1. Introduction and Review of literature

1.1 Breast cancer: Leading cause of death Worldwide

Almost all developing and underdeveloped countries are majorly affected by different types of cancers yearly. Breast cancer second most diagnosed cancer after lung cancer in women. There have been increasing incidences of breast cancer worldwide, even though there have been notable advancements in treatments and diagnosis. Data show an increasing trend of 0.5% every year starting from 2010 to 2019. Majorly diagnosed breast cancer was hormone receptor-positive and localized [1]. It is estimated that19.3 million new cancer cases (18.1 million excluding non-melanoma skin cancers) and death of 10.0 million cancers (9.9 million excluding non-melanoma skin cancers) occurred in 2020. In females, breast cancer is the most diagnosed cancer with 2.3 million (11.7%) new cases, followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancer. In breast cancer increasing the ratio of relapse due to drug resistance and metastasis in various organs with primary tumors leads to more than 90 % mortality in cancer [2]. There remains a challenge for the advancing research and development in diagnosis and treatment options. However, the different physiological conditions, origins, metastasis, and re-occurrences of breast cancer in female is still not understood.

1.2 Breast cancer types and treatment

Breast cancer is the most diagnosed cancer in women. Based on the tissue involved in breast cancer is divided into two parts ductal and lobular. Progression and migration of cancer cells to near-about tissue are considered an *invasive* type of cancer and if localized called *in situ*. Based on the cellular characterization of particular cells involved in cancer development, It is further divided into three types of breast cancer luminal type, Baso-luminal, and basal type of breast cancer [3]. Based on the receptor expression breast cancer is divided int to three-part Estrogen/Progesterone receptor expression, HER2 expressing and TNBC (Triple-negative breast cancer. About 60-75% of breast cancer expressing ER (estrogen receptor), which is the driver of cancer growth. Particularly targeted therapy as a Selective estrogen receptor modulator and selective receptor degrader and aromatase inhibitor, further sub-type predominant expressing HER2 and EGFR this type of breast cancer diagnosed with 20-15%. Blockers as antibodies are majorly used as anti-EGFR and HER2.last type of breast cancer occurrence wise less than 10-15% but not expressing any receptor so it is known as a Triple negative type of breast cancer. Due to

the unavailability of targeted therapy still, this cancer depends on surgery, radiation, and chemotherapy.

1.3 Tumor microenvironment and modulation of immune response for anti-cancer therapy

The tumor microenvironment (TME) of solid tumors plays a critical role via inducing angiogenesis for nutrient supply, overcoming the hypoxic condition, and generating an immune suppressive environment, that supports tumor cells to evade from the anti-tumor immune response [4]. TME consists of a heterogeneous cell population other than tumor cells, like fibroblast, stromal cells, and immune cells. Interestingly TME prevents immune cell-mediated clearance of tumor cells by several possible mechanisms like down-regulation of MHC(Major histocompatibility) on the cell surface and poor antigen presentation to T cells [5]. Tumor cells and other cell types in the TME generate inhibitory cytokines that suppress T cell activity [6]. Cells other than T cells, like NK cells also play a vital role in the recognition and killing of tumor cells. NK cell directly interacts with tumor cells and exocytosis of cytotoxic granules that contain perforin (a membrane-disturbing protein) and granzyme (proteolytic enzymes) and kill the tumor cells. Activated NK cells also produced IFN-γ and TNF-α and chemokines like CCL3,4 and 5 (Chemokine C-C motif ligand) that further shapes innate immunity [7].

The classical method of therapy for cancer patients like surgery, radiation, and chemotherapy are prevalent, however, a significant number of tumors of different origins still do not respond to react to these treatments [8]. Hence there has been an attempt for alternative and novel treatments like cancer immunotherapy and cell-based therapies [9]. Interestingly the immune checkpoint inhibitor (ICI), anti-cytotoxic T lymphocyte antigen 4 (CTLA-4), Anti PD/PD-L1blocker, and CAR T cell (Chimeric antigen receptor T cells) emerged as newer therapeutic modalities in the tumor microenvironment[10][11].

Immune checkpoint inhibitors play a critical role in cellular immunity against cancer. Generally, the immune checkpoint (IC) protects normal tissue during the pathogenic infection and keeps the balance between normal and infected cells via co-stimulation and inhibitory signals. CD28, CD137, and ICOS (Inducible T-cell co-stimulator)fall into co-stimulatory protein, and CTLA-4, PD-1, and VISTA (V-domain Ig suppressor of T cell activation)are considered as inhibitory of T cells antigen recognition functions [12]. Cancer cells cleverly modified these checkpoint proteins to escape from the cellular

immune response. Cancer cells and TME produces cytokines that upregulate PD-L1 like IL-6, IL-4, IL-10, and IL-1β. Over expression of immune check point protein helps tumor cells escape from cellular immune response via blocking tumor cell and T cell interaction activation [13]. Insufficient infiltration of T cells and NK cells makes TME cold and helps escape of the tumor cells [11]Lack of T cell infiltration into the TME is considered as cold tumor and these tumors do not respond to immune checkpoint inhibitors (ICI) therapy. Immunotherapy becomes more efficient as type-1interferon in TME enhances immune cells infiltration of the tumor and the 'cold tumor' is converted into a 'hot tumor' [14]. Infiltration of T cell and NK cell majority is driven by interferon [15]. Interestingly interferon-alpha therapy has been approved by FDA for hairy cell leukaemia, and later for chronic myelogenous leukaemia (CML), follicular non-Hodgkin lymphoma (NHL), melanoma, and AIDS-related Kaposi's sarcoma in 1986 [16]. Interferon alpha sensitizes the treatment of chemotherapeutic agent as 5 fluoro uracil in hepatocellular cancer [17].

Interferon plays a critical role in the anti-tumor activity as interferon α and β upregulate the MHC I, II and enhance antigen presentation of tumor cells, and enhance the potential of anti-tumor response [18]. Type-1 interferon response includes interferon α, β and TNF-α, which induce infiltration and activation of immune cells [19]. Type-1 interferon activates the infiltration of immune cells to the tumor site and helps in the antigen cross-priming to tumor-associated dendritic cells, Polarization of macrophage, upregulation of NK receptors, and inhibits the MDSCs (Myeloid-derived suppressor cells)[19][15].

Viral infection and activation of innate immune response show similarity to the activation of similar pathways during tumorigenesis, while this response is generally suppressed or it is modulated for the tumor growth by suppression of immunity [18]. Generally, during viral infections, their genomic content is recognized by various pattern recognition receptors (PRRs) receptors that activate chemokines as type-1 interferon response and cytokines as the mediator immune cell-mediated innate immune response [20][21]. Different stimuli activating immune response can be broadly classified into Pathogen-associated Molecular Patterns (PAMPs) and Damage-associated Molecular Patterns (DAMPs) [22]. The immunogenic molecules may be released by stressed tumor cells in the TME which may be recognized by different sensors like Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs) in the extracellular milieu, and intracellular

DAMPs are recognized by RIG-1(Retinoic acid-inducible gene I), IFI16 (Interferon-inducible protein), AIM2 (absent in melanoma 2) NOD-like receptors (NLRs) [23][24]. The release of DNA in the cytoplasm under stress conditions can be sensed by PRR, which is part of molecular sensing and immune response. However, all cytoplasmic DNA sensors mediated downstream pathways are not universally active in all cell types [25]. Majorly, cytoplasmic DNA is sensed via cGAS (cyclic GMP AMP synthase) and catalytic activity of cGAS generates 2'3'cGAMP that further activates STING and downstream proteins as IRF-3 and NF-κB. Activation of the cGAS-STING pathway leads to downstream activation of type-1 interferon and inflammatory cytokines [26]. The chronic activation of this pathway in TME had been observed in highly proliferating tumor cells however, these pathways had been intense focus of research during viral infections. However, its implication in tumor progression and chronic inflammatory conditions had been observed recently.

1.4 Activation of cGAS-STING pathway:

Cytosolic DNA can be recognized as DAMP and activates a Type-1 immune response [27]. Infection of DNA viruses, retroviruses, intracellular bacterial infections, damaged cells, exosomes, damaged mitochondria, chromosomal instability, and DNA-damaging drugs may release the DNA in the cytoplasm and stimulate the cGAS-STING. Under normal conditions, cellular DNA is confided in the nucleus and mitochondria [28]. The increased level of DNA during viral infection or intracellular bacterial DNA in stress conditions in the cytoplasm is detected by cGAS (cyclic GMP-AMP synthase) [29], which activates the further downstream inflammatory and type-1 interferon pathway.

cGAS is a cytoplasmic enzyme of 59 kDa molecular weight. cGAS protein has disarranged N-terminal and the extreme of the N-terminal and C-terminal pack against each other in a specific helix bundle arranged to finally form the CD-NTase cage-like structure creating a dipped pocket for catalyze nucleotide signal formation [30]. The N-terminal lobe in the catalytic domain includes a highly twisted β sheet and two helices, all catalytic residues are located in this domain. The catalytic activity is located at the C-terminal of cGAS, which is known as nucleotidyl transferase (NTase) [31]. The helical bundle at the C-terminal contains a conserved zinc-ion-binding module that facilitates DNA binding and cGAS dimerization. The DNA binding site is perfectly opposite to that of the catalytic site on cGAS. Two clefts at the C-terminus of cGAS form a cleft (Pocket

that binds to the substrate cGAS binds both ATP and GTP and catalyzes the formation of 2′3′-cGAMP in two steps. The first step is the generation of a linear dinucleotide 5′-pppG (2′-5′)pA using ATP as the donor and the 2′-OH on GTP as the acceptor. This generates an intermediate product and then flips over in the catalytic pocket, placing the GTP moiety at the donor position and the AMP moiety at the acceptor position for the formation of the second, 3′-5′ phosphodiester bond [30]. Two DNA-binding sites in cGAS lead to the formation of a 2:2 cGAS-DNA complex. 2:2 cGAS DNA complex further forms oligomerization and activates potent IFN (Interferon) response.

2'3'cGAMP is a natural ligand of the dinucleotide sensor STING (Stimulator of interferon gene) protein [32]. STING is a butterfly-like protein residing at the endoplasmic reticulum. (STING; also known as TMEM173, MITA, ERIS, and MPYS). STING, a 40 kDa protein, is having four transmembrane domains and is localized at the endoplasmic reticulum membrane [33]. The C-terminal tail (CTT) of STING interacts with the TBK (Tank binding kinase). The ligand bind domain always remains in a dimer form that generates a pocket for binding of 2'3'cGAMP and related molecules [34]. The ligand binding domain is generated by four α helices with five β helices. Helices α 1-3 generate the dimer that gives a STING butterfly-like shape [31]. All cyclic-di-nucleotides take a 'U' shape while interacting with the STING ligand binding domain pocket. Phosphate and ribose moieties of CDNs (Cyclic dinucleotides) bind at bottom of the LBD pocket [35]. After binding of CDNs to the LBD (Ligand binding domain) interacting amino acid under gores to a conformational change leading to the closure of the ligand binding pocket [36].CDNs other than 2'3' cGAMP, di-nucleotide derived from bacteria also similarly activates STING while non-nucleotide synthetic agonists can activate STING in an open conformation [37].

After binding of CDNs to STING, it forms a complex with Coat Protein Complex II and ER-Golgi intermediate compartments (ERGIC) and activated STING translocate from ER to Golgi [38]. Further STING recruits TBK-1 at the C-terminal, which in turn phosphorylates STING at many residues, including Ser^{366} (human STING). The phosphorylation of this sequence motif provides an interacting site that recruits IRF-3 through binding to a positively charged surface of IRF-3 [34]. The latest cryo-EM study shows that the dimer of STING interacts with the dimer of TBK1 at CTT of STING [35]. Oligomerization of STING brings other TBK1 into close proximity for

transphosphorylation [31]. This suggests that the oligomerization of STING is required for subsequent activation of TBK1 and further TBK1 phosphorylate IRF-3. Interestingly, after activation of STING, TBK1 and its homolog IκB kinase epsilon (IKKε) can activate IKK complex, which then further activates the transcription factor nuclear factor κB (NF-κB)[34]. Following activation of IRF-3, type-1 interferons, including IFN alpha and beta and NF-κB signaling generate pro-inflammatory cytokines including TNF-alpha [39]. cGAS-STING activation pathways have been summarized in Fig 1.

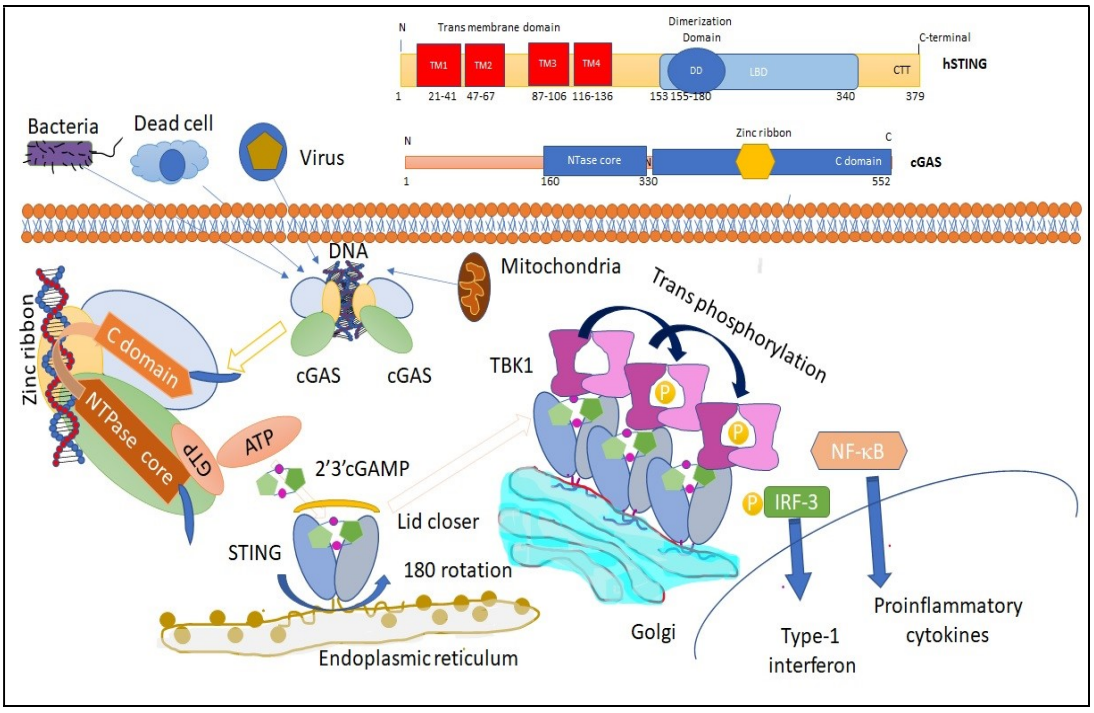

Figure 1 Schematic diagram of the linear protein structure of cGAS, STING, and overview of the cGAS-STING pathway

During pathogenic infection and during stress conditions nuclear and mitochondrial DNA is released as dsDNA in the cytoplasm. cGAS binds to dsDNA to form a 2:2 cGAS-dsDNA complex, generating a second messenger 2'3'cGAMP, after binding 2'3'cGAMP to dimer STING, transferred from the endoplasmic reticulum via ERGIC to the Golgi apparatus and generates oligomerization of STING to facilitate binding of TBK1 at C Terminal and further oligomerization allows TBK1 trans phosphorylation of self. Modified STING recruits TBK1 and activates IRF-3 and IKK, triggering the transcriptional activation of

NF-kB. Ultimately, it regulates the expression and secretion of pro-inflammatory cytokines and IFN.

1.5 cGAS: Emerging association with cancer

DNA sensing is attributed as a major function of cGAS, however, another distinct role is cGAS-mediated regulation of replication fork [40]. Further, cGAS can also translocate to the nucleus and regulate the speed of DNA replication. Nuclear cGAS interacts with DNA replication forks and slower down replication activity. Loss of cGAS in untransformed cells and cancer cells results in uncontrolled DNA replication, aggressive proliferation, and genomic instability [41]. Interestingly, cGAS regulates senescence during DNA damage generated via radiation and DNA-damaging agent [42]. Cellular senescence is an important factor regulating tissue homeostasis and had been implicated in various patho-physiological conditions like cancer, premature aging, and neuronal-related disease. The expression of cGAS regulates cellular senescence and the loss of cGAS in mouse embryonic fibroblast cells becomes immortalized. [42].cGAS has the ability to bind at negatively charged acidic patches formed by histones H2A and H2B via its second DNA-binding site 19. This high-affinity nucleosome binding conformation prevents double-stranded DNA binding and maintains cGAS in an inactive conformation [43]. Generally, DNA damage induces the activity of DNA-PK (DNA-dependent protein kinase), which phosphorylates PARP-1(Poly(ADP-ribose) polymerase 1) on Thr^{594} and facilitates PARP-1 translocation to the cytoplasm where it PARylates cGAS on Asp^{191} [44]. The conformational changes due to PARylation inhibit the cGAS binding to ds DNA. The inhibition of PARP-1 activates the cGAS activity via inhibition of PARylation of cGAS and activation of cGAS. PARP1 inhibitors show significant upregulation of type-1 immune response through the cGAS-STING pathway in both *in-vitro* and *in-vivo* tumor models and prolonged treatment generates anti-tumor memory[45][46]. Phosphorylation of cGAS at Y^{215} by lymphoid tyrosine kinase (LBK) retains cGAS in the cytoplasm in an inactivated condition[44]. The interaction of cGAS and PARP1 inhibits homologous recombination. During DNA damage conditions, cGAS translocate to the nucleus through importin-α and is recruited at the site of dsDNA break and interacts with PARP1[44]. cGAS prevent homologous recombination-mediated DNA repair and promote tumor growth. Carcinogenic compound, 12dimethylbenz(a)anthracene (DMBA), generates nucleosomes into the cytoplasm and activates the cGAS-STING pathway which develops

skin tumors in mice [47]. Another study showed the interaction of cGAS and PARP which inhibited the activity of DNA repair and supports chromosomal instability and malignant transformation and accelerates metastasis in lung cancer [44].

cGAS also play important role in colitis-induced tumors in colon cancer. [48]. cGAS expression in tumors shows an enhanced level of 2' 3'cGAMP production, which can be transported through gap junction to other tumor cells and dendritic cells [49]. Enhanced level of IFN leads to the recruitment of CD8+ T cells to the tumor termed as 'Hot tumor'. Loss of cGAS in the tumor leads to poor production of 2'3'cGAMP and loss of CD8+ cells migration into the tumor side is seen which is also known as a 'cold tumor' [11].

The activity of cGAS is still controversial in the context of cell growth as it is not an independent phenomenon and leads to the activation of the cGAS-STING pathway which may have a different outcome at the different stages of tumor growth and type of TME of different origins. Expression of cGAS and breast cancer development corss-talk still not reported well.

1.6 cGAS-STING; Master regulator of innate immune response during chromosomal instability and DNA damage conditions

Chromosomal instability (CIN) is frequently associated with erroneous chromosome mis-segregation during cell division, which is frequently observed in many cancers. Chromosomal instability majorly drives metastasis and aggressiveness of the tumor [50]. Highly proliferating cells show high CIN and formation of micronuclei which can release DNA in cytoplasm, which is detected by the cGAS-STING pathway. This further supports chromosomal instability and leads to cellular survival and cancer-supportive conditions [51]. A recent study showed significant upregulation of NF-κB via the cGAS-STING pathway during chromosomal instability showing higher proliferation and aggression of metastasis in TBNC cancer as well cell lines [50][52].

Cytoplasmic DNA generated during the chromosomal instability suppress induces STING mediated alternative pathway as NF-κB rather than IFN signalling [53]. A non-canonical STING activation is defined as the activation of STING independent of cGAS, which mainly activates the NF-κB pathway but not the IRF-3 pathway. During DNA damage, DNA nick formation activates PARP and ATM, which further activate the complex of STING, IFI16, p53, and E3 ubiquitin ligase TRAF6. That further catalyzes the formation

of K63-linked ubiquitin chains on STING, leading to the activation of the transcription factor NF-κB [54]. In the canonical pathway, 2'3' cGAMP binds to STING and generates the type-1 interferon response through IRF-3 instead of NF- κB [55].

DNA-PK (DNA-dependent protein kinase) belongs to serine/threonine protein kinase. DNA-PK recruited at DNA strand break and activated by Ku complex and plays a critical role in nonhomologous end joining for DNA repair [56]. High expression of STING promotes chromatin-bound DNA-dependent Protein kinase (DNA-PK) complex [57]. It has been demonstrated that the STING resides at the nuclear membrane and helps protein kinase (PK) binding to chromatin. This non-canonical activation of STING suggests its role in the regulation of inflammatory pathways during DNA damage conditions in tumor cells [58]. Chromosomal instability in triple-negative breast cancer (TNBC) activates NF-κB leading to enhanced IL-6 [53]. Hyper activated IL-6 STAT3 pathway further supports cancer progression and metastasis. IL-6 binds to its receptor IL-6R and activates the IL-6 STAT3 pathway which is pro-tumorigenic and helps in cancer progression[59][60]. The blocking of the IL-6R pathway using tocilizumab, in tumors with high chromosomal instability exhibiting higher expression of IL-6R showed improvement in survival and metastasis[53].

The study from our group also showed that doxorubicin-induced DNA damage in MDA-MB-231, an estrogen-negative cell line with high expression of STING, induces NF-κB activation and further IL-6 expression. IL-6 binds to the IL-6 receptor and activates the downstream IL-6-STAT3 pathway [61]. Furthermore, STAT3 induces PD-L1 expression, and higher PD-L1 expression inhibits T-cell-mediated clearance of tumor cells hence this pathway is of major interest for immunotherapy for different tumors [62]. Inhibition of STAT3 using HJC0152 sensitizes CDNs mediated activity via STING [63]. DNA damaging agents like doxorubicin and STAT3 inhibitors such as HJC0152 reduce the survival of TNBC cells and suppress PD-L1 expression, activating and enhancing T-cell-mediated death [61]. It has been demonstrated that the treatment of camptothecin induces pro-tumorigenic production of IL-6 in STING expressing NCI60 panel of cancer cell lines and that are often independent of type-1 IFN response further inhibition of ERK1/2 sensitize the DNA damaging agent in anti-tumor response [64]. These reports suggest that cGAS STING may be differently activated in tumor cells having different chromosomal

instability levels from different origins. Particular chromosomal instability in TNBC as a basal type of cancer open ups different paradigms in therapy.

1.7 Factors modulating cGAS activity during tumorigenesis:

1.7.1 dsDNA; cGAS does not differentiate between self and non-self-DNA

Self-DNA activates cGAS and leads to the production of CDN as a signalling molecule leading to the induction of IFN activity. The length of dsDNA is a considerable factor in the activation of cGAS. Short dsDNA has a lower binding affinity for cGAS, in contrast, longer dsDNA facilitates multiple binding sites for cGAS and thereby higher production of 2'3'cGAMP [65]. Further, DNA fragments smaller than 20bp of DNA fail to activate cGAS [66], however, the dsDNA sequence of nucleotide is not important to the activation of cGAS [67]. In addition, longer dsDNA causes liquid-liquid separation of cGAS and DNA complex and induces a strong immune response. The generation of droplet-like liquid-liquid separation is more dependent upon the concentration of cGAS and dsDNA [32]. TREX1 (Three prime repair exonuclease1)is a cytoplasmic exonuclease that clears cytoplasmic DNA during stress and hence negatively regulates the activity of cGAS [52]. Rapid cGAS-DNA complex formation and liquid-liquid phase separation does not allow TREX-1 activity, hence cGAS and TREX maintain a balance between the production of 2'3'cGAMP and regulation of cGAS activity [68]. Therefore, the micro nucleoli generated during chromosome mis-segregation, the endoplasmic reticulum interacts with this micro nucleolus and facilitates ER-associated TREX1for the nuclease activity. When the envelope of micro nucleoli ruptures, the endoplasmic reticulum also plays a critical role in the TREX1-mediated inhibition of cGAS activity [69]. All these pieces of evidence suggest the differential regulation of cGAS and downstream STING pathway-mediated IFN activation. (Different pathways of cGAS had been summarized in figure-2).

1.7.2 Phosphorylation of cGAS:

Post-translational modification of cGAS differently affects its activity. In normal conditions, self-DNA is not assessable to cGAS but the nuclear envelope dissolves during the cell division and cGAS activity is inhibited via mitotic kinase CDK1 [70]. Consequently, CDK1 cyclin B complex phosphorylates human cGAS at S305 or mouse cGAS at S291, and phosphorylation of cGAS suppresses the cGAS-STING pathway during cell division. After cell division, the PP1(Protein phosphatase1) enzyme

dephosphorylates cGAS and restores the cGAS activity [70]. Therefore, cGAS activity is strictly regulated via phosphorylation during cell division[43]. Similarly, the phosphorylation of cGAS by AKT at S305 and S291 inhibits its catalytic activity and inhibits the production of 2'3'cGAMP [71]. Different growth factors activate the AKT pathway and cell division. PTEN loss is majorly associated with cancer and regulates Akt, PI3K, and mTOR proteins. However, PTEN is a negative regulator of AKT[72]. Loss of PTEN induces activation of AKT inhibiting cGAS activity via phosphorylation thereby indirectly regulating the immune response in cancer. HER2, EGFR signalling also activates AKT hence cGAS-STING activity is inhibited leading to suppression of immune response in the tumor [71].

Hence high AKT activation and its phosphorylation and inhibition of catalytic activity may prevent unwanted inflammation and cell death. This may be a general mechanism that may prevent cGAS-STING activation during cell division however needs to be tested in different cancer model systems like breast cancer.

1.7.3 Ubiquitination of cGAS:

Post-translational modification through ubiquitination may regulate the turnover and activity of proteins. Ubiquitination is mediated by three-step ubiquitination and up with protein degradation. Enzyme E3 Ligase recognizes the substrate and facilitates ubiquitination [73]. Several E3 Ligases have been now reported to modulate the cGAS activity by mediating different types of ubiquitin chains through different K linkages preferably either K63 linked or K48. TRIM56, E3 ligase, induces mono-ubiquitination at lys^{335} and enhances dimerization and catalytic activity leading to increased production of 2'3'cGAMP and IFN-α and β [74]. However, some members of TRIM proteins, TRIM14 negatively regulates cGAS levels by mediating K48-linked ubiquitination and degradation during infections [75]. RNF185 induces GAS activity via K27-linked ubiquitination chains on the K137/384 site of cGAS and induces cGAS-mediated IFN response [76]. This evidence suggests that ubiquitination of cGAS plays an important role in the regulation of cytosolic DNA sensing mechanism and inflammation however its implication in tumorigenesis of breast cancer and other pathophysiological of chronic disease conditions are not well understood.

1.7.4 Other post-translational modifications of cGAS:

Several ubiquitin-like proteins like Small Ubiquitin-related Modifier protein (SUMO) also known as irreversible post-translational protein modification [77]. SUMOylation of cGAS at K 335/372/382, which suppresses three major cGAS activities including DNA binding, oligomerization, and nucleotidyltransferase activities. 'Specific protease 7' (SENP7) plays a critical role in the de-SUMOylation of cGAS and restores the activity of cGAS [78]. Carboxypeptidase CCP5 or CCP6 plays a critical role in regulating type-1 immune response via polyglutamylation of cGAS [79].TTLL6 (Tubulin mono-glutamylase TTLL6) obstructed its DNA-binding ability, whereas TTLL4 (Tubulin mono-glutamylase TTLL4) via mono-glutamylation of cGAS blocked its synthase activity. CCP6 removes the polyglutamylation of cGAS, whereas CCP5 hydrolyzes the mono-glutamylation of cGAS, which together led to the activation of cGAS [80]. CCP5 and CCP6 are very important for the establishment of DNA virus infection. In addition, mice with loss of CCP5 and CCP6 are more susceptible to DNA virus infection [79].The monoglutamylase TTL4 and the polyglutamylase TTL6 are associated with cGAS in BMDMs (Bone marrow-derived Macrophages). Lack of TTL4 and TTL6 in BMDMs from mice lacked glutamylation of cGAS, producing more type I IFN [81]. The role of these PTMs in the regulation of cGAS activity and their implication in innate immune response and their implication in breast cancer tumorigenesis needs to further established.

1.7.5 Acetylation of cGAS:

Acetylation is another posttranslational modification that is important for various activities of proteins. Acetylation of cGAS at Lys^{384}, Lys^{394}, or Lys^{414} retains the enzyme in an inactive state [82]. Enforced acetylation of cGAS via aspirin renders its inactive form and does not activate the IFN pathway even in the presence of cytoplasmic dsDNA, and suppresses the cGAS-STING pathway for a type-1 immune response [83]. Histone Acetyltransferase, KAT5, regulates GAS activity via acetylation. KAT5 is a positive regulator of cGAS and overexpression of KAT5 enhances the IFN-1 pathway while loss of KAT5 suppresses the IFN-1 pathway. KAT5 acetylates multiple lysine residues in the N-Terminal of cGAS [84].

Aspirin has already been reported to overcome the resistance of genotoxic drugs such as docetaxel in the TNBC subtype of breast cancer [85]. TNBC has higher chromosomal instability and STING agonist may not work in this condition because chronic activation

of the cGAS-STING pathway support proliferation and metastasis via activation of NF-κB and IL-6 [53]. In this condition, aspirin-mediated acetylation of the cGAS-STING pathway may inhibit the production of IL-6 and inhibit survival and metastasis. This suggests the epigenetic silencing of the genes of the cGAS/STING pathway in specific tumors and will determine the intactness of the IFN pathway in different tumors. It is important to investigate the promoter of these genes and their epigenetic status to develop specific therapies targeting different tumor types including the triple-negative type of breast cancer.

1.7.6 Mn^{2+} as a cofactor of cGAS:

Metals have been considered vital elements as co-factor for numerous enzymes including those involved in different cellular processes like metabolism and oxidoreductase activity. Interestingly nutritional deficiency of some trace elements has also been shown to be important for immunity and their deficiency leads to compromised immunity [86]. Typically, cGAS as NTase also belong to metalloenzymes as cGAS catalyzes nucleophilic substitution reactions and is divalent cation-dependent. The catalytic core structures of NTases, usually share a common structural fold and similar active sites harbouring a highly conserved catalytic triad (hG[GS], [DE]h[DE]h, and h[DE]h, having a hydrophobic amino acid), which is available for the catalytic metal coordination [87].

Manganese is important for many cellular processes however its role in the regulation of the immune system is less understood. The experimental studies established the role of Mn^{2+} in the regulation of the cGAS/STING pathway in the innate immune response during both infection and cellular stress conditions. $Mn^{2+}$is sequestered in mitochondria and is released in the cytoplasm and binds to cGAS to different motifs to induce activation activity of the cGAS-STING pathway [88]. Mn^{2+}also induces enzymatic activity even in the monomeric condition of cGAS in absence of dsDNA. Therefore, the allosteric coupling of Mn^{2+} with dsDNA controls the enzymatic catalytic activity [89]. It has been reported that C57BL/6 with mouse melanoma cell B16F10 model deprivation of Mn^{2+} is more prone to tumorigenesis and tumor invasion. Treatment of Mn^{2+} induces infiltration and activation of T cells, NK cells, and maturation of DC with antigen presentation [90]. Further, immune checkpoint inhibitors as anti-PD-1 and Mn^{2+} show a synergistic response in anti-tumor activity in C57BL/6 with mouse melanoma cell B16F10 model and in patients with advanced ovarian cancer [90][91]. Meanwhile, Mn^{2+} with STING agonist-

loaded nano particles injected intratumorally and intravenous shows effectively higher fold induction of the cGAS-STING pathway as compared to STING agonist alone which show anti-tumor response in a murine model [92].

Considering that Mn^{2+} has the potential to activate the cGAS-STING pathway and synergetic effect with STING agonist shows therapeutic implications in the future. Mn^{2+} and STING agonist-loaded different formulations of delivery may be developed in the future for activation of the sGAS-STING pathway [93]. Mn^{2+} may also add a profound impact on tumor vaccine-related immune response in patients [94].

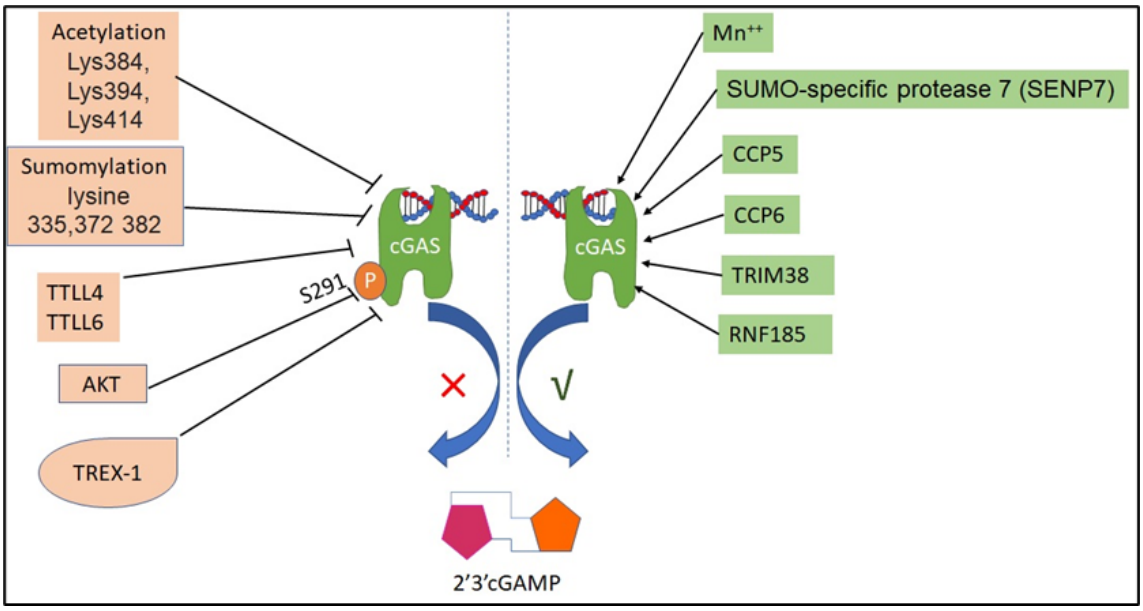

Figure 2 Schematic representation of network regulating cGAS

Cytoplasmic cGAS binds to dsDNA and generate 2'3'cGAMP, a natural ligand for STING. The binding of STTNG with 2'3'cGAMP induces type-1 interferon response. A schematic network of proteins regulating cGAS had been shown. The red box with a blocking arrow suggests the inhibitory mechanisms for the cGAS: some inhibitory mechanisms such as Acetylation, Sumomylation, and phosphorylation inhibit the activity. The green box represents positive regulators asCCP5/6, TRIM38, SENP7, and RNF185 promote the activity of cGAS by deglutamylation, SUMOylation, deSUMOylation, ubiquitination of cGAS, respectively.

1.8 2'3'cGAMP and other cyclic dinucleotides: Critical factor regulating cGAS and STING pathway:

2'3'cGAMP is a natural ligand of STING. Canonical activation includes the activation of cGAS synthesis via 2'3'cGAMP, the dinucleotide, while non-canonical activation of cGAS forms dinucleotides such as 2'5' and 3'3'cGAMP [95]. Dinucleotides are known to be synthesized by prokaryotes as well as diverse eukaryotic cells. There are several types of CDNs and they can act as important intracellular signalling molecules, act as an agonist, and can activate downstream signaling pathways leading to innate immune response or other stress response pathways [96]. The different types of dinucleotides secreted from different bacteria can activate STING. c-di-AMP from gram-positive *Listeria monocytogenes* and *Staphylococcus aureus,* c-di-GMP secreted from *Mycobacterium tuberculosis* activates STING in a manner similar to 2'3'cGAMP but with different binding potency [97][96]. Similarly, 3'3'cGAMP, synthesized by *Vibrio cholerae* also activates STING. 2'3'cGAMP is stronger and highly potent for the activation of STING as compared to 3'3'cGAMP [96][98]. Studies from different bacterial-derived CDNs activating STING have been summarized in Table 1.

Thus, different dinucleotides from self and bacterial derived potentially activate STING and many of them have been modified and used for anti-cancer therapy.

Table 1: Different dinucleotides that activate the STING pathway in different pathogenic conditions

Bacteria	**Activation modes**
Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis	Bacterial c-di-GMP mediated STING activation[99]
Staphylococcus aureus	Bacterial c-di-AMP mediated STING activation[100][101]
Listeria Monocytogenes	
Vibrio cholerae	Bacterial c-di-AMP mediated STING activation[102]
Streptococcus pheumoniae, Streptococcus agalactiae, Streptococcus pyrogenes, Streptococcus pneumonia	Bacterial dsDNA during intra cellular infection[103][104][105][106] Bacterial c-di-AMP mediated STING

Chlamydia muridarum, Chlamydia trachomatis	activation[107]
Brucella abortus	

1.9 STING Activation and regulation:

1.9.1 Ubiquitin-mediated regulation of STING:

Ubiquitination plays an important role to make homeostasis in many cellular processes. Different E3 ligases like TRIM56, TRIM32, and AMFR have been reported to catalyze K63 or K27-linked polyubiquitination of a number of lysine residues in STING[108]. Tripartite motifs (TRIM) containing proteins play a critical role in the regulation of various cellular processes and cancer development by regulating immune response[109]. TRIM protein has three domains like RING, BOX, and Coil-Coil domain. TRIM56 has a critical role in DNA damage-mediated interferon stimulation. It has been reported that TRIM56 in the HEK293 cell line strongly upregulates interferon-β during DNA damage conditions [74]. TRIM56 interacts and initiates K63-mediated ubiquitination of STING leading to dimerization and activation of STING that recruits TBK1 and produces a type-1 immune response [110]. On the other end, TRIM29 negatively regulates type 1 immune response. TRIM29 level is elevated in macrophage and dendritic cells during several DNA virus infections [111]. The cGAS-STING pathway is predominantly activated in TRIM29 knockdown cells and as STING degradation is inhibited [112]. TRIM30α interacts with STING and promotes the degradation of STING via K48-linked ubiquitination at Lys^{275}[113]. STING activation induces TRIM30α and IFN response is amplified and knockdown mice are resistant to viral infection. This suggests that TRIM30α interacts with STING and promotes the degradation of STING via K48-linked ubiquitination at Lys^{275}[113].

These reports suggest that different TRIMs interact with STING and may act in two different ways; either enhancing the turnover of stabilizing or inducing the transcription of the TRIMs which specifically regulate STING activation in specific stimuli. This also suggests that different TRIMs may be expressed in specific tumor types and may modulate this pathway which may determine the outcome of the growth of the tumor as well as therapeutic responses.

1.9.2 Autophagy-mediated regulation of STING-mediated inflammation:

Autophagy plays important role in innate immunity during bacterial and viral infections. Interestingly innate immunity, regulation of autophagy during tumorigenesis, and their implication in tumor cell metabolism is not well understood [114]. Interestingly, STING plays an important role in the induction of autophagy, and it is its primordial role. It is observed RG domain of STING that interacts with LC3 is well conserved from lower eukaryotes to higher eukaryotes including humans [115]. The acquisition of the C-terminal domain that interacts with TBK1and activation of IRF-3 is a newly acquired property. [116]. Interestingly DNA transfection or infection with DNA viruses and not with RNA viruses induces autophagy [117].Which not only degrades DNA but also resolves inflammation through the degradation of STING [118]. It is observed that STING is ubiquitinated by different E3 Ligases which may be cell-specific and stimulus-specific conditions and ay be recognized by p62 which had a Uba domain and recruits this in autophagosomes and degrades STING. [119].

STING regulates iron-dependent apoptotic cell death also called Ferroptosis. This mode of cell death is caused by an imbalance in the antioxidant system and mitochondrial-mediated oxidative stress [120]. In pancreatic cancer, zalcitabine (an antiviral drug) induces oxidative mtDNA (mitochondrial DNA) is fragmented and released in the cytoplasm, activating the cGAS-STING pathway, which induces ferroptosis which is mediated through autophagy and suppresses pancreatic tumor growth in mice [121]. This suggests that autophagy, the primordial function of STING, and its turnover through autophagy may play a critical role in the regulation of innate immune response in stressed TME of solid tumors [122]. STING-mediated autophagy and its role in tumor cell proliferation and its relation with innate immune response in different cancer including different stress conditions specifically during chemotherapy is not well understood and needs to be explored further in breast cancer.

1.9.3 Negative regulators of STING-mediated pathways:

Type-1 interferon during viral infection mounts antiviral response, however, some viral infections overcome or modify the type1 interferon response via their virulence factors [123]. During HIV-1 infection, NLRX1 interacts with STING and inhibits the NF-κB and IFN pathways hence downstream anti-viral pathways [124]. HPV16 virus also follows a similar mechanism and escapes the STING-mediated interferon response. HPV16 also

regulates the NLRX1, by preventing the interaction of STING with TBK1[125]. NLRC3(Nucleotide-binding leucine-rich repeat-containing protein) directly interacts with STING and inhibits the interaction of TBK1 thereby lowering the type-1 IFN response. HSV-1 infection regulates NLRC3, it directly interacts with the STING and inhibits the TBK1 interaction, and reduces the type-1 immune response[126]. Our lab also reported that the NLRX1 regulates the cross-talk of mitochondria and lysosomes under the TNF-alpha treatment and maintains invasiveness and metastasis potential in a triple-negative subtype of breast cancer cells [127]. However, NLRX1 and STING interaction and determining the inflammation and metabolism in tumor cells are not well understood.

1.9.4 Metabolic reprogramming and STING:

Immune response and metabolic reprogramming during infection and chronic inflammatory conditions gained interest after the discovery of several adaptor proteins on the mitochondria and its contact sites like ER and peroxisome. These interactions are dynamic and important for metabolism however their mechanism and regulation had not been well investigated. Interestingly STING had been observed on the contact site on mitochondria and ER junctions [128]. The emerging reports suggest the important role of STING in metabolic adaptation and inflammation [129]. Interestingly brown adipose tissue which is having a density of mitochondria provides an interesting site where mitochondria and inflammation and metabolism are altered. It had been observed that a deficiency of Trx2 leads to enhanced ROS and mitochondrial dysfunction and STING activation leading to inflammation and insulin resistance [130]. It has been reported that the expression of STING stabilizes the mRNA of the Nr12 level [131]. Nr12 activation decreases the expression of STING and responsiveness of STING agonist molecules. 4-octyl-itaconate (4-OI) or the Nr12 inducer sulforaphane is sufficient to repress STING expression and type-1IFN production in cells from patients with STING-dependent interferonopathies[131]. On the other end, during bacterial infection STING is activated and HIF-1 alpha is stabilized. HIF-1 alpha plays a crucial role in oxidative phosphorylation and increases glycolysis during infection [132]. During bacterial infection, STING regulates a transcriptional program that controls the generation of reactive oxygen species (ROS), and STING loss alters ROS homeostasis to reduce DNA damage and cause therapeutic resistance [133]. Several pieces of evidence strongly suggest the crosstalk of STING and inflammation and metabolism whereas it had not been

systematically studied in tumor progression and pathogenesis of different cancer conditions including breast cancer.

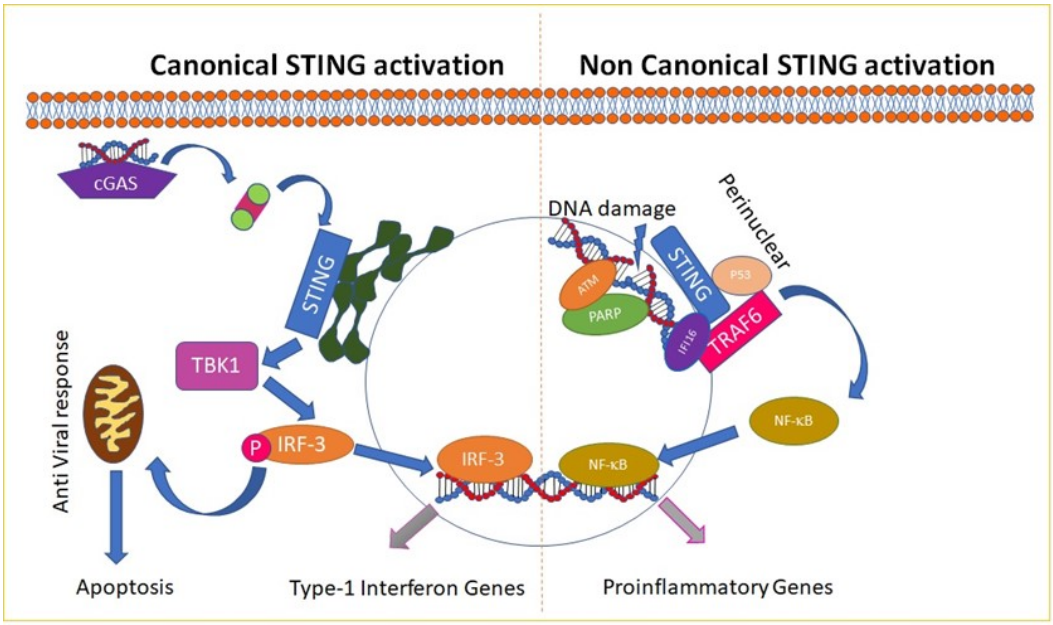

Figure 3 Mechanisms of STNG activation

Cytoplasmic dsDNA is sensed by cGAS and activation of cGAS produces 2'3'cGAMP and which activates STING mediated downstream pathway, known as a canonical pathway. Further activated IRF-3 beyond its role as transcription factor translocate to mitochondria and induces mitochondrial-mediated apoptosis or translocate to nuclei to activate type I interferon genes. During DNA damage, ATM and PARP are recruited at the site and further recruit p53and IFI16, and TRAF6 forming a multiprotein complex that activates STING. This activation is considered as non-canonical STING pathway majorly NF-κB is activated that leads to proinflammatory genes expression

1.9.5 Phosphodiesterase regulates STING activation

Phosphodiesterase as Ecto-nucleotide pyrophosphatase 1 (ENPP1) had been discovered as a type-1 transmembrane protein that regulates the pyrophosphatase and phosphodiesterase activity but recently, their new role identified as a degrader of 2'3'cGAMP as a natural ligand for STING [134].ENPP1 is located on rough ER in the hepatocyte and Ca+ activates ENPP1 and hydrolyzes the cyclic dinucleotide (CDN) [135]. The ectonucleotidases CD39 and CD73 both hydrolyze ATP to AMP modulating the immune response which creates an immunosuppressive microenvironment [136]. ENPP1inhibitor

is also used as an indirect agonist as inhibition of 2'3'cGAMP hydrolysis leads to enhanced activation of cGAS [134]. The knockdown of ENPP1hasa higher tendency to activate the IFN pathway [137]. ENPP1 is abundant in extracellular in the tumor microenvironment of TNBC. ENPP1 hydrolyses all extracellular 2'3'cGAMP and immune cells and suppresses the recruitment of immune cells in TME [138]. There are two possible ways to overcome the ENPP-mediated inhibition: first modifying the phosphate bond between nucleotides and second generating an ENPP1 inhibitor. Both strategies are under preclinical evaluation. STING agonist as ADU S-100 is a modified dinucleotide of c-di-AMP that is resistant to ENPP1, c-di-AMP with phosphodiester bond between dinucleotide is venerable to ENPP1 while modification of this bond with phosphorothioate diester linkages generates stable ENPP1 resistant CDNs [139][140]. The ENPP1 inhibitor TXN10128 exhibits nanomolar potency both *in vitro* and *in vivo*, and its effectiveness has been studied in combination with the immune checkpoint inhibitor anti-PD-L1 in MC38 syngeneic murine model of colorectal carcinoma. The combination showed improved anti-tumor effect and infiltration of lymphocytes and tumor regression [141]. Another ENPP1 inhibitor, RBS2418, which is an oral medication, has entered a phase 1 clinical trial in conjunction with anti-PD-1 therapy in a patient with metastatic adrenal carcinoma [142]. Further effectiveness of ENPP1 inhibitor monotherapy and in combination with natural dinucleotide as STING agonist in different cancer still need to be further investigated.

1.10 Differential expression of STING in tumors determines type-1 interferon response in TME:

Several proteins are required from the initiation of the activation of the cGAS/STING pathway and activation of IFN in the TME. Therefore, it is important to check the intactness of the pathway in cancer. Ishikawa et.al. reported poor expression of STING in tissue skeletal muscle, brain, kidney, small intestine, colon, and liver [143]. The incidence of melanoma is correlated with low levels of STING. Interestingly decreased expression of STING was observed in melanoma cell lines like G361, MeWo, SK-MEL-5, SK-MEL-2, SK-MEL-28, and WM115 [144]. Further, estrogen receptor-expressing cell lines like MCF-7, T47D, and ZR-75 have a low or undetectable expression of STING as compared to HER2 and TNBC subtype of breast cancer cell lines as MDA-MB-231 and MDA-MB-468 [145]. The STING signaling was suppressed in colorectal carcinoma and unable to generate cytokines and type-1 interferon production, loss of T cell priming in the tumor

during DNA damage condition [144]. In KRAS mutated lung cancer shows loss of LKB1 and silencing of STING expression, which is insensitive to cytoplasmic DNA [146]. cGAS STING pathway activation induces a type-1 interferon response that activates T Cell-mediated immune response in head and neck carcinoma cell lines. [147]. Interestingly, the same study also demonstrated a link between the decreased intratumorally CD8+ T cell infiltration and downregulated cGAS-STING in Head and Neck Squamous Cell Carcinoma (HNSCC) [147]. Cancer cells develop deficiencies in the cGAS-STING pathway[148] and the intactness of this pathway is important for the inhibition of tumor growth. RIG-1 regulates the expression of STING, a further agonist of RIG1 induce IFN-β which amplified IFN signalling as the autocrine pathway is activated via binding with IFN β receptor, activation STAT1/2, leading to IRF-7 and 9 translocations to the nucleus. STAT1 also binds to the promoter of STING and induces transcription of STING the up-regulation of STING is reported in oesophageal cancer cell lines [149].

These studies suggest differential expression of the cGAS-STING pathway in different tumors and this variation determines modulation of anti-tumor response. The understanding of differential expression and its implication in different tumors will be important for its therapeutic modulation.

1.11 Modulation of cGAS–STING pathway for tumor cell-specific apoptosis:

Programme cell death is a key process for the maintenance of cellular homeostasis during development and the adult body. Several physiological and pathological stimuli and conditions can trigger apoptosis via extrinsic and intrinsic pathways mediated by mitochondria [150].

Activated cGAS generates natural molecules such as 2'3' cGAMP can induce the proinflammatory cytokine from NF-κB and type-1 immune response from the transcriptional activity of IRF-3[39]. Proapoptotic function depends on the signaling strength of activation of the cGAS-STING pathway [151]. It is observed that the proapoptotic function of the activated cGAS -STING pathway is majorly contributed by IRF-3 and p53. Activated cGAS-STING pathway positively regulates apoptotic genes like NOXA and Puma [151]. It has been reported that the c-di-AMP with prolonged treatment induces the cGAS-STING pathway and also induces a type-1 IFN response and mitochondrial-mediated apoptosis in breast cancer cell lines [145]. Similarly, c-di-GMP

induces growth inhibition and apoptosis in the mouse breast cancer cell line as 4T1cell line [152], Further 3'3'cGAMP as cyclic dinucleotide also reported induced apoptosis in B cell lymphoma lines via mitochondrial caspase 9 mediated apoptosis [153]. Paclitaxel induces the cGAS and STING pathway-mediated secretion of interferon and leading to paracrine activation of the STAT1 pathway which mediated apoptosis in neighbouring cells. Blocking of BCL-XL is required to sensitize the paracrine apoptotic signal generated during paclitaxel treatment *in vitro* and *in vivo* breast cancer model [154].

These reports suggest the possibility to divert or modulate the pro-survival pathways of cGAS/STING toward induction of apoptosis in breast cancer and another model system. This however needs to be further investigated *in vivo* model systems using different combinations of therapeutic drugs.

1.12 cGAS-STING: Exploitation in onco-immunotherapy

The tumor microenvironment comprises several immune escape mechanisms like a low expression of MHC expression in tumor cells and presentation of tumor antigen leads to the escape of tumor cells from the immune system [5]. Myeloid-derived suppressive cells (MDSCs) are considered as pathological activated Neutrophils and Monocyte type of hematopoietic cells having immune suppressive activity. An increased number of MDSCs rather than classical terminally differentiated neutrophils, monocytes, and dendritic cells is observed in chronic pathological conditions like cancer and inflammatory conditions. MDSCs show weak activation signals and show anti-inflammatory properties[155]. The increase in the number of MDSCs had been observed in many cancer conditions including breast cancer [156].

Moreover, these MDSCs produce certain cytokines like TGF-beta and IL-10, and MMPs(Metalloproteinase) support cell growth and metastasis, which helps in tumor progression [157]. On the counter role of type-1 interferon play a critical role in antitumor activity. Type-1 immune response increases MHC expression on tumor cells and upregulates NK cell ligands[158]. The further type-1 immune response also supports DC (dendritic cells) to the prime tumor-associated antigen to T cells, maturation., Polarization of macrophages further augments the cytotoxicity activity of NK cells and IFN- gamma production[159].

cGAS-STING pathway significantly contributes to the activation of the type-1 interferon response. Direct activation of STING via agonist becomes a preferred target for activation onco-immunotherapy for cancer [160]. Activation of the cGAS-STING pathway leads to type-1 interferon response and proinflammatory cytokines like CXCl-10,9. Proinflammatory cytokine induces the infiltration of CD45-positive cells to the tumor site and covert 'cold tumor' into 'hot tumor' [92]. Fragmented DNA from dead cancer cells are present in TME and can be detected by dendritic cells (DC) which can be sensed by the cGAS-STING pathway and activated IFN response. Further, this event leads to the maturation of DC. Enhanced DC maturation at the local site enhances tumor-specific T cells response. Defective in IFN-α, β receptor fails to activate T cells. Hence, this suggested that IFN plays a significant role in DC-mediated anti-tumor response [161].

Enhanced secretion of type-1 IFN and consequent activation of autocrine and paracrine pathways supports the cross-priming of tumor-specific CD8+ cytotoxic T lymphocytes (CTLS)and increase NK cells cytotoxicity activity and inhibits the activity of MDCSc (Myeloid-derived suppressor cells). STING activation shows enhanced infiltration of T cells to the tumor site [19][162].

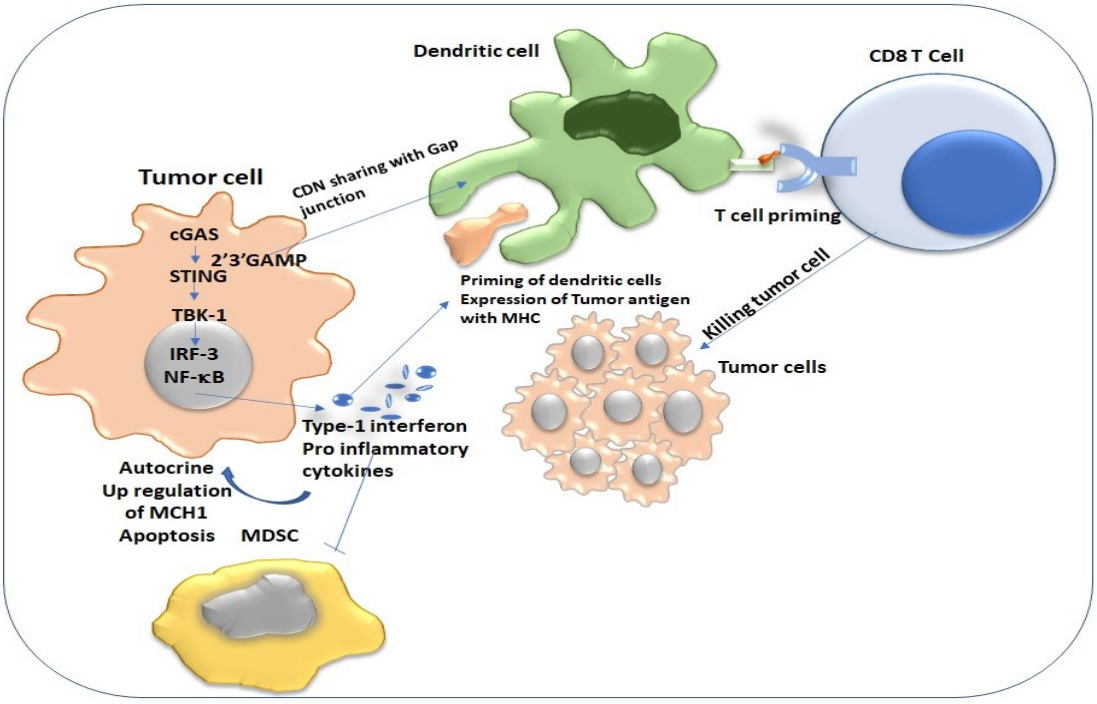

Figure 4 Role of the cGAS-STING signaling pathway in antitumor immunity

The DNA is recognized by cytosolic DNA sensor cGAS to produce 2'3'cGAMP/or STING agonist for STING activation generates type-1 interferon, pro-inflammatory cytokines response, Type-1 interferon inhibit the activity of MDSCs (Myeloid-derived suppressor cells).Type-1interferon stimulates the maturation of DCs and stimulates the cross-presentation of tumor-associated antigens (TAA) to CD8+ T cells, which exhibit an antitumor immunity after proliferation and infiltration into the tumor microenvironment.

1.13 STING agonist: Natural cyclic dinucleotide modified or synthetic:

Promising type-1 interferon as anti-tumor therapy, it is important to device newer strategies to generate the type-1 interferon by tumor cells itself like viral infected cells. Intense efforts have been made in this direction and in a short span several STING agonists had been developed showing promising anti-tumor activity in preclinical models and reached to clinical trials. Initially, the CDNs from bacteria were used as natural CDNs and further modified as per need. Based on the structure STING agonists are further divided into two cyclic dinucleotide and non-cyclic dinucleotide.

1.13.2 Cyclic dinucleotide:

Cyclic dinucleotide synthesized by cGAS is a potent activator of the STING, hence intense focus had been to develop several agonists, however, CDNs have been favourite as it is a natural ligand and is highly potent [163]. Further other natural and synthetic STING agonists have entered clinical development with the first generation of intra-tumor delivered cyclic dinucleotides however in the *in vivo* conditions it had shown modest activity hence there is a need to further modify them to develop a new generation of CDNs.

Firstly, the FAA (flavone acetic acid) was investigated as STING agonist but it failed to show enhanced efficacy in the murine tumor model hence it was further modified into DMXAA (5,6-dimethylxanthenone-4-acetic acid) [164]. DMXAA shows significant activation of mouse STING, however, it failed to activate hSTING [110], [165].

ADU-S100, another agonist developed by Aduro biotech, is a CDNs analog of the endogenous agonist 2,3'-cGAMP containing 2-3'- and 3-5-thiophosphodiester linkages on the c-di-AMP scaffold [139]. Modification of phosphodiester bond showed resistance to ENPPI-mediated degradation. The modification showed extended activity and was

resistant to ENPPI-mediated degradation. This advantage gives it long-lasting activity and higher stimulation of hSTING is seen which is also known as (ML RR-S2 CDA, also ADU-S100) [166].

MK-1454 is another dinucleotide developed by Merk and Co. for the treatment of advanced/metastatic solid tumors or lymphomas. Currently, it is undergoing in phase I trial as monotherapy and in combination with PD-1 inhibitor pembrolizumab.MK-1454 Clinical trials are also undergoing for NCT03010176 [167].

BMS-986301 was first developed by IFM therapeutics and was later acquired by Bristol-Myers Squibb. In the CT26 model, the combination of BMS-986301 with an anti-PD-1 monoclonal antibody provides 80% complete regressions, while no regressions was achieved when treated alone with the anti-PD-1. It was further observed that in CT26 mice with complete tumor regressions generated immune memory as fresh tumor cells were rejected without further therapy. Currently, BMS-986301 is in phase I clinical trial (CA046-006, NCT03956680).

SB-11285 is a small molecule-nucleic acid hybrid STING agonist developed by Spring Bank Pharmaceuticals. SB-11285 structure has not been disclosed yet. Nevertheless, it shows significant potency *in vitro* and *in vivo*. Based on the preclinical data, a phase fa/Ib trial in patients with advanced solid tumors including melanoma and head and neck squamous cell carcinoma was planned to begin in the USA to examine the efficacy of SB-11285 in combination with nivolumab in September 2019 (NCT04096638)[168].

IMSA-101 is an analog of cGAMP developed by Immune Sensor Therapeutics as the small molecule STING agonist. In preclinical studies, IMSA-101 show highly effective anti-tumor activity. Clinical trials are ongoing for IMSA-101 alone or in combination with ICIs as dose escalation (phase 1) and dose expansion (phase Ila) in patients with advanced solid tumors (NCT04020185).

JNJ-67544412 is a cyclic dinucleotide (CDN) developed as a STING agonist. JNJ-4412 can bind both mouse and human STING and is more potent in binding all the major human STING alleles than most other STING CDN agonists[169].

Bi-STING (BI-1387466) molecule was developed by Boehringer Ingelheim and has similarities to the natural ligand of STING (NCT04147234).

1.13.3 Non-dinucleotide:

GSK-3745417 had been developed by GlaxoSmithKline and belongs to a synthetic non-CDN STING agonist with a dimeric ABZI scaffold suitable for systemic administration [170]. Intravenous administration of GSK-3745417 to immunologically active mice bearing syngeneic colon tumors provokes strong tumor immunity with complete and lasting regression of tumors. Since 2019, GSK-3745417 has been launched by GlaxoSmithKline in phase I clinical trial in 300 participants with refractory/relapsed solid tumors to assess the safety, tolerability, and preliminary clinical efficacy, as well as to establish an optimal intravenous dosage for GSK3745417 alone or co-administered with pembrolizumab (NCT03843359).

MK-2118 is a STING agonist of unreported structure, developed by Merck, is being tested as an intratumorally or subcutaneous injection alone or in combination with pembrolizumab in patients with advanced solid tumors or lymphomas (NCT03249792).

SNX281 is a novel small molecule therapeutic developed as a STING agonist. It had been developed by Stingthera, Inc. which is active against all isoforms of human STING and has stable drug properties thus permitting systemic delivery. In pre-clinical models, a single intravenous dose of SNX281 in mice bearing CT26 colorectal tumors resulted in complete regression of tumors. SNX281 also synergized with anti-PD1 agents in inhibiting tumor growth and improving the overall survival of tumor-bearing mice. In an ongoing phase 1 clinical trial, the safety, tolerability and maximum tolerated dose of systemic SNX281 will be assessed in patients with advanced solid tumors and lymphomas. This trial is comprised of two treatment arms, in which intravenous SNX281 is given either as monotherapy or in combination with pembrolizumab, in a dose escalation followed by dose expansion phase to determine the recommended dose for phase 2 studies (NCT04609579).

TAK-676 is another small molecule STING agonist, which is modified structure of natural dinucleotide. This molecule developed by collaboration of Takeda and Pfizer. TAK-676 is now under clinical investigation in a phase I dose escalation study. This trial aims to determine the safety and tolerability of intravenous TAK-676 as monotherapy and in combination with pembrolizumab in patients with advanced or metastatic solid tumors (NCT04420884).

E7766 belongs to the novel class of macrocycle-bridged STING agonists (MBSAs). MBSAs show superior in vitro activity against all major human STING genotypes. E7766 binds to both human and mouse STING protein and show activity against a broader range of human STING genotypes compared to reference CDNs. A single intratumoral injection of E7766 was shown to significantly reduce the growth of subcutaneous tumors in mice [78]. In mice bearing CT26 tumors in both subcutaneous tissue and liver, a single intratumoral injection of E776 led to resolution of tumors in 90% of treated mice with no recurrence over 8 months [79]. Intravesical administration of E7766 in mice with BCG-unresponsive non-muscle invasive bladder cancer was associated with robust IFN-β gene induction and a dose-dependent anti-tumor response [80]. The clinical efficacy of intratumoral injection of E7766 is being evaluated in a phase 1/1b clinical trial as a monotherapy in patients with advanced solid tumors or lymphomas (NCT04144140).

RVU3128603 is a small molecule compound that belongs to non-nucleotide and shows STING-mediated immune response, as well as up-regulation of STING is reported.

Considerable progress had been made to develop as STING agonist that generates a specific anti-tumor response through type-1 interferon in the tumor microenvironment, by recruiting tumor-associated dendritic cells. Further cytokines attract T cells and NK cells to the tumor microenvironment and are activated via tumor antigen. Activated T cell and NK cells kills the tumor cells. It is important to investigate the intactness and activation of the cGAS-STING pathway in different cancers and determine the optimal combination therapy for anti-cancer therapy.

1.14 The exploitation of cGAS-STING pathway with combination treatment for anti-cancer therapy

Anti-cancer therapy became more important after lots of heterogenicity and the low response from classical treatment. Onco-immunotherapy became a central part to treat the tumor. In onco-immunotherapy immune checkpoint, inhibitors are major blocks hence, the cGAS-STING pathway became a preferred target for anti-cancer therapy. Major emphasis had been to develop STING agonists and many natural and synthetic molecules have already reached to the clinical trial stage in a short span. Some potent molecules have been identified in preclinical models and clinical trials are ongoing for the same. STING agonists activates T cells mediated immune response against the tumor cells but many of tumor expressing higher level of immune checkpoint inhibitors like PD-L1 are resistant.

Preclinical data showed that the carboplatin-induced higher expression of PD-L1 and the combination of STING agonist 2′3′-c-di-AM (PS)2 with anti-PD-1 shows superior efficacy in C57BL6 -ID8p53$^{-/-}$ ovarian mouse model [171]. Other combination as c-di-GMP loaded in liposomes and anti-PD-1 was effective in the immune check point resistant model as the B16-F10 mouse melanoma model [172]. MSA-2 is an orally available STING agonist that shows a synergistic effect with bispecific antibody (TGF-β/PD-L1) treatment in a mouse model of CT26, F10, H22, EMT6, and B16 models [173]. The further liposome of 2'3'cGAMP with anti-PD-L1 shows the superior anti-tumor effect in B16-F10 and FRAF mutated melanoma murine model [174]. In 4T1 breast cancer mouse model combination of atezolizumab (anti-PD-L1) and STING agonist shows profound anti tumor response [175]. Further nano particle loaded STING agonist with anti-PD-L1 efficacy was evaluated in an orthotopically-transplanted model (C3(1) Tag model) for basal-like triple-negative breast cancer (TNBC) and a spontaneous genetic engineered mouse (GEM) model of basal-like TNBC (C3(1) Tag GEM). This TNBC model was resistant to anti-PD-L1 treatment but in combination with STING agonist shows upregulation of type-1 interferon, macrophage polarization and infiltration of T cells [176]. STING agonist, anti-PD-L1, and anti- GITR (Glucocorticoid-induced TNF receptor) show remarkable efficacy in the lymphoma model [177]. Considering all almost all clinical trials included PD-1/PD-L1 combination therapy. higher all STING agonists evaluated with PD-L1 or PD-1 blocker [178] showed effective anti tumor response. Interestingly all immune checkpoint inhibitors with STING agonists show a broad spectrum in different preclinical and clinical models.

As metal ion Mn^{2+} has ability to induce cGAS sensitization to dsDNA and Mn^{2+}able to activate cGAS independent of ds DNA and generate 2'3'cGAMP. Mn^{2+} and STING agonist loaded liposome elicit higher fold induction of type-1 interferon in CT26 mouse model [92]. PEG nanoclusters of Mn^{2+}activate the immature bone marrow-derived dendritic cells (DCs) and upregulates 57.3- and 13.3-fold higher production of interferon β and interleukin-6 as compared to free cGAMP, MnP-PEG nanocluster in combination with a checkpoint inhibitor (anti-PD-1)produce to significant tumor regression in the B16F10 murine melanoma model without any significant toxicities [93]. Further Mn^{2+} is required for the anti-tumor activity through the cGAS STING pathway same also reflected in clinical trials with platinum-resistant ovarian and refractory metastatic breast

cancer patients [179]. The efficacy improved in presence of anti-PD-1. In addition to irradiation of the tumor and intratumorally injection of Mn^{2+} increase the activity of the cGAS-STING pathway that leads to 90% of inhibition of the tumor[180]. Hence Mn^{2+} is an indirect STING agonist so further investigations are required to develop combinatorial therapy with different immune check point inhibitors as well as with STING agonists.

Depending upon tumor type it has been established that there is a higher expression of ENPP1 enzyme present in the extra cellular matrix so it is difficult to stabilize the natural STING agonist [181]. Natural STING agonist as 2'3'cGAMP and other CDNs have phosphodiester bond between dinucleotide that is hydrolyzed by ENPP1 enzyme. Inhibition of ENPP1 enzyme became an indirect agonist via stabilization of CDNs and 2'3'cGAMP[182]. Combination with ENPP1 inhibitor and natural CDNs may generate a profound immune response. Many inhibitors of ENPP1 are currently under development [183].

cGAS-STING pathway is differently regulated in cancer types of different origins. Chromosomal instability reported higher in TNBC, shows non-canonical NF-κB leading to higher IL-6 production. IL-6 binds to its receptor and downstream activates STAT3 mediated survival pathway [184]. During DNA damage conditions induced by the genotoxic agent also generate IL-6generated via non-canonical STING activation [61]. Interestingly Inhibition of STAT3 via HJC0152 shows an improved response of c-diAM(PS)2 and the future shows the synergistic response in vivo condition in the 4T1 breast cancer mouse model [185].In prostate cancer cell line with high IL-6 and STAT3 was unresponsive to STING agonist while inhibition of JAK2/STAT3 restore the responsiveness of STING agonist in the TRAM-C2 mouse model [186]. Inhibition of this pathway in a different way like directly blocking of IL-6 receptor as Tocilizumab or blocking of the downstream pathway as JAK2 inhibitor like Ruxolitinib, Upadacitinib further STAT3 inhibitor as HJC0152, Niclosamide, in combination with STING agonist may improve therapy [187][188].

HER2 expression also affects the cGAS-STING pathway [189]. The higher expression shows downregulation of STING protein further HER2 recruits AKT1 and inhibits STING signalling [190]. Higher HER2 expression showed downregulated expression of interferon-stimulated genes (IGS) [191]. Hence combinatorial therapy of blocking of

HER2 receptor via Trastuzumab or AKT inhibitor may show a synergistic effect during DNA damaging agent or STING agonist treatment.

PARP inhibitor shows promising clinical activity and patient with BRCA mutation shows significant improvement in treatment. PARP inhibitor as Olaparib triggers local and systematic immune activation through STING. Olaparib generates a micro-nucleus in the cytoplasm that activates cGAS-STING in mice bearing BRCA1-deficient ovarian tumors [46]. Moreover, STING agonist with PARP inhibitor shows macrophage polarization and CD8 T cell migration in BARCA mutated tumors [192]. PARP inhibitor shows cGAS-STING mediated macrophage polarization and CD8 T cell infiltration in BRCA-1 deficient TNBC syngeneic FVB/129P mice tumor [193]. In addition to that PARP inhibitor shows a positive correlation with cGAS STING and loss STING decrease potential of PARP inhibitors.

Other molecules like TLR7/8 agonists are also reported to show synergistic effects with STING agonists via upregulation of IFN-β [194]. As immunotherapy IL-2 with STING agonist act as superkine immunotherapy in MHC-1 deficient tumor [195]. Treatment of IL-15 with ADU-S100 as a STING agonist shows potential immunotherapeutic effects in prostate cancer[166]. IDO inhibitor with STING agonist shows improved combination therapy in mouse colorectal carcinoma model [196].

Different regulations of the cGAS STING pathway behave differently in tumors of different origins hence it is better the explore the combination therapy to exploit the cGAS STING pathway in anti-tumor treatment some of the examples listed as table no:2

Table 2 Combination therapy for cGAS-STING pathway as anti-cancer therapy

Combination therapy	Problem	Impact	References
Direct			
STING agonist with PD-1/PD-L1	High PD-L1 expression is majorly associated with many tumors and inhibit T cell-mediated killing	Using the PD-1/PD-L1 blocker provides the scope of T cell engagement with tumor cell	[172][174][197][198]

STING agonist with $MnCl_2$	STING alone may not sufficiently activate the Type-1 immune response	$MnCl_2$ activates cGAS even in absence of cytoplasmic DNA and activates STING. Combination with STING agonist to generate the exaggerated immune response	[91][90][93][92] [180]
STING agonist with ENPP1 inhibitors	ENPP1 is present in an extra tumoral matrix as well in the tumor cell leads to hydrolysis of natural di nucleotide, STING agonist	Inhibition of ENPP1 increase the stability of di nucleotide use for STING agonist	[134][135][137][202][183]
IL6/STAT3/JAK2 inhibitor with STING agonist	High activity of IL-6/STAT3 was reported in many tumors and that suppresses the cGAS-STING pathway	Inhibition of STAT3 sensitizes the cGAS-STING pathway and moreover enhances the STING signalling	[200][187] [201][185]
HER2 inhibitor with STING agonist	HER2 regulates cGAS activation via AKT and inhibits the interaction of STING and TBK1	Inhibition of HER2 or AKT inhibitor supports the cGAS activation and STING-TBK1 interaction	[202][203][189] [204][191][205]
PARP inhibitor	cGAS PARylated via PAPP inhibit the activation of cGAS	PARP1 inhibitor shows impressive response in combination treatment of STING agonist *in vitro* and *in vivo*	[45][206][46]
Indirect way			
STAT3/JAK2 inhibitor with DNA damaging agent	During non-canonical activation of STING leads to shifting of pathway	Inhibition of STAT3 in DNA damage conditions shifts toward IFN	[207][53][61]

	predominantly to NF-κB, IL6 /STAT3 pathway and suppress IFN pathway		
ATM inhibitor with DNA damaging agent	ATM required to activate non-canonical activation of STING	Inhibition of ATM leads to inhibit the non-canonical activation of STING	[208][209][210]
AKT inhibitor with ENPP1 and $MnCl_2$	Loss of PTEN types have highly upregulated AKT activity that inhibits the cGAS	AKT inhibitor enhances cGAS activity and will be upregulated in the presence of $MnCl_2$ and ENPP1inhibitor	[71][211][205]
STING agonist with other recombinants protein	IL-2 and IL-15 considered as further supportive for NK-cells and T cell activation	IL-2 maintain activation of T cells and IL-15 support for NK-cells mediated response with STING agonist	[195][212]