

## Review

## Pharmacological Modulation of the STING Pathway for Cancer Immunotherapy

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The advent of immunotherapy in recent years has shown the potential to revolutionize the treatment of cancer. Unleashing antitumor T cell responses via immune checkpoint blockade has led to remarkable responses in previously untreatable tumors. The master regulator of interferon-mediated antiviral responses – stimulator of interferon genes (STING) – has now emerged as a critical mediator of innate immune sensing of cancer, and is a promising target for local immunostimulation, promoting intratumoral inflammation, and facilitating antitumor T cell responses. Pharmacological activation of the STING pathway can lead to T cell-mediated tumor regression in preclinical tumor models, and novel STING activating small molecules are now being tested in clinical trials. Here we will introduce the STING pathway and review the current state of drug development.

**Cancer Immunotherapy**

The concept of immunity against disease was first proposed by Thucydides in the 5th century BC, while the earliest recognized attempt to intentionally induce immunity was in the 10th century in China. It is, however, only recently that the close link between cancer and the immune system has become evident, although the benefit of infecting tumors with pathogenic organisms (e.g., Coley's toxins) was reported in the 19th century.

It is comparatively very recently that immune evasion has been clearly established as a hallmark of cancer [1], and harnessing the power of the immune system in the battle against cancer has only been widely recognized as an approach with curative potential in the last few years. Indeed, the impact of cancer immunotherapy has been acknowledged by the award of the 2018 Nobel Prize in Physiology or Medicine for the discovery of **immune checkpoint** (see [Glossary](#)) blockade as a cancer therapy. This approach is based on pioneering work aimed at releasing the brakes on antitumor T cells by using monoclonal antibodies to target the cytotoxic T lymphocyte antigen 4 (CTLA-4) [2] and the programmed death 1 (PD-1) surface receptors [3], which both prevent T cells from launching all-out immune attacks aimed at tumors. Anti-CTLA-4 and anti-PD-1 therapies have yielded impressive results in clinical trials, but only a fraction of patients initially respond to these agents, and there is growing clinical evidence indicating that a significant proportion of initial responders ultimately relapse with lethal, drug-resistant disease [4–6]. Toxicity of combinations of checkpoint blockers may also limit their application, and immune-related adverse events occur in a majority of patients [7–9].

At the present time, more than 50 Phase III trials in cancer immunotherapy have been initiated, most of them based on anti-CTLA-4, anti-PD-1, and anti-PD-L1 monoclonal antibodies that have already demonstrated clinical success [10]. These have been recently reviewed in detail elsewhere [11]. The immuno-oncology therapeutic space also includes promising data using immunostimulatory peptide vaccines, chimeric antigen receptor (CAR)

**Highlights**

The host STING pathway has been identified as a crucial mechanism of innate sensing of cancer and tumor growth.

Activation of the cGAS-STING-IRF-3 cascade leads to the priming and infiltration of CD8<sup>+</sup> T cells through type I IFN production and triggers potent antitumor immunity.

The STING protein is now well characterized and ligand-bound crystal structures are available, allowing for the design of novel agonists.

*In vitro* and *in vivo* data using CDN and non-CDN STING agonists suggest a robust and sustained antitumor effect.

Accumulating *in vitro* and *in vivo* evidence shows the potential of STING agonists as promising immunotherapies for cancer patients, which will be applied in the near future.

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T cell therapy, **dendritic cell (DC)** therapies, and small molecule immune checkpoint inhibitors (reviewed in [12]). CAR T cell therapies have produced remarkable results in patients with refractory leukemias and lymphoma [13–15], but the striking antitumor effects are associated with life-threatening cytokine release syndrome and neurotoxicity [16]. DC-based tumor vaccines have been used in lymphoma and melanoma patients since the 1990s [17], and autologous DCs have been employed in prostate cancer, malignant glioma, and renal cell carcinoma (RCC) with mixed responses [18]. Small molecule inhibitors of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) [19] seem to have hit a wall, and clinical trials have failed because of lack of efficacy.

These approaches have illustrated the potential of immunotherapy for the treatment of cancer, but have also shown its limitations, with the necessity for further developments to meet the needs of more patients and cancer types. Besides serious side effects, especially with anti-CTLA-4, which can trigger inflammatory destruction of thyroid, pituitary, and adrenal glands, with the need for lifelong hormone replacement, the response rates warrant the development of novel therapies. The frequency of durable responses to anti-CTLA-4 therapy in metastatic melanoma is 15% [11], and for the initial trial of PD-1 blockade comprising melanoma, RCC, and non-small cell lung cancer (NSCLC), six out of 16 patients had objective responses [20].

One of the crucial parameters that is necessary for a favorable response to immunotherapies is pre-infiltration of the **tumor microenvironment (TME)** by **CD8<sup>+</sup> T cells**; in other words, a ‘hot’ or inflamed tumor, in which negative feedback mechanisms, notably through PD-1:PD-L1 interaction, limit the action of the immune system [21,22], which in turn would be reactivated by checkpoint blockade. This has also been established by the use of oncolytic viruses to generate the pre-existing inflammation in the tumor via the type I **interferon (IFN)** response, thus enabling improved responses to anti-PD-1 therapy [23,24]. It is promising that response rates are increased by dual treatment with anti-CTLA-4 and anti-PD-1, or by local oncolytic viruses and anti-PD-1 [23,24], illustrating the hope of extending the application of immunotherapy by employing combination approaches. The poor response to immune checkpoint blockade in KRAS-LKB1 lung cancer mutants lacking STING expression and thus T cell infiltration, highlights also the beneficial effects of STING activation in combination immunotherapy [25].

### Local Immunostimulatory Approaches to Overcome Barriers to Antitumor Immunity

One of the major areas of interest in expanding the effectiveness of immunotherapy approaches is indeed to find ways to render immunologically ‘cold’ tumors ‘hot’, and therefore overcome local immunosuppressive mechanisms, increasing the potential for approaches like immune checkpoint blockade to work effectively. These types of approach include the use of immunostimulatory monoclonal antibodies (e.g., anti-OX40, an agonistic monoclonal antibody against receptor OX40, or BB1, targeting CD74) to directly stimulate T cell activity, and oncolytic viruses [26], which as well as replicating selectively and specifically lysing tumor cells, establish an inflammatory microenvironment.

One strategy for local immunostimulation which is gaining increased interest involves activation of the STING pathway, a key sensing system that allows the **innate immune system** to respond to infections as well as tumor growth, to coordinate immune responses. Later we will summarize our most up-to-date understanding of the STING pathway, survey the development of small molecule STING agonists, review current clinical trials in this area, and discuss prospects and challenges for the future development of this approach.

### Glossary

#### **Adaptive immune response:**

principally involving B and T lymphocytes, the adaptive immune system produces memory cells following interaction with antigens, leading to long-lasting and efficient responses. The primary response drives the production of antigen-specific antibodies by B lymphocytes and generates antigen-specific T cells. The secondary response following encounter with the same antigen is faster and reactivates memory cells that were first produced.

#### **Antigen-presenting cells (APCs):**

include macrophages, dendritic cells (DCs), and B cells. Their role is to display antigens (via MHC class II binding) after pathogen engulfment and to promote immune response by recruiting T helper cells.

**CD8<sup>+</sup> T cells:** these are cytotoxic T cells responsible for eliminating pathogen infected cells and damaged cells. T cells directly bind to antigens presented by MHC class I on the cell surface.

**Dendritic cells (DCs):** potent APCs that link the innate immune system to its adaptive arms by inducing activation and differentiation of naive T cells, by expression and presentation of the antigen on MHC class II, priming immature CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

**Immune checkpoints:** key regulatory elements which normally function to limit the immune response. Hijacked by cancer cells to avoid T cell-mediated killing.

**Innate immune system:** the innate immune defense consists of external barriers (such as the skin and mucosal membranes), various white blood cells and soluble proteins, which act rapidly and nonspecifically against the entry and spread of pathogens in the body.

**Interferons:** these are secreted proteins and a subclass of cytokines produced by host cells in response to pathogens (bacteria, viruses, microorganisms, parasites) or tumor cells. Their role is to mediate crosstalk between different immune system components and activate macrophages, natural killer cells, and APCs.

**Regulatory T cells (Tregs):** these are a type of antigen-specific T

### STING, a Novel Player in the Field of Cancer Immunotherapy

The immuno-oncology field has been ‘stung’ by recent advances in our understanding of the structure and function of the adaptor protein STING (official gene ID: TMEM173) [27]. STING was discovered a decade ago, as a 28 kDa endoplasmic reticulum (ER) dimeric adaptor protein that acts as a master regulator of type I IFN (IFN $\alpha$  and IFN $\beta$ ) production by the innate immune system in response to viral or bacterial infection [27]. Critically, in addition to sensing infections, the STING pathway has now clearly been identified as a crucial mediator of innate immune sensing of cancer [28]. Activated STING ultimately promotes antitumor responses essentially by ‘heating up’ the TME via secretion of IFNs and other cytokines [29–35]. This initial innate immune sensing of tumors leads to the recruitment, activation, and expansion of CD8<sup>+</sup> T cells (Figure 1, Key Figure).

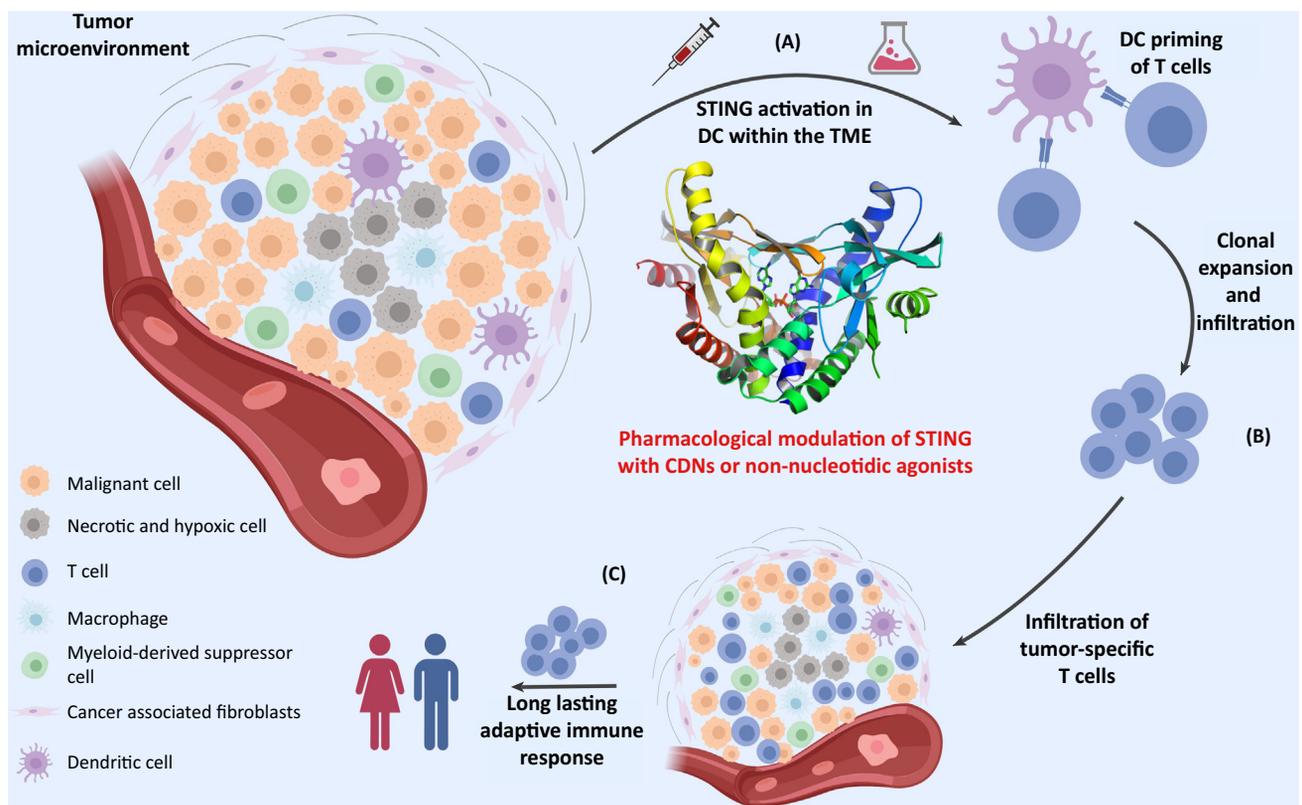
lymphocytes whose role is to suppress or downregulate induction and proliferation of effector T cells.

**Tumor infiltrating lymphocytes (TIL):** these are lymphocytes which migrate from the blood circulation into the tumor microenvironment.

**Tumor microenvironment (TME):** comprises the environment surrounding the tumor. It includes: extracellular matrix, signaling proteins, immune cells, blood vessels, and fibroblasts.

### Key Figure

STING-Mediated Priming of CD8<sup>+</sup> T Cells, Clonal Expansion, and Long Lasting Adaptive Immune Response Following the Use of Therapeutic Agonists



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**Figure 1.** (A) Stimulator of interferon genes (STING) activation by agonists triggers a type I interferon (IFN) response that leads to T cell priming by tumor-associated dendritic cells (DCs). (B) Expansion and infiltration of CD8<sup>+</sup> T cells into the tumor. (C) Long lasting adaptive tumor response by circulating tumor-specific T cells.

### The cGAS–STING Pathway

The innate immune system detects molecules from bacterial and viral pathogens using pattern recognition receptors to trigger immune activation [36,37], and it is now understood that the STING pathway plays a key role in pathogen detection by sensing bacterial dinucleotides and by the surveillance of cytosolic double-stranded DNA (dsDNA) through cyclic GMP–AMP synthase (cGAS) [38]. In addition to the innate response to pathogens, where viral DNA (either directly or through reverse transcription for retroviruses) or bacterial DNA activate the host STING cascade, DNA released by dying cells or tumor cells can trigger STING activation [39]. Also in tumors, it has been reported that mitochondrial DNA instability may lead to cytosolic mtDNA leakage and STING activation in neighboring phagocytic cells [40].

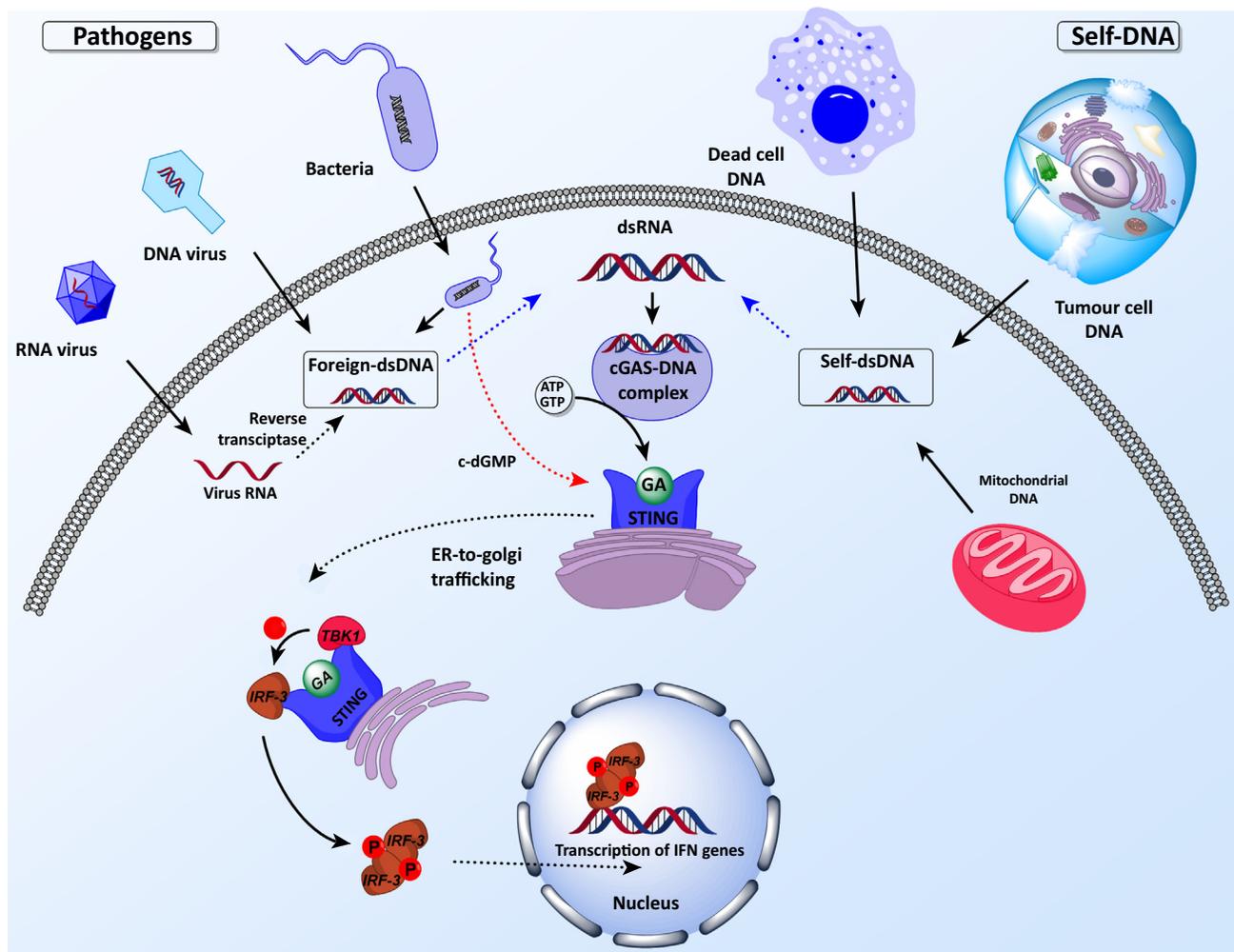
The upstream sensing of cytosolic dsDNA itself is done by the enzyme cGAS, that catalyzes the formation of the non-canonical cyclic dinucleotide (CDN) cyclic GMP–AMP {more precisely, cyclic [G(2',5')pA(3',5')p]; cGAMP}. cGAS binds dsDNA through its phosphate backbone, therefore making the binding nonsequence dependent [38,41,42]. The produced endogenous cGAMP ligand in turn binds the STING dimer [43], inducing conformational changes and the trafficking of the protein into perinuclear Golgi vesicles [44]. Palmitoylation of STING on Cys residues and further phosphorylation events take place in the Golgi. These changes are inhibited when ER-to-Golgi trafficking is abolished by brefeldin A [45]. Furthermore, treatment with the palmitoylation inhibitor 2-bromo-palmitate abolishes the type I IFN response to STING activation [45]. Besides cGAMP-mediated activation, STING can be activated directly by bacteria-derived CDNs. Thus, CDNs are both endogenous and pathogen-derived potent activators of the STING pathway, and as such, they function as ubiquitous second messengers in prokaryotic species [46] and within the immune system of eukaryotes [47].

After activation by CDN binding, STING recruits the TANK binding kinase 1 (TBK1) by interaction through its highly conserved C-terminal tail, which leads to the dimerization and phosphorylation of interferon regulatory factor 3 (IRF-3). The phosphorylated IRF-3 dimer ultimately translocates to the nucleus and activates the transcription of interferon-associated genes [48–52]. IFN- $\beta$  induction in response to dsDNA or cGAMP is completely abolished in cells expressing short hairpin (sh)RNA directed toward STING [43], indicating the critical role of this signaling mechanism. Furthermore, the Goldenticket mutant mouse strain, that comprises a single nucleotide variant, leading to a T596A mutation in the Sting protein, fails to produce detectable STING and type I IFN response to CDNs and *Listeria monocytogenes* *in vivo* [53].

Besides IRF-3, the STING cascade can also control the activation and nuclear translocation of NF- $\kappa$ B [54,55], further participating to the induction of cytokines and proteins. Its activation may be predominantly controlled by TBK1, as for IRF-3, but also involves the I $\kappa$ B kinase complex IKK $\alpha\beta$  [56,57]. The cGAS-STING-IRF-3 pathway is summarized in Figure 2.

### Structure of the STING Protein

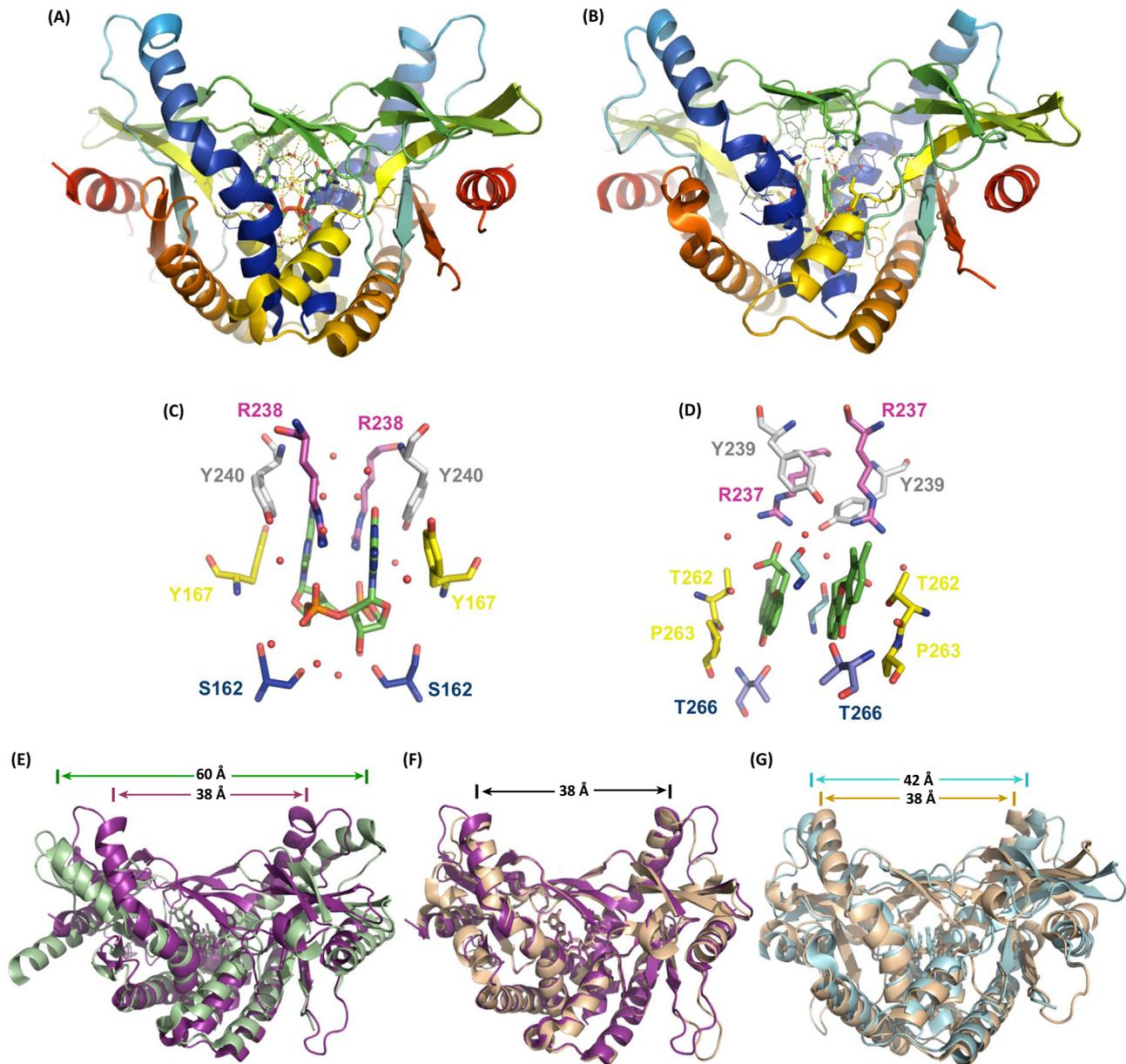
The human STING protein contains an N-terminal domain that folds into four transmembrane helices (aa 1–154) and a cytosolic C-terminal tail (aa 342–379) separated by a central globular domain (aa 155–341), which can together be enclosed within the C-terminal domain (140–379) [50]. Major allelic variants were identified as the R232H in humans (hSTING<sup>H232</sup> is the reference sequence) and the R231A variant of mouse STING (mSting<sup>A231</sup>). The H232 STING allele was the first to be characterized, although it was later found to be a minor variant, while R232 is the major one, especially in the American population [58,59]. Human and mouse STING exhibit 68% amino acid identity and 81% similarity [60]. High resolution ligand cocrystallized structures of both human and murine STING were solved in 2013, and these seminal contributions



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**Figure 2.** The Host cGAS-STING-IRF3 Pathway is Activated by the Presence of Either Self or Foreign dsDNA in the Cytosol. Double-stranded DNA (dsDNA) is first sensed by cyclic GMP–AMP synthase (cGAS), catalyzing the formation of cyclic GMP–AMP (cGAMP), which then binds the stimulator of interferon genes (STING) dimer on the endoplasmic reticulum (ER). The complex is trafficked to perinuclear vesicles, where phosphorylation and dimerization of interferon regulatory factor 3 (IRF3) by TANK binding kinase 1 (TBK1) takes place. This phosphorylated dimer translocates to the nucleus and finally induces the transcription of interferon (IFN) genes through binding to consensus sites in IFN gene promoters.

delivered important insights into the binding of CDNs within the large pocket formed at the interface of the dimeric receptor [43,50]. Analysis of the bound and free structures shows a conformational transition between an inactive ‘open’ state and an active ‘closed’ conformation upon host–guest interaction with agonists [43,50]. The conformational change, which propagates over the entire structure of the symmetric dimer, involves the formation of a four-stranded  $\beta$ -pleated sheet cap, which acts as a lid over the CDN binding pocket (Figure 3A,B) [50,61]. The CDN ligand is positioned in a U-shaped cavity with its sugar–phosphate backbone at the bottom and the purine bases aligned parallel upward and further anchored by the closing lid. The phosphate moieties bind the base of the cavity through the S162 sidechain hydroxyls, with additional direct R238 contacts and water-mediated hydrogen bonds to Y240 and T267 [50]. On the sides of the cleft, the Tyr residues form brackets that enclose the purine rings through  $\pi$ -stacking while the sidechain from R238 engages in hydrogen bonds with the N7



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**Figure 3. Structural Insights into the STING Dimer and the Binding of Agonists.** (A) X-ray structure of the hSTING<sup>H232</sup> dimer cocrystallized with the c[G(2',5')pA(3',5')p] dinucleotide (PDB 4LOH) and (B) DMXAA (5,6-dimethylxanthenone-4-acetic acid) in complex with the mouse dimeric mSting<sup>R231</sup> (PDB 4LOL) [50]. (C) Close-up showing details of important interactions for the binding of c[G(2',5')pA(3',5')p] to hSTING<sup>H232</sup> and (D) two DMXAA molecules to mSting<sup>R231</sup>. (E) Aligned structures of the hSTING<sup>H232</sup>/cGAMP (magenta) and hSTING<sup>H232</sup>/c[di-GMP] complexes showing the inability of the bacterial c[di-GMP] to induce a full conformational 'open-to-closed' transition. (F) Closed conformations of mSting<sup>R231</sup> in complex with cyclic GMP-AMP (cGAMP) (magenta) and DMXAA (beige), emphasizing the similar conformational transition between the endogenous ligand and the synthetic analog. (G) Superposition of cGAMP in complex with mSting<sup>R231</sup> and hSTING<sup>H232</sup>, highlighting the close similarity between the two structures.

position of the nucleobases and makes further contacts with the phosphates (Figure 3C,D). G230 takes part at the edges of the overhead cap and thus participates in the closing of the complex. It is important to note that binding of the bacterial c[di-GMP] and the endogenous 2',3'-cGAMP do not equally induce the conformational change and the active closed state. Indeed, c[di-GMP] binds the STING dimer, but it results in a V-shaped complex that keeps a rather open conformation with disordered R238 sidechains and 60 Å spacing between the edges of the  $\alpha 2$  helices, in comparison with the more compact U-shaped complex where these helices come closer together (38 Å, Figure 3E–G). These differences may well explain why the bacterial c[di-GMP] binds with a relatively high  $K_d$ , but poorly induces IFN- $\beta$  production [43].

The same group investigated the binding of DMXAA (5,6-dimethylxanthenone-4-acetic acid) to mSting<sup>A231</sup> and the reason for its selectivity toward the mouse variant [62,63], for which it triggers a similar 'open-to-closed' transition [61]. The authors identified a few key residues, Q266I and S162A in the binding cavity and G230I in the lid region that, upon mutation, could confer hSTING sensitivity toward DMXAA. If two of these mutations gave a similar sensitivity of hSTING to DMXAA as the mouse variant, the triple mutant would render hSTING even more sensitive to the mouse-selective compound [61], as evidenced by IFN- $\beta$  induction. These critical features of the binding of the natural agonist to STING shed light on the putative design of novel small molecules for cancer immunotherapy through STING pathway activation.

### STING Expression in Cancer

STING is found in a variety of tissues, including the lung, ovary, heart, spleen, thymus, placenta, and smooth muscle, but is poorly expressed in the brain, skeletal muscle, colon, small intestine, liver, and kidneys [27]. Within the immune system, STING is present in various **antigen-presenting cells (APCs)**, such as DCs and macrophages as well as in T cells [44]. Perhaps reflecting its varied expression in different tissue types, its expression in cancer has been revealed to be either upregulated or downregulated, depending on cancer type and stage, which suggests that the success of STING therapies may be tumor dependent [64]. In four breast cancer cell lines (MCF-7, T47-D, HBL100, and MDA-MB-23) STING expression was also found to be lower in malignant cells than in the non-tumorigenic cell line MCF-10A [65], and a similar observation was reported in several cancerous melanoma cell lines [66]. Defective or low STING signaling activity, as described in a variety of human colorectal adenocarcinoma lines generated from cancers diagnosed at various stages, was related to more advanced Dukes' tumor stage (an early colorectal cancer classification) [67]. In patients, downregulation of STING was observed in human hepatic carcinoma and was associated with advanced tumor-node-metastasis (TNM) stage and poor survival [68]. In gastric cancer patients, low expression of STING was associated with tumor progression and lower overall survival [69]. As mentioned earlier, robust STING silencing has been uncovered in KRAS-driven lung cancer, following the loss of the LKB1 tumor suppressor gene [25]. Although current data tend to show a downregulation of STING expression in cancer, a significant increase of STING expression was observed in tongue squamous cell carcinoma patients, in comparison with normal epithelial tissue [70].

### STING in Cancer Immunotherapy

Spontaneous T cell responses (i.e., infiltration of the tumor by effector T cells) are of great prognostic value for cancer patients. Tumor composition and **tumor infiltrating lymphocytes (TIL)** are indeed better indicators for cancer progression and relapse than the classical TNM staging, and T cell responses are correlated with favorable prognosis in diverse malignancies and predict positive clinical outcome [71–74]. Such infiltration of tumors by immune cells has also been shown to be a

prerequisite for optimal response to immune checkpoint blockade [21,75–77]. Type I IFN signaling is critical in this process, and there is now clear evidence that this IFN response, controlling spontaneous regulation of tumor growth, is regulated by activation of the host STING pathway [78]. T cell priming depends on host type I IFN production, via crosspresentation by CD8 $\alpha$ <sup>+</sup> DCs [28,79], and the critical role of the STING pathway in this process has now been clearly demonstrated both *in vitro* and *in vivo* [78]. Tumor-derived DNA has been observed to be transferred to host APCs; *in vitro*, DNA was the sole compound to trigger an IFN- $\beta$  response, and this response was mediated by cGAS, STING, and IRF-3 [78]. The transfer of tumor-derived DNA to host APCs, identified as CD45<sup>+</sup>/CD11c<sup>+</sup> DCs, activates the STING pathway [78], leading to subsequent type I IFN production. This is predominately induced by CD11c<sup>+</sup> DCs within the TME, which in turn promotes intratumoral accumulation of CD8 $\alpha$ <sup>+</sup> DCs and finally leads to the activation of CD8<sup>+</sup> T cells [79]. Murine data shows that CD8<sup>+</sup> priming is blunted in STING<sup>-/-</sup> and IRF3<sup>-/-</sup> animals, which are no longer capable of rejecting methylcholanthrene-induced sarcoma, as well as B16.SIY tumors [78]. Therefore, there is no doubt that the host STING pathway is a critical element involved in the immune sensing of cancer and the immune control of tumor growth.

Clinical responses to immunotherapeutic strategies using anti-CTLA-4 and/or anti-PD-1 correlate with preexisting CD8<sup>+</sup> infiltration [80,81], and these infiltrates can upregulate IDO, PD-L1, and **regulatory T cells (Tregs)** as a negative feedback mechanism [81]. STING activation after tumor-derived DNA recognition (or treatment with a STING agonist) leads to the production of CXCL9 and CXCL10, which are key cytokines responsible for T cell recruitment, but these recruited CD8<sup>+</sup> T cells in turn can upregulate different immune inhibitory pathways (PD-L1, IDO, FoxP3), leading to the failure of spontaneous tumor elimination [82]. These findings explicitly warrant the combination of STING agonists with current CTLA-4 and PD-1 checkpoint blockade, because the promotion of inflammation by STING activation would overcome suppressive mechanisms, while checkpoint blockade would release the brakes on antitumor T cells. Indeed, immune checkpoint blockade loses its efficacy in STING deficient mice [78].

#### Pharmacological Modulation of STING – Nucleotidic Agonists

CDNs have been recognized as mediators of cell signaling for decades. The canonical 3',5'-bridged c[di-GMP] (see Table 1) was first discovered as an important second messenger in *Acetobacter xylinum* in the late 1980s [83], where it plays key roles in bacterial intracellular signaling, including the regulation of biofilm formation and motility [84]. C[di-AMP] has also been found in prokaryotic cells [85] and actively participates in a broad spectrum of cellular processes [47]. The potential anticancer activity of these naturally derived dinucleotides was first tested in 2005 with c[di-GMP], which inhibited basal proliferation of human colon cancer cells *in vitro* [86]. It was then discovered that CDNs were potent immunostimulatory compounds that induce type I IFN responses in bone marrow macrophages [87,88] via the direct activation of STING [89]. Intravenous injection of a CDN/liposome delivery system [YSK05-Lip/c(di-GMP)] induced a striking decrease of metastatic lesions in the B16F10 mouse melanoma model with almost 40% of mice showing full protection against tumor rechallenge, suggesting the induction of a memory **adaptive immune response** [90]. Biopolymer implants were also used to co-deliver c[di-GMP] with CAR T cells, resulting in potent tumor regression and a fivefold increase in survival in mice bearing pancreatic tumors [91]. Recently, it was reported that the combination of cytotoxic cationic silica nanoparticles and c[di-GMP] showed marked tumor regression and prolonged survival after a single intratumoral injection in the B16 melanoma mouse model [92]. C[di-GMP] led to a drastic reduction of metastases and tumor size in the metastatic breast cancer 4T1 model in mice immunized with an attenuated *Listeria monocytogenes*-based vaccine [93].

Table 1. A Summary of STING Agonists for Cancer Immunotherapy

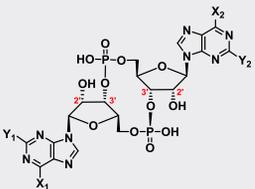
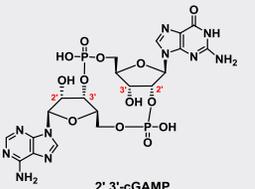
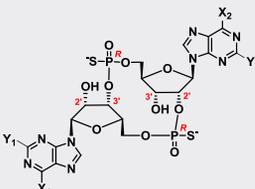
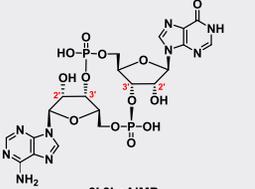
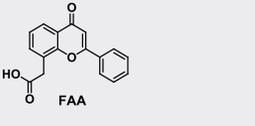
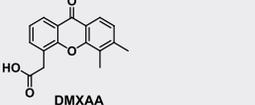
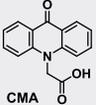
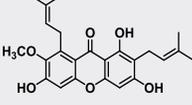
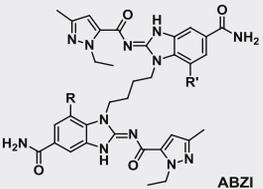
Natural CDN agonists	
 <p>c-di-GMP : X<sub>1,2</sub> = O; Y<sub>1,2</sub> = NH<sub>2</sub>  c-di-AMP : X<sub>1,2</sub> = NH<sub>2</sub>; Y<sub>1,2</sub> = H  3',3'-cGAMP: X<sub>1</sub>, Y<sub>2</sub> = NH<sub>2</sub>; Y<sub>1</sub> = H; X<sub>2</sub> = O</p>	<p>Prokaryotic CDNs</p> <p>c(di)-GMP</p> <ul style="list-style-type: none"> <li>• Antitumor <i>in vitro</i> activity against H508 cells at 50 μM [86]</li> <li>• Used in cancer vaccines as adjuvant against 4T1 and B16 mouse models [34,93]</li> </ul> <p>3',3'-cGAMP</p> <ul style="list-style-type: none"> <li>• Higher binding affinity to mSting compared with hSTING [50]</li> </ul>
 <p>2',3'-cGAMP</p>	<p>Eukaryotic CDNs</p> <p>2',3'-cGAMP</p> <ul style="list-style-type: none"> <li>• Natural hSTING ligand with a higher affinity for hSTING than its linkage isomers [50]</li> <li>• Reduced tumor growth and size, increased survival in numerous <i>in vivo</i> models or in association with radiation in MC38 [120]</li> </ul>
Synthetic CDN agonists	
 <p>ML-RR-S2-cDG : X<sub>1,2</sub> = O; Y<sub>1,2</sub> = NH<sub>2</sub>  ML-RR-S2-cDA : X<sub>1,2</sub> = NH<sub>2</sub>; Y<sub>1,2</sub> = H  ML-RR-S2-cGAMP: X<sub>1</sub>, Y<sub>2</sub> = NH<sub>2</sub>; Y<sub>1</sub> = H; X<sub>2</sub> = O</p>	<p>ML-RR-S2-cGAMP</p> <ul style="list-style-type: none"> <li>• Resistant to ENPP1 hydrolysis [97]</li> <li>• Higher affinity for hSTING than natural CDNs [35]</li> <li>• Activates all five allelic variants of hSTING [35]</li> </ul> <p>ML-RR-S2-cDA (ADU-S100)</p> <ul style="list-style-type: none"> <li>• Higher affinity for hSTING than natural CDNs [35]</li> <li>• Activates all five hSTING alleles [35]</li> <li>• Potent antitumor activity associated with tumor regression in B16F10, 4T1, and CT26 mouse models. Induction of 'long lasting immune protection' [35]</li> <li>• Intratumoral injection inhibits growth of distant metastatic lesions in B16F10 mice [35]</li> </ul> <p>ML-RR-S2-cDG</p> <ul style="list-style-type: none"> <li>• Similar antitumor potency than ML-RR-S2-cDA in B16F10 models but associated with side effects (open wounds) and decreased overall survival [35]</li> </ul>
 <p>3',3'-cAIMP</p>	<ul style="list-style-type: none"> <li>• Active through type I IFN induction against HSV2 both <i>in vitro</i> in human cells and <i>in vivo</i> in mice [101]</li> </ul>
Non-CDN agonists	
 <p>FAA</p>	<p>Flavone 8-acetic acid (FAA)</p> <ul style="list-style-type: none"> <li>• Potent antitumor activity associated with tumor regression in various mouse models but dropped in Phase I trials [124]</li> </ul>
 <p>DMXAA</p>	<p>DMXAA</p> <ul style="list-style-type: none"> <li>• First discovered as a vascular disrupting agent through TNF-α induction. Related to FAA [105–109]</li> <li>• Potent antitumor activity associated with tumor regression on various <i>in vivo</i> models [35,112–114] in a STING-dependent fashion [30]</li> <li>• mSting agonist with no affinity for hSTING both <i>in vitro</i> [62,63] and <i>in vivo</i> [35]</li> </ul>

Table 1. (continued)

 <p>CMA</p>	<ul style="list-style-type: none"> <li>Failed in Phase III clinical trial in combination with chemotherapy in NSCLC patients due to lack of efficacy [110]</li> </ul>
 <p><math>\alpha</math>-mangostin</p>	<p>CMA</p> <ul style="list-style-type: none"> <li>No activation of hSTING [116]</li> <li>Antiviral activity in murine models [116]</li> </ul>
Amidobenzimidazoles	
 <p>ABZI</p>	<p><math>\alpha</math>-Mangostin</p> <ul style="list-style-type: none"> <li>Natural product with antitumor and antiviral properties [125]</li> <li>Better type I IFN inducer for hSTING than mSting [117]</li> </ul> <ul style="list-style-type: none"> <li>Submicromolar STING activation and IFN induction; systemic <i>in vivo</i> efficacy against CT26 tumors [119]</li> <li>Does not provoke the 'open-to-closed' conformational transition [119]</li> </ul>

2',3'-cGAMP (the endogenous product of cGAS) demonstrated potent activity after intratumoral injection in the CT26 murine colon adenocarcinoma model, reducing tumor size and increasing survival [94]. Delayed and reduced tumor growth was also observed after intratumoral injection of cGAMP in 4T1-luc (mouse breast cancer), B16F10, mSCC1 (murine squamous cell carcinoma), and CT26 tumors [95]. Another study reported similar observations after injection of 2',3'-cGAMP in mice bearing subcutaneous B16F10 tumors with concomitant reduction of lung metastases [32]. cGAMP-loaded nanoparticles have been disclosed to enhance STING activation in the tumor and sentinel lymph nodes, both after intratumorally and systemically administration, and showed synergistic effects with immune checkpoint blockade. Treatment with the nanoparticles triggered a shift to a 'hot', T cell infiltrated TME, and a third of mice bearing well-established and relatively large B16.F10 subcutaneous tumors completely rejected the intratumorally treated tumors. Moreover, these cured animals rejected contralateral flank tumors in rechallenge experiments, indicating the establishment of memory antitumor immunity [96].

However, limitations to the use of CDNs in anticancer therapy lie in their chemical features: CDNs are prone to enzymatic hydrolysis by phosphodiesterases in host cells or in the bloodstream and their anionic and polar profile severely hampers membrane diffusion and cellular uptake. Synthetic CDNs with more favorable properties are thus needed and newly designed compounds are now entering trials. To increase enzymatic stability, sulfur has been used to replace the nonbridging oxygen from the phosphodiester linkages to make phosphorothioates. The resulting compound, 2',3'-cG<sup>S</sup>A<sup>S</sup>MP, is more resistant to degradation by the ecto-nucleotide pyrophosphatase ENPP1 (identified as the major 2',3'-cGAMP hydrolase), prolonging its systemic half-life while maintaining high affinity for hSTING [97]. Another 'dithio' CDN analog made of two AMP moieties cyclized via 2',5'- and 3',5'-phosphodiester bonds (known as

ML-RR-S2-CDA, MIW815, or ADU-S100), shows improved IFN- $\beta$  responses and tumor regression in established B16 tumors when compared with 2',3'-cGAMP [35]. Antitumor efficacy was also observed after intratumoral injection of ML-RR-S2-CDA in 4T1 and CT26 mouse models with significant and durable tumor regression [35]. In addition, this CDN analog induced lasting immune-mediated tumor rejection in long-term survivors when rechallenged with the same tumor cell line [35]. These striking preclinical results provided support for the use of ML-RR-S2-CDA in clinical trials for patients with advanced metastatic solid tumors or lymphomas (NCT03172936, NCT02675439), and the first results are expected in 2020. It has just been reported that the dose of the compound affects both local clearing of 4T1 mammary flank tumors and, following a bell-shaped curve, tumor-specific T cell activation and durable antitumor immunity [98]. To improve the intratumoral delivery of ML-RR-S2-CDA, an injectable peptide hydrogel (STINGel) was developed and tested against MOC2-E6E7 tumors (a murine orthotopic head and neck squamous cell carcinoma model). This demonstrated potent efficacy, with a significant decrease in tumor growth or complete tumor regression and prolonged survival [99]. Merck is currently investigating a CDN compound in solid tumors and lymphoma (NCT03010176), although the structure of this molecule has not yet been disclosed. Other novel synthetic c[AMP-CMP] CDNs have been investigated for their induction of type I IFN, but failed in comparison with natural CDNs [100]. Mixed adenosine and inosine dinucleotides have also been disclosed and 3',3'-cAIMP was found to have promising antiviral activity against herpes simplex virus, but has not been tested in cancer [101,102].

Despite these very encouraging preclinical results with CDN STING agonists, demonstrating *in vitro* and *in vivo* efficacy with striking tumor regressions and long lasting systemic immune responses, their nucleotidic and anionic nature warrant the development of molecules with improved drug-like qualities, with simple chemical synthesis and more favorable pharmacokinetic profiles.

#### Pharmacological Modulation of STING – Non-CDN Agonists

Additional non-CDN STING agonists are also under investigation. The first of these to be studied was the flavone-8-acetic acid derivative DMXAA [103], which was first reported for its *in vivo* antitumor activity in CT38 cells in mice [104]. Also known as Vadimezan or ASA404, it was initially considered as a vascular disrupting agent and a TNF- $\alpha$  inducer [105–109]. Encouraging preclinical data brought the molecule to the clinic, however, DMXAA failed to deliver any significant patient benefit in Phase III trials in combination with carboplatin and paclitaxel for the treatment of non-small cell lung cancer [110]. Its failure is explained by the fact that it specifically activates the STING-IRF-3 pathway [111] as a competitive and selective mSting agonist, but has poor affinity for hSTING [62,63]. In animals, DMXAA has shown growth inhibitory effects on the gastroenteropancreatic BON model [112], the murine glioma GL261 model, for which it largely prolonged survival [113], the murine acute myeloid leukemia C1498.SIY (with a STING-dependent increase in survival) [30], and the murine lung cancer model 344SQ-ELuc [114]. Intratumoral injection of DMXAA in mice bearing B16 melanoma tumors induces potent tumor regression and a striking total rejection in most of the treated mice, while STING knockout mice were unresponsive to the compound [35]. These results strongly suggest that potent antitumor activity can be achieved through pharmacological activation of the host STING pathway using small molecule agonists, and constitutes a proof of concept for the design of novel non-nucleotidic analogs for the human STING protein. In this regard, C7-functionalized DMXAA derivatives for targeting the human protein were recently designed from *in silico* prediction based on the structural findings of Gao *et al.* [61], but without significant success [115]. A similar scaffold derivative, 10-carboxymethyl-9-acridanone (CMA), was also identified as a specific mSting agonist that is inactive toward human cells [116].  $\alpha$ -Mangostin, a natural molecule structurally related to DMXAA, was able

to better activate hSTING than mSting [117]. A rather unexpected identification of a type I IFN inducing effect of ganciclovir has revealed that it can probably activate STING, especially when in dimeric form by the use of a polyethyleneglycol linker [118].

A very recent study from GlaxoSmithKline disclosed potent amidobenzimidazole (ABZI) agonists with *in vivo* efficacy following systemic administration in mice [119]. A linking strategy between two ABZI units using a short alkyl chain allowed for strong STING binding with an EC<sub>50</sub> for IFN- $\beta$  induction in the micromolar to submicromolar range. The crystal structure of the complex reveals that ABZI agonists unexpectedly activate STING function while maintaining its open confirmation. The lead derivative was tested against subcutaneous CT26 tumors by intravenous injection and elicited significant tumor growth inhibition and improved survival, with 80% of the treated group remaining tumor free at the end of the study. This effect was reversed by depletion of CD8<sup>+</sup> T cells. To the best of our knowledge, this is the first non-CDN molecule showing high hSTING selectivity and *in vivo* efficacy. This discovery illustrates the opportunity for further drug development based on this novel mechanism.

#### Pharmacological Modulation of STING – Combination Approaches to Increase Efficacy

STING agonists appear to be excellent candidates for combination therapies with other immunotherapeutic or chemotherapeutic drugs. For example, the combination of cGAMP and 5-fluorouracil (5-FU) in the CT26 mouse model showed enhanced antitumor activity and reduced 5-FU toxicity [94]. Intratumoral injection of 2',3'-cGAMP combined with radiotherapy considerably reduced tumor size in mice with MC38 tumors compared with radiation or 2',3'-cGAMP alone, and complete tumor rejection was observed in about 70% of the combination group; the response being logically potentiated as radiation-induced antitumor immunity also relies on the cGAS–STING pathway [120]. Similar observations were made when combining radiotherapy with RR-S2-CDG in murine pancreatic adenocarcinoma, producing systemic immune responses and a significant survival increase [121].

In addition to combination with standard therapies, STING agonists may enable reinitiation of immune responses in non-immunogenic tumors. The STINGVAX vaccine, which comprises the dithio-CDN RR-S2-CDA and GM-CSF, enhanced antitumor efficacy in subcutaneous B16 and TRAMP (murine prostate adenocarcinoma) models in comparison to the parent c[di-AMP], but with marked PD-L1 upregulation. No response was observed to PD-1 blockade alone in established B16 and CT26 tumor models, but the STINGVAX and anti-PD-1 combination induced tumor regression and even cured all treated CT26 tumor-bearing mice [33]. Recently, RR-S2-CDA was tested against the murine ovarian carcinoma ID8 model in combination with carboplatin and anti-PD-1, and showed improved survival compared with a combination of a STING agonist and carboplatin only [122]. The CDN agonists ML-RR-S2-CDA and RR-CDG were recently formulated into cationic nanoparticles to increase cytosolic accumulation in THP1-Blue human monocytes. When these were coadministered with anti-PD-1, increased survival and reduced tumor growth in B16 melanoma mice was observed in comparison with the PD-1 antibody or CDNs alone [123]. These results indicate that by triggering inflammation in the TME, STING agonists could overcome the inability of 'cold' tumors to respond to immune checkpoint blockade [24,25]. This explicitly encourages the combination of anti-PD-1 with STING agonists.

#### Concluding Remarks

The burgeoning interest in restoring immunity within the TME, using the patient's own immune cells as weapons against cancer, is obviously extremely appealing and the recent clinical achievements have garnered much attention from the scientific community. Success from

#### Outstanding Questions

Is the reactivation of the deficient innate sensing of a tumor, a way to render non-immunogenic tumors visible to the immune system?

Will STING agonists, alone or in combination, induce long lasting immune defense against secondary tumors?

What are the possible side effects of STING agonist therapy? Would STING activation induce a prolonged proinflammatory state, leading to specific undesired side effects?

Is systemic delivery of STING agonists going to match the promising intratumoral efficacy?

Will STING agonists expand the fraction of responding patients when used in combination with checkpoint blockade?

checkpoint blockade therapies and the recent Nobel award highlight the significance and the hopes that immunotherapy has recently brought to the oncology field. The search for continuous improvement and the goal of seeing responses in a vast majority of patients has brought our attention to a novel player in the field, STING. Since its discovery as an important sensor of pathogens in innate immunity, it has gained its place as the central pathway in the immune sensing and control of tumor growth through activation by tumor-derived DNA leading to T cell priming and infiltration. This is a very central point: the presence of CD8<sup>+</sup> T cells in the TME has been shown not only to be of tremendous importance for the outcome of the disease, but also for the success of current cancer immunotherapies by checkpoint blockade. The goal of STING activation is therefore twofold: (i) restoring and/or triggering the immune response within the tumor, and (ii) potentiate other immunotherapeutic modalities through a restored infiltration of activated T cells.

In recent years, intense research has shed light on STING and its pathway. Upstream, cGAS and its product 2',3'-cGAMP, which is the endogenous ligand for hSTING following sensing of tumor-derived DNA, have been well characterized, both from structural and functional perspectives. Downstream, the type I IFN response and its involvement in the activation of the immune system toward tumor growth is also now well established as a critical mechanism. The STING adaptor protein itself and the cascade of events following agonist binding is now well understood, and high-resolution cocrystallized structures are available, paving the way for the design of small molecule agonists by medicinal chemists, hopefully feeding clinical trials with new drug candidates with better pharmacokinetics and potency in the near future. Recent data also encourage the use of STING agonists for expanding the range of patients that respond to checkpoint blockade, and hopefully could make non-immunogenic tumors recognized by the immune system (see Outstanding Questions); as such, everything is now in place to bring to clinical trials, new immunotherapeutic drugs that could be used in combination with anti-CTLA-4, anti-PD-1, or conventional chemotherapies if necessary. This will bring hope for patients who do not respond to current immunotherapies and may also provide opportunities to improve outcomes in aggressive and incurable cancer types, such as glioblastoma and pancreatic cancer.

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