparticle-based STING agonist and motivates the design of carriers that minimize liver accumulation and/or hepatic STING activation. For example, the biodistribution profile of NPs can be tuned to some degree to bias delivery to other organ sites (*e.g.* spleen, lungs, bone marrow) by altering the physicochemical properties of the particles (*e.g.* charge, size, *etc.*)[548, 549] or through the addition of targeting ligands[550], and liver preconditioning strategies have also been employed to reduce Kupffer cell uptake and liver accumulation of nanomedicines[551]. It will be interesting and important to determine if such approaches can widen the therapeutic window of nanoparticle STING agonists. Nonetheless, developing nanoparticle-based platforms that allow for more tumor-selective STING activation, either through molecular targeting or environmentally-responsive release, will be an important future direction to pursue.

Another important consideration in using nanoparticles for intravenous delivery of STING agonists, is their inefficient capacity to deliver drug cargo to tumor sites. Though nanoparticles can preferentially accumulate in human metastatic tumors[407-411], only a small percentage of an intravenously injected drug dose reaches the tumor[552, 553]. Indeed, in their now notorious article, Chan and co-workers have estimated that less than 1% of nanoparticles reach tumor sites in preclinical tumor models[554]. However, the shortcomings of inefficient tumor delivery have largely been manifested in applications where efficacy is dependent on delivery of high drug doses to the vast majority of tumor cells at a majority of tumor sites (*e.g.* chemotherapy, siRNAs against cancer cell targets). We maintain that this may not be as critical of a barrier, and may even be an important opportunity, for nanoscale STING agonists and other nanoparticle-based immunostimulants, where robust therapeutic responses may be achieved via delivery to a relatively small subset of cancer or stromal cells capable of initiating endogenous programs of systemic antitumor immunity[555-558]. This important distinction has been nicely highlighted by the Karathanasis group, who have demonstrated that intravenous administration of both liposomal[420, 421] and silica-based nanocarriers[459] results in CDN accumulation primarily in perivascular regions where STING activation in local cell populations results in the recruitment of antitumor effectors into the TME. Nonetheless, nanoparticle delivery strategies that can further enhance the tumor accumulation and penetration of STING agonists require continued development, particularly for cases where the passive targeting may not be appreciable.

While targeting STING agonists to the TME is a rational and likely effective strategy for expanding the therapeutic window, this may not be a necessity provided systemic inflammatory effects can be adequately controlled. While their pharmacological mechanisms have not been fully described, it is likely that systemically administered small molecule STING agonists work at least in part via the induction of a peripheral inflammatory response initiated by diverse cell populations. A STING-driven cocktail of circulating proinflammatory cytokines can act on the TME via multiple mechanisms in manners comparable to systemic cytokine therapies. Moreover, such a systemic response can mobilize antitumor effector cells in immune reservoirs (*e.g.* bone marrow, spleen, *etc.*), causing them to migrate into tumors. For example, using a cationic liposomal formulation, Nakamura *et al.* demonstrated that cytokines secreted into the circulation by liver macrophages triggered the activation of NK cells in the spleen, which then led to the elimination of melanoma metastases in the lung following the migration of the activated NK cells[559]. Therefore, a complementary approach to tumor targeting is to develop molecules and/or delivery platforms for optimizing the magnitude and duration of systemic STING activation. However, as discussed in ***Section 5.3***, it is also not yet known what the ideal pharmacokinetics for STING agonists (and innate immune agonists more generally) should be, nor is it known whether a slow and sustained or a fast "on/off" profile is optimal for therapeutic efficacy. Notably, while chronic STING activation is often associated with certain autoimmune and chronic inflammatory diseases[203], acute STING activation might also be problematic if the magnitude and distribution of STING signaling is too intense. The half-life of STING agonists is relatively short, ranging from several minutes for CDNs to several hours for SR-717, which results in a spike in blood levels of proinflammatory cytokines typically 2–8 hours post administration[338, 388]. The pharmacokinetics of nanoparticle-based STING agonists has not been widely described beyond our report on STING-NPs[388], but the half-life of PEGylated liposomes, for example, can be on the order of several days[560]. Therefore, it will be important to better understand the pharmacokinetic-pharmacodynamic relationships that underlie antitumor immunity and therapeutic efficacy of STING agonists and to develop strategies to more precisely tune their circulation half-life and other key pharmacological properties.

In addition to strategies for tumor targeting and controlling systemic inflammation, a third approach to improving the safety and efficacy of STING agonists is to deploy adjunctive therapies that do not directly activate the pathway, but instead increase sensitivity to STING agonists and/or modulate the inflammatory response. While the discovery of STING pathway potentiators, such as those described in ***Section 7***, is still in its infancy, therapeutic strategies that co-deliver STING

pathway agonists with Mn^{2+} have already emerged and have demonstrated preclinical efficacy[352, 519, 522]. Likewise, the studies by Hou *et al.*, which demonstrate that selective inhibition of specific NF-κB pathway components (*e.g.* non-canonical NF-κB) can increase the STING-driven IFN-I response[238], offer a pharmacologically tractable target for potentiating STING signaling. Intriguingly, though not yet explored for STING agonists, the Esser-Kahn group has demonstrated that inhibiting non-canonical NF-κB signaling can minimize overproduction of TNF-α and IL-6 (*i.e.* "wasted inflammation") induced by CpG ODN (*i.e.* agonist of TLR-9)[529, 561]. This not only reduced the toxicity associated with excessive systemic release of these particular cytokines, but also boosted antibody responses elicited via vaccination. Therefore, it will be important to elucidate whether such "wasted inflammation" exists for STING agonists in the context of cancer immunotherapy, and if so, how to target the signaling pathway to maximize production of factors that are critical for efficacy while minimizing those that contribute to inflammatory toxicities. As these and other pathway modulators are identified and further defined, it will be increasingly important to develop therapeutics as well as drug delivery systems that maximizes their ability to synergize with STING agonists. Indeed, an important advantage of drug carriers is their capacity to deliver multiple agents in precisely balanced ratios[562], a strategy that has achieved clinical success in liposomal delivery of chemotherapeutics (*e.g.* Vyxeos)[563]. Applying similar strategies for co-delivery of STING agonists and pathway modulators holds promise for further improving their efficacy and safety when administered systemically.

Finally, as is often the case in immunology, the STING pathway is a double-edged sword with potential to exert dichotomous effects that are dictated by numerous factors (*e.g.* biological context, magnitude of STING-driven gene expression, kinetics of signaling, *etc.*) and must be considered when optimizing the design and/or delivery of STING agonists. In response to STING activation, a number of immunosuppressive factors may also increase as a regulatory mechanism to dampen the inflammatory response. STING activation can result in the production of IDO-1[398], the infiltration of immunosuppressive MDSCs[564], and the upregulation of PD-L1[449] and other immune checkpoints (*e.g.* B7-H3[565]), amongst other counter regulatory mechanisms[11] that may inhibit antitumor immunity or even drive tumor progression. Accordingly, the development of rationally designed immunotherapy combinations that target these acquired resistance mechanisms is likely to be necessary to fully realize the potential of STING agonists. Which mechanisms to target, however, may also depend on specific cancer types or patient subpopulations, and additional research is needed to more completely understand these mechanisms and to develop biomarkers to predict which patients may be more likely to respond

to STING agonists. Such efforts are ongoing in the setting of other immunotherapy agents and combinations, and the clinical data emerging from the ongoing and future clinical trials will be critical for beginning to fill this knowledge gap. It will be important to consider this emerging information in the design of next-generation STING agonists and delivery technologies. Several groups have already developed systems that allow for co-delivery of STING agonists and therapeutics that can target other therapeutic pathways[352, 420, 456, 476, 521, 566]. While such dual-delivery approaches may prove critical for optimally combining STING agonists with other agents, caution should be taken in considering whether or not two agents that target different pathways and/or cell types need to be chemically or physically coupled, especially in cases where one of the agents is already approved for clinical use. This not only adds additional complexity to manufacturing and regulatory approval, but may also not yield the optimal sequencing or dose of each agent, which may be more readily achieved by simply adjusting the administration regimen of each component independently.

Research over the past decade has led to an enormous leap in our knowledge of the cGAS/STING pathway and its complex but critical role in cancer immune surveillance. Despite early clinical setbacks, the importance of the STING pathway in antitumor immunity is increasingly clear, and STING agonists continue to hold great promise as pharmacological agents for the treatment of cancer, with many now entering clinical trials. The outcomes of these trials, coupled with continued mechanistic investigations in preclinical models, is expected to accelerate our understanding of the immunopharmacological mechanisms and shortcomings of a diversity of STING agonists as well as drug delivery technologies. As this information emerges, the recent and continued advancement in chemical and biomolecular strategies for STING pathway activation, including many described herein, will be critical to realizing the full clinical potential of this promising immunotherapeutic target.

Conflicts of Interest:

J.T.W. is an inventor on U.S. Patent 10,696,985 “Reversibly Crosslinked Endosomolytic Polymer Vesicles for Cytosolic Drug Delivery” and on U.S. Patent Application PCT/US2019/058945 “Graft Copolymers, Methods of Forming Graft Copolymers, and Methods of Use Thereof” which both describe drug delivery technologies that have been used for STING agonist delivery.

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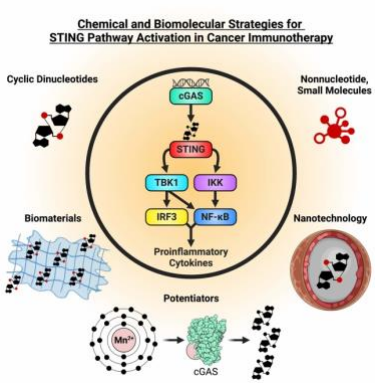
Kyle M. Garland received his B.S. degree in Bioengineering with a focus in Biopharmaceutical Engineering at Lehigh University in 2016. He then joined the Department of Chemical and Biomolecular Engineering at Vanderbilt University, where he studied Immunoengineering under the direction of Dr. John T. Wilson. In 2017, he was awarded the Pediatric Oncology Student Training (POST) Grant from Alex's Lemonade Stand Foundation, which allowed him to pursue STING activation strategies in the context of pediatric neuroblastoma. In 2020, he received the Russell G. Hamilton Graduate Leadership Institute Dissertation Enhancement Grant, which enabled him to conduct research that focused on developing a novel cGAS agonist. He earned his Ph.D. degree in Chemical Engineering at Vanderbilt University in 2021. His dissertation describes the development and use of innate immune activators for local cancer therapy applications.

Taylor L. Sheehy received a B.S. degree in Biochemistry in 2019 at Duquesne University, where she pursued research studying the effects of macromolecular crowding on enzyme kinetics. Upon graduation, she began pursuing her Ph.D. in Biomedical Engineering under the direction of Dr. John T. Wilson. Taylor received a National Science Foundation Graduate Research Fellowship Program (GRFP) award in 2020 to support her work in molecularly engineered STING agonist delivery technologies to improve cancer immunotherapy. These strategies focus on tumor and immune cell-specific targeting regimes. Although this work can be applied to numerous cancer models, Taylor also received a Pediatric Oncology Student Training (POST) award from Alex's Lemonade Stand Foundation in 2020 to pursue projects that concentrate on treatments specifically for pediatric neuroblastoma.

John T. Wilson is an Associate Professor of Chemical & Biomolecular Engineering at Vanderbilt University. Dr. Wilson earned his Ph.D. in Bioengineering from the Georgia Institute of Technology and completed his postdoctoral training at the University of Washington prior to launching his independent Immunoengineering Laboratory in 2014. His research lies at the interface of molecular engineering and immunology, bringing together polymer and organic chemistry, nanotechnology, pharmaceutical science, and immunobiology to develop new technologies for immune modulation, with applications in next-generation vaccines for infectious diseases, cancer immunotherapies, and therapies for autoimmune and chronic inflammatory diseases.

Table of Contents (TOC) Graphic:



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