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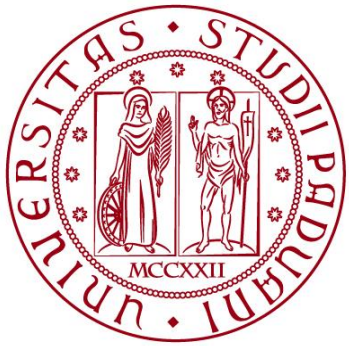

ELABORATO DI LAUREA

ROLE OF THE CGAS-STING PATHWAY IN CANCER IMMUNITY AND IMMUNOTHERAPY: THE STING PATHWAY COMBINED WITH CELL THERAPY

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SUMMARY

1 Introduction

Cancer is a genetic disease caused by mutations in cellular DNA. Mutations can be random or induced, while some individuals show a predisposition to develop the disease. According to the Cancer Journal for Clinicians, in 2020 there were about 19.3 million new cases of cancer in the world and about 10 million deaths from the disease. Therefore, it is increasingly essential to identify effective therapies that consider individual variability. Recently research is focusing on the modulation of the host's immune system to reprogram it and activate it powerfully against tumor cells. The following review will analyze the potential role of the cGAS-STING pathway in immunotherapy combined with cell therapy against cancer.

The cyclic GMP– AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway is a mechanism of innate immunity aimed at protecting the host from viral and microbial infections, such as bacterial pneumonia, tuberculosis, and sepsis. The main proteins involved in the pathway are conserved in mammals and bacteria, suggesting its origin as a bacterial defense system. However, the STING pathway can be also identified in other species, such as the anemone Nematostella vectensis and the fly Drosophila melanogaster.
Cytosolic DNA sensors are the molecules capable of identifying foreign double-stranded DNA (dsDNA) and to consequently activate a signal cascade promoting the release of type I Interferon (IFN), antimicrobial response, and autophagy. These proteins can recognize different types of DNA, both exogenous (viral or bacterial), and endogenous (tumoral). Cytosolic dsDNA activates the cGAS (cyclic GMP-AMP Synthase) enzymes determining the formation of cGAMP (cyclic GMP-AMP). In response to cGAMP, STING - a transmembrane adapter protein located in the endoplasmic reticulum (ER) - activates TBK1 (TANK-binding kinase 1) which phosphorylates and activates the transcription factor IRF3 (Interferon Regulatory Factor 3). IRF3 in turn induces the expression of type I IFNs genes.
Nevertheless, the cGAS-STING enhances both the innate inflammatory response as well as the adaptive response.

1.1 The cGAS-STING pathway and intracellular signaling

Upstream the signaling cascade is characterized by the fundamental interaction between cGAS' C-terminal domain and dsDNA, followed by the conformational change of the enzyme leading to an increase in affinity for ATP and GTP substrates and the formation of the catalytically active 2:2 cGAS-DNA complex. The dimerization and consequent activation of cGAS occurs only if the enzyme

interacts with sufficiently long DNA molecules. This mechanism explains the tolerance of the pathway towards self-genomic DNA. 2',3'-cGAMP is then sensed by STING. The transmembrane protein is characterized by four functional domains: N-terminal transmembrane domain with four helices, connector region, cytosolic ligand-binding domain (LBD) and C-terminal tail (CTT). As a homodimer, STING interacts with cGAMP and tetramerizes. The transduction signaling is activated solely after STING has been translocated from the ER to the Golgi through the ER-Golgi intermediate compartment (ERGIC). Upon reaching the Golgi, STING undergoes palmitoylation and promotes the recruitment of TBK1 and the formation of autophagic vesicles. The interaction between STING and TBK1 is mediated by CTT and consent kinase's activation and the pursuance of intracellular signaling.

Activated TBK1 dimerizes and trans-phosphorylates STING's C-terminal domain. The STING-TBK1 complex recruits the transcription factor IRF3 which also undergoes phosphorylation by TBK1 and dimerizes. IRF3 translocates to the nucleus and induce the transcription of various genes including genes encoding type 1 interferon as well as genes encoding pro-inflammatory cytokines (such as IL-6, IL-1 and IL-2).

Secondly, STING is involved in the transcriptional activation of NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), which regulates the expression of pro-inflammatory genes coding for cytokines (TNF, IL-1, IL-6), chemokines (CCL2, CXCL8), endothelial adhesion molecules (E-selectin) and costimulatory molecules (CD80, CD86). The release of chemokines mediates the placement of cellular effectors into the inflamed tissue.

Additional signaling pathways are still being studied, but it is necessary to mention that under physiological conditions the signaling cascade induced by STING activation may also promote apoptosis of the infected cell. The mechanisms underlying programmed cell death are various: the induction of cell cycle inhibitors such as p21 and the activation of the pro-apoptotic proteins BAX and BAK by IRF3 are undoubtedly the processes better understood. On the other hand, the induction of type I IFNs such as TNF promotes necroptosis.

Interestingly, the release of cytokines, chemokines, proteases, and growth factors also promotes cellular senescence.

Finally, SURF4 adapter protein allows for the subsequent retrograde transport of STING and its degradation in lysosomes.

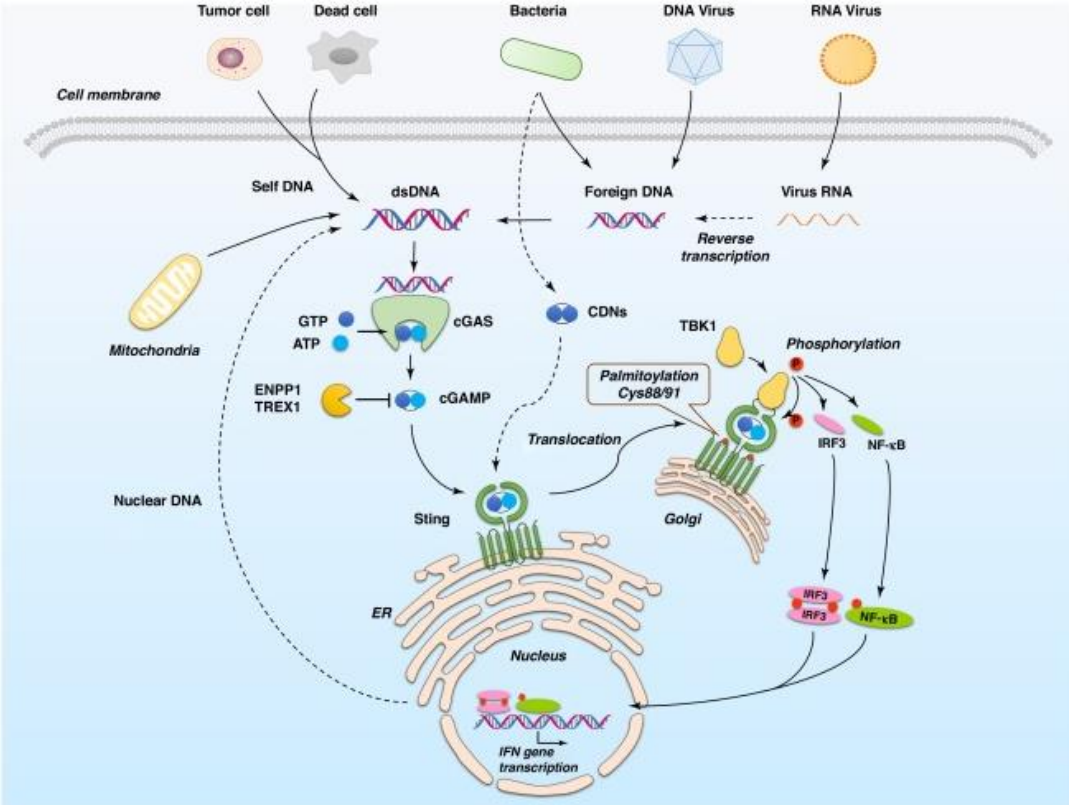


Figure 1: cGAS-STING intracellular signaling (Feng et al., 2020)

1.2 The role of cGAS-STING pathway in cancer progression

STING's role in tumor proliferation is twofold: despite the induction of apoptosis and the stimulation of both innate and adaptive immunity; the creation of an immunosuppressive microenvironment favored by the activation of the pathway can contribute to carcinogenesis.

First and foremost, it is described (Kwon et al., 2020) that tumor cells contain free cytosolic dsDNA, that, activate the STING pathway to control and limit tumor cell proliferation, as shown in figure 2. The release of pro-inflammatory cytokines and chemokines is essential for the infiltration into the inflamed tissue of important cellular mediators of innate (Natural Killer cells) and adaptive (T cells) immunity, whose role will be investigated in more in-depth later in this thesis. On the other hand, it is known that the chronic inflammation greatly favors the carcinogenesis.

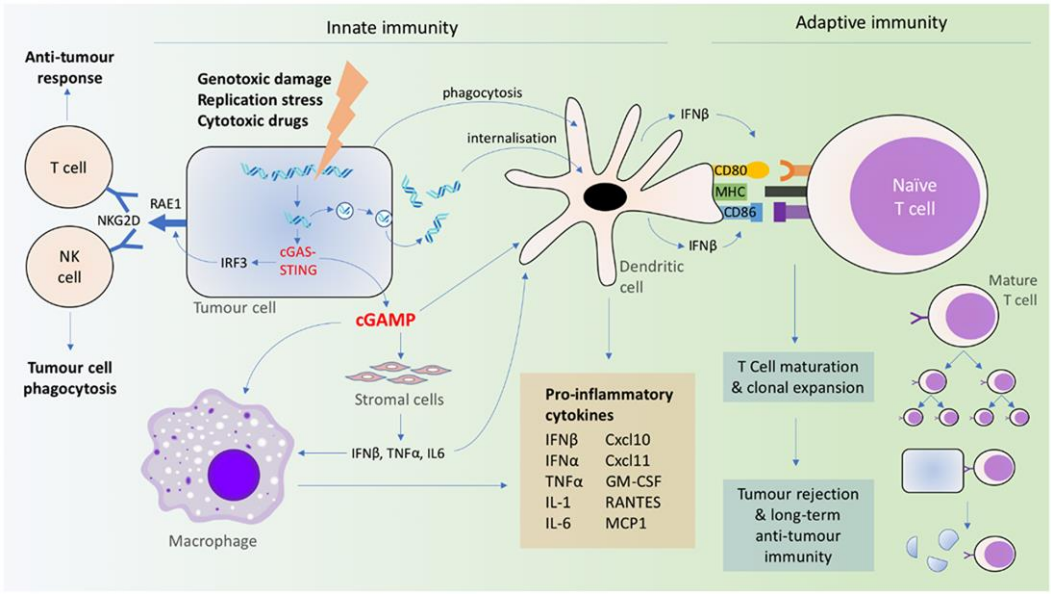


Figure 2: Cooperation of innate and adaptive immunity in response to STING activation (Yi Da Hoong et al., 2020)

The sources of cytosolic DNA are sundry, first being the chromosomal instability (CIN) typical of the tumor cells. Interestingly, an increase of CIN correlates with chronic activation of STING and a major probability of tumor progression. Additionally, cells with unstable genome suppress the production of type I IFNs. Furthermore, in cells that uncontrollably proliferate, the mitochondria are severely damaged and their consequential loss of membrane, leads to the release of mitochondrial DNA into the cytosol. Besides, recent studies (Dou et al., 2017) have shown that DNA devoid of histones - and therefore of steric hindrance - such as mitochondrial DNA, is more efficient in activating the STING pathway due to a higher affinity for cGAS. Agents of a chemical and physical nature (as radiation) can also irreversibly damage the DNA which, if not repaired, will find itself free in the intracellular environment.

The activation of STING can also follow non-canonical pathways. For instance, the DNA repair proteins ATM and PARP-1 and the DNA binding protein IFI16 promote the activation of STING in response to DNA damage induced by etoposide, an antineoplastic drug.

Peculiarly, cGAS can act non-canonically thanks to its ability to translocate to the nucleus through a yet unknown mechanism. Nuclear cGAS is not activated in response to genomic DNA but has been shown to promote carcinogenesis through inhibition of homologous recombination (HR) repair of DNA. In normal infected cells, this activity induces cell death; however, in genetically unstable cells, inhibition of HR can lead to further damage to cellular DNA.

It must be also considered that cGAMP transport is mediated by gap junctions. Tumor cells produce cGAMP which is secreted and detected by antigen

presenting cells (particularly dendritic cells) through the folate transporter SLC19A1. cGAMP can be presented by APCs to CD8 T cells, leading to their activation and allowing adaptive immunity to cooperate with innate immunity. This greatly amplifies the inflammatory response with potentially harmful effects.

1.3 Tumor evasion mechanisms to immune response

The dichotomy in the consequences of STING's activation can also be explained through multiple mechanisms exploited by the tumor to escape the host's immune system.

There are various evasion mechanisms, one of which is the methylation of the cGAS and STING promoters. Tumor cells, however, maintain cGAS and STING synthesis, albeit at reduced levels. On the other hand, evasion of adaptive immunity appears to be closely related to the expression of inhibitory molecules such as PD-L1, also induced by STING activation.

It is also interesting to highlight the peculiar role of autophagy in tumor progression. As previously mentioned, autophagy is one of the strategies used by the cell to overcome viral or bacterial infection following the activation of the STING pathway.

Although macroautophagy can constitute an efficient barrier in preventing neoplastic progression, it is highly probable that it can act together with the endoplasmic reticulum stress response to facilitate tumorigenesis.

Lastly, in metastatic breast cancer for example, the tumorigenic cells communicate with astrocytes through the release of cGAMP. cGAMP is released into the extracellular space through gap junctions and induces STING's astrocytes activation and the subsequent release of pro-inflammatory cytokines promoting cancer metastatic proliferation in the brain. This response suggests that cGAMP released by tumor cells could be an element capable of promoting tumor metastasis.

2 How cGAS-STING pathway triggers adaptive immunity

2.1 CD8 T cell activation

As mentioned above, the activation of STING in antigen presenting cells (APCs) can stimulate the release of cytokines promoting the maturation and differentiation of T cells. APCs - in particular dendritic cells - are triggered by free dsDNA which is recognized by pattern recognition receptors (PRRs).
T cells are induced to differentiate into two main classes: helper CD4+ cells and cytotoxic CD8+ cells. The latter are involved in the elimination of intracellular pathogens and tumor cells. Particularly, CD8+ T cells can infiltrate inflamed tumor microenvironment (TME).
The presence of activated CD8+ T cells in TME seems to have positive prognostic effects in cancer-bearing patients: although the activity of T cells is not sufficient to eliminate the tumor, it reduces its progression.
Studies suggest that there is also a correlation between type I IFNs, T-cell infiltration, and clinical outcomes (Woo et al., 2015). CD8+ T cells are indeed involved in the up-regulation of two factors modulating central tolerance and autoimmunity: PD-L1 and indoleamine-2,3-dioxygenase (IDO) induced by IFN-γ. Tregs, on the other hand, produce the chemokine CCL22 which upregulates Foxp3. Paradoxically, patients with melanoma expressing high levels of these inhibitory molecules responded better to therapeutic vaccines. These results suggest that these factors are markers of inflammation and indicate the ability of T cells to effectively infiltrate tumor tissue.

The administration of poly-ADP-ribose polymerase (PARP) inhibitors, such as olaparib, promotes the infiltration of activated CD8+ T cells into the tumor microenvironment of BRCA1-deficient triple-negative breast cancer (TNBC) *in vivo* (Pantelidou et al., 2020).
The recruitment induced by PARP inhibitors is mediated by the activation of the STING pathway, which however has only been observed in BRCA1-deficient, but not BRCA1-proficient mouse tumor cells. Also, significant tumor progression was observed by treating immunocompetent mice with olaparib and anti-CD8+ antibodies.
Immunohistochemical analyzes showed that olaparib enhances the expression of granzyme B in CD8+ T cells and NK cells and promotes the infiltration of CD8+ and CD3+ T cells into tumor tissue.
In human BRCA1-deficient MDA-MB-436 cells it was observed that the STING pathway is more efficiently activated in HR-deficient TNBC cells, as DNA damage occurs.

2.2 Release of type I IFNs

Notably, the release of type I IFNs through the activation of the STING pathway has been observed to have beneficial effects in breast cancer (Ascierto et al., 2012). In type I IFNR$^{-/-}$ mice, the carcinogen methylcholanthrene induces a higher development of tumors and the response of T cells was significantly weaker. Notably, STING$^{-/-}$ and IRF3$^{-/-}$ mice were equally unable to effectively activate T cells against tumor antigens.

In glioma models STING$^{-/-}$ mice had a shorter life expectancy. The exogenous delivery of cyclic dinucleotides as STING agonists - whose role will be investigated in detail later in this thesis - allowed to observe interesting beneficial effects. However, in other models the activation of STING and the release of type I IFN favors carcinogenesis, for example, STING$^{-/-}$ mice did not develop tumors following injection of the tumorigenic 1,3-Dimethylbutylamine to the skin (Ahn et al., 2014. Lemos et al. (2016) reports instead how the inactivation of STING promotes the infiltration of CD8+ T cells in Lewis lung carcinoma (CLL), suggesting a context-dependent role for STING in cancer progression.

Overall, these studies lead to the conclusion that enhancing the infiltration of cytotoxic T cells into the tumor microenvironment most likely have beneficial effects.

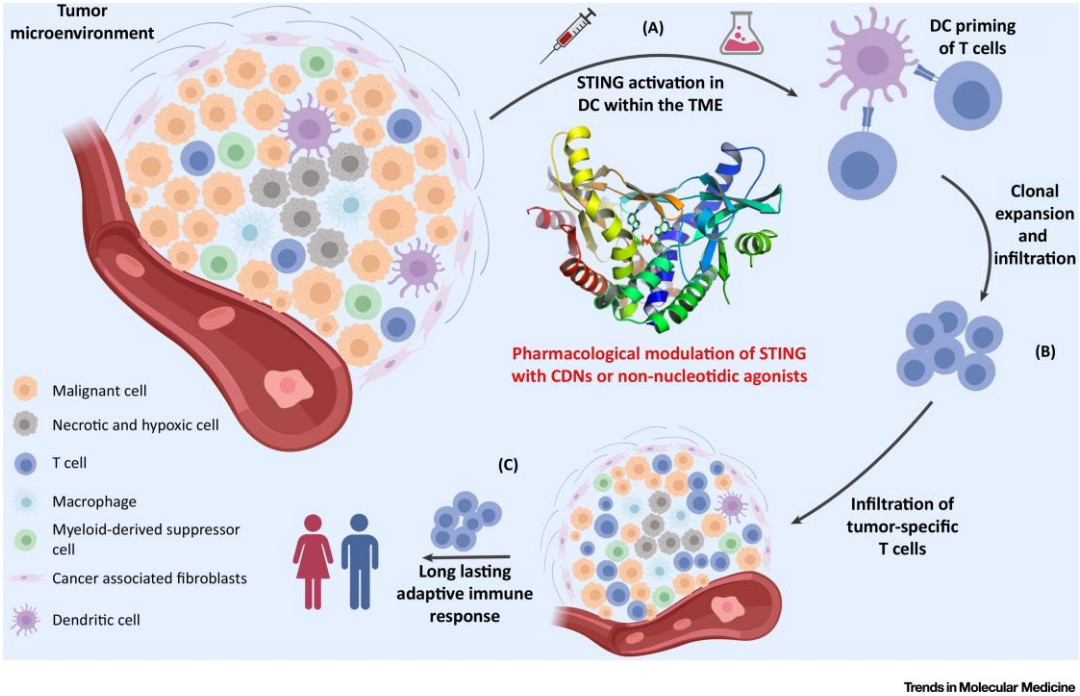


Figure 3: Pharmacological modulation of STING pathway in cancertheraphy (Berger et al., 2019)

2.3 CAR-T cell therapy

Chimeric antigen receptor (CAR) cell therapy combined with the administration of STING agonists is also achieving interesting results. CAR- T cells are lymphocytes whose TCR is modified to recognize tumor antigens. These cells maintain the effector function of T cells, while assuming the typical specificity of antibodies produced by B cells. The administration of STING agonists, such as DMXAA and cGAMP (Xu et al., 2020), favors the infiltration of CAR-T cells into TME in breast cancer, as well as the production of cytokines. However, the ability of CAR-T cells to kill tumor cells is severely limited by tumor heterogeneity and the immunosuppressive milieu of TME and effective tumor regression was achieved only by combining cell therapy and the administration of STING agonists with anti-PD1 and anti-GR-1.

2.4 CD4 T cell activation

The STING pathway is involved in the differentiation of naïve CD4 T cells into IFN-γ-producing Th1 cells and interleukin-9 (IL-9) secreting Th9 cells. The latter seem to have potent antitumor activity. Th9 cells are induced by IL-4, transforming growth factor β (TGF- β) and pro-inflammatory cytokines such as IL-1 β and tumor necrosis factor α (TNF-α).

cGAMP was administered to evaluate the efficacy of cGAMP as an antitumor agent *in vivo* in the MC38 murine colon cancer model (Benoit-Lizon et al., 2022). Following administration, an increase in the production of the cytokines IFN-γ and IL-9 was found. To demonstrate that CD4+ T cells contribute to the antitumor activity of STING ligands, $Rag2^{-/-}$ mice were crossed with STING-deficient mice and reconstituted with wild-type or STING-deficient CD4 T or CD8 T cells. The MC38 tumor cells were then engrafted into mice subsequently treated with cGAMP. The treatment was effective only in mice reconstituted with wild type CD4 T cells, indicating their potential antitumor role along with cGAMP.

STING's activation by cGAMP administration also increases IFN-γ production by the same CD4+ T cells *in vivo*. In MC38 model it was also observed that the neutralization of IFN-γ reduces the beneficial effects of cGAMP, effectively inhibiting its ability to control tumor growth. However, this ability is not completely abolished, suggesting that other cytokines play a role in promoting the antitumor activity of cGAMP. Lastly, corroborating these results, IL-9-neutralizing antibodies reduce the antitumor activity of cGAMP.

3 How cGAS-STING pathway triggers innate immunity

3.1 Natural Killer cells

Natural Killer cells are cytotoxic cells of innate immunity capable of recognizing tumor or virus-infected cells or cells that have lost class I MHC, in a fundamentally non-specific manner. NK cells are activated by type I IFN and their effector functions are deeply promoted by two cytokines, IL-15 and IL-15Rα.
NK cells are also able to release cytokines and chemokines which attract dendritic cells allowing the consequent activation of adaptive immunity.

The participation of Natural Killer cells following STING pathway activation could have an important antitumor function.

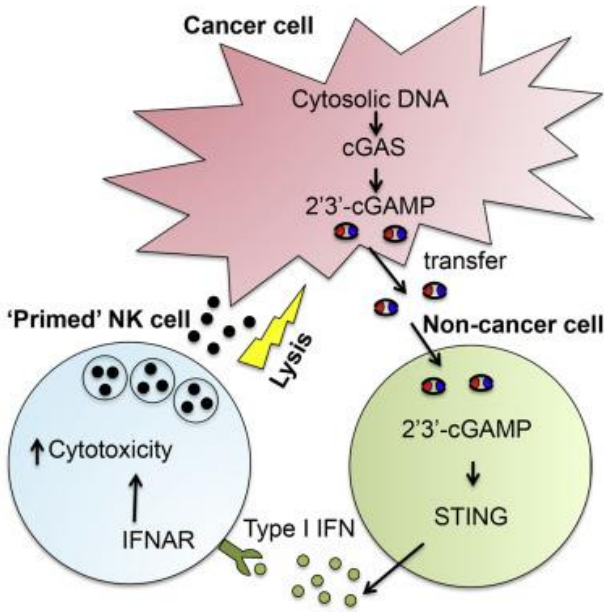


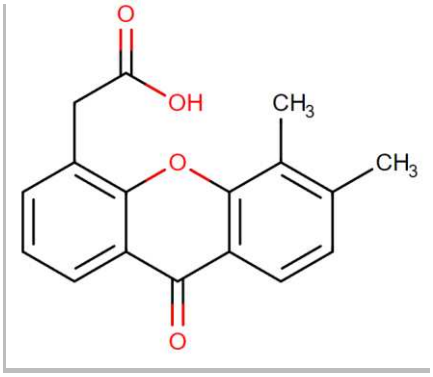
Figure 4: Natural Killer cells priming through STING activation (Marcus et al.,2018)

Here, I will analyze the role of STING agonists in the activation of NK cells and in the subsequent treatment of cancer since numerous studies have been completed in this regard.

4 The potential role of STING's agonists in immunotherapy

Being cGAS-STING's role in tumor proliferation clear, it is evident that the development of efficient STING agonists could represent an interesting new approach in the treatment of cancer. The direct pharmacological activation of STING also using synthetic and hydrolysis-resistant cGAMP, seems to reduce tumor growth and favor the antitumor activity of T cells, especially if concomitant with the administration of anti-PD-1 and anti-CTLA-4 antibodies. Moreover, STING agonists induce apoptosis of target cells and the expression of tumor antigens that allow the activation of T cells.
The role of STING agonists in anticancer therapy was initially hypothesized thanks to studies involving mice bearing colon adenocarcinoma. The exogenous and intratumoral administration of the cyclic agonist 2',3'-cGAMP - produced in mammalian cells by cGAS-cGAMP synthase in response to dsDNA in the cytoplasm - allowed to obtain positive results in limiting the progression of cancer. Equally encouraging results have been obtained in the treatment of melanoma. Furthermore, endothelial cells respond to STING agonist administration with the production of IFN- γ within the TME.

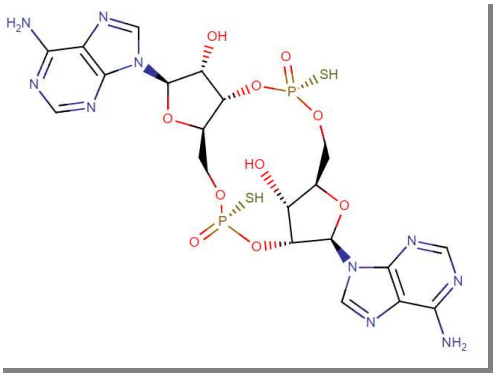
4.1 DMXAA



The STING agonist dimethyloxoxanthenyl acetic acid (DMXAA) potently induces TBK1 phosphorylation in DCs resulting in their activation and maturation. STING ligand DMXAA in mice enhances Th9 differentiation but may trigger cell death. A mild activation of STING can favor the differentiation of T cells, without causing cell death. However, previous studies have shown that DMXAA does not bind to human STING and thus does not activate the human protein (Conlon et al., 2013).

An alternative to DMXAA as a non-cyclic dinucleotide STING agonist is represented by amidobenzimidazole (ABZI) that, administered together with 4-carbon butane linker, has demonstrated a higher affinity for human STING. However, the safety of ABZI has yet to be determined (Ramanjulu et al., 2018).

4.2 ADU-S100



STING pathway activation was evaluated in glioblastoma (GBM) models by measuring the downstream cytokine CXCL10 produced following type I IFN release (Berger et al., 2022). Human GBM models don't show a functioning STING pathway, nevertheless the administration of STING agonists such as the synthetic cyclic dinucleotide ADU-100 favors the activation of the pathway and the production of different cytokines.

Particularly, administration of 2',3'-cGAMP or ADU-S100 to immunocompetent mice with glioblastoma (GL261 and CT-2A strains) showed positive results in activating the innate immune response and increasing the myeloid population. Infiltration and increased population of CD4+, CD8+ and NK cells, increased expression of PD-L1 and expansion of myeloid granulocytic cell population were also highlighted 7 days after administration. In this study ADU-S100 was administered as a bolus. This approach allowed to observe a profound remodeling of the TME and a potent activation of the inflammatory response. Overall, treatment of GL261 and CT-2A with biodegradable gel-beased implants resulted in long-term immunity for GL261 and a higher survival rate for CT-2A.

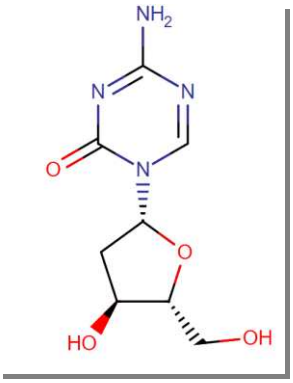
Analyzing the multidimensional data obtained by t-distributed stochastic neighbor embedding (t-SNE) coupled to FlowSOM - which allow to cluster the populations and to visualize the global shift in the immune population - a considerable activation of the innate cell population, consisting of NK cells and immature myeloid-derived innate immunity cells.
These results corroborate the hypothesis that Natural Killer cell activation has an important role as an antitumor effect following activation of the STING pathway.
Moreover, analysis of the transcriptome of murine glioblastoma cells showed that the administration of STING agonists induces the activation of many genes involved in the inflammatory response, including the *Ifit1* (IFN-induced protein with tetratricopeptide repeats 1B-like 1) genes, *Ifi47*, *Tnf* and *Mx2*, implicated in IFN signaling. The administration of ADU-S100 intensifies the production of type I (IFN α and IFN β) and type II (IFN γ) IFNs. Furtheremore, one of the most up-regulated genes following ADU-S100 administration is *NCR1*, a major activator of NK cells. Also, interestingly, STING agonist-treated tumor cells express high levels of the cytokines IL-15 and IL-15Rα.

To evaluate the antitumor activity of NK cells, independently from the CD8+ T cells, several types of tumor cell line with low class I MHC levels were generated and injected into syngeneic mice and subsequently treated with ADU-S100. The results showed that, even in the absence of CD8+ T cells, the tumor is still rejected.
Conversely, treating mice with anti-NK antibodies, implanting the tumor, and carrying out a therapy with STING agonist, leads to a rapid growth of the tumor.
NK cells in tumors treated with STING agonists express higher levels of IFN-γ, CD107a, Sca-1 and granzyme B, and proliferate significantly. Administration of STING agonists also appears to favor systemic NK cell activation, which is also independent of T cells. Also, NK cell depletion in GL261 mice results in a total loss of efficacy of ADU-S100. In $Rag2^{-/-}$ mice that lack NK, T and B cells, the administration of ADU-S100 initially slowed the tumor growth, even if, there was no tumor regression and the mice did not survive.

ADU-S100 can also be administered intratumorally to increase the local concentration of the agonist and promote the development of immunological memory by lymphocytes infiltrated in the TME. Systemic administration of high doses of the agonist following local administration could also contribute to distant antitumor activity. However, overly high doses of the agonist induce cell death.
A phase I clinical trial involved 47 patients with solid tumors or lymphomas in advanced/metastatic stages (not in the central nervous system) and cutaneous, subcutaneous, or nodal lesions, unresponsive to standard therapies and aged between 26 and 80 years (Meric-Bernstam et al., 2021). Patients were treated with weekly intratumoral injections of increasing doses of the agonist (3-weeks-on/1-weeks-off schedule) until toxicity, disease progression, or patient refusal to prosecute therapy was noted. The aim of the study was to determine the effective and nontoxic dose of the agonist, as well as its pharmacokinetic and pharmacodynamic properties. ADU-S100 was rapidly absorbed systemically. Its degradation was also rapid (2 hours).
The main side effects noted were anemia, fatigue, nausea, pyrexia, injection site pain, and chills. No relationship between dose and toxicity was found and deaths encountered during treatment were attributable to disease progression.
Three patients experienced a partial systemic response, although only one of the responses was confirmed, while in most of the treated individuals it was evidenced a reduction in the size of the lesions. In 18 patients the disease did not progress.
Biopsy analysis of treated and untreated lesions did not show a change in PD-L1 expression levels or CD8+ T cells activity. Interestingly, higher levels of CD8+ T

cells were detected in the patient who showed a confirmed partial response. A nonsignificant increase in expression levels of NK cell markers was also found in some patients. Cytokines such as IFN-β, IL-6 and CXCL10 showed an increase in their levels in the 6 hours following injection.
The results suggest that higher doses are needed to enhance STING activity. Furthermore, the lack of a clear dose-effect relationship prevented determining the most effective dose. In any case, the results obtained were encouraging, especially in the treatment of melanomas.

4.3 5AZADC



Reversal of promoter DNA hypermethylation by 5-aza-2'-deoxycytidine (Decitabine o 5AZADC), a Food and Drug Administration-approved methyltransferase inhibitor (DNMT) may have an important role in cancer treatment, since silencing of tumor suppressors by DNA methylation is a mechanism commonly exploited by the tumor to proliferate. Particularly, several studies have linked DNA methylation to colon cancer, lung cancer, and melanoma. Therefore, the use of methyltransferase inhibitors could be extremely useful in cancer therapy.
Interesting studies are being conducted especially in the treatment of triple-negative breast cancer (TNBC) (Yu et al., 2018). DNMT levels have been shown to correlate with patients' response to decitabine. Moreover, the administration of the inhibitor is followed by the degradation of the methyltransferases DNMT1, DNMT3A, DNMT3B, over-expressed in a significant number of tumors.

Administration of high levels of decitabine is commonly associated with toxic effects, but it is effective as an anticancer agent. Low levels of the inhibitor could exert more interesting results, although few studies have been completed in this regard. In the study, the results below were achieved analyzing breast patient-derived xenograft (PDX) mouse models tumors. Although the relationship between DNMTs and decitabine is still quite controversial, it seems that high levels of DNMT protein correlate with greater sensitivity to decitabine.

A different study used a combination of decitabine and anti-PD-L1 antibodies in the treatment of TNBC with MYC overexpression in Balb/c immunocompetent mice (Wu et al., 2021). The product of the MYC gene is in fact a protooncogene promoting neoplastic proliferation if over-expressed. In this context, MYC

upregulates DNMT1 transcription in TBCN which methylates the 5' UTR of *STING*, inhibiting its transcription.
Overall, the treatment potently inhibited tumor growth without causing changes in the weight of the mice, suggesting a promising safety treatment. Moreover, decitabine promotes the creation of an inflamed TME and up-regulates PD-L1 which represses T cell activation, hence the need to administer the inhibitor together with anti-PD-L1 antibodies.
Flow cytometric analysis of the cells infiltrating TME showed that treatment with decitabine and anti-PD-L1 induces an important activation of CD8+ cells and, favors the production of granzyme B in lymphocytes. Furthermore, the expression of cytokines such as CCL5, CSCL10 and IFN−β is increased.
According with these observations, in STING knockdown mice, the administration of decitabine has much more limited effects, thus confirming its role in cancer.

4.4 Further applications

STING could also be employed as a cancer vaccine adjuvant (Fu et al., 2015). STINGVAX contains tumor cells secreting granulocyte-macrophage colony-stimulating factor (GM-CSF) and cyclic dinucleotides. In the treatment of B16 melanoma and various mouse tumor models, the vaccine has proved quite effective, particularly in promoting the infiltration of activated T cells into the TME. A second vaccine, SatVax combined with anti-PD-L1 therapy has yielded even more encouraging results in xenograft model (Tan et al., 2018). CD8+ T cell activation was promoted while inhibiting the immunosuppressive effects of STING.
Alternative therapies propose to use antibodies targeting T-cell inhibitor receptors along with STING agonists. Combining the administration of anti-CTLA-4 (negative regulator of T-cell activation) with anti-PD-L1 and anti-CD137 (member of the tumor necrosis factor receptor superfamily) resulted in bilateral tumor regression in 40% of treated mice (Ager et al., 2017). The percentage reached 75% by combining the therapy with the administration of STING agonist cyclic di-GMP.

5 Concluding remarks

Despite the positive results obtained in the treatment of tumors with STING agonists, the chronic activation of the pathway represents a serious problem as it favors the formation of metastases. Future therapies must, consequently, take this aspect into consideration and develop combined approaches that see the use of STING agonists and antagonists. Antagonists could limit the involvement of the pathway in tumor progression and indeed a covalent palmitoylation inhibitor of STING may effectively reduce the formation of metastases (Hansen et al., 2018).

The antitumor activity of NK cells activated by STING in solid tumors is too still being investigated. Positive results were obtained in the treatment of MHC I-high MC-38 tumors expressing high levels of NKG2D ligands (Nicolai et al., 2020). Therapies that aim to amplify NK cell activation and mobilize T cells could have clinically interesting results. In designing a therapy, STING agonists and the release of type I IFN may also play a role, which intensifies the antitumor activity of the STING agonist itself as well as the efficacy of T-cell responses. Nevertheless, the role of type I IFN *in vivo* remains unclear: it could favor the release from the tumor itself of cytokines involved in the activation of NK cells.

Other genes' interaction with *STING* and their potential capability of activating the pathway should also be investigated. Gene regulation is still a poorly understood process, but grasping the impact that other signaling pathways have on STING activation could prove extremely useful in defining tumor progression.

Lastly, the different efficacy that the treatments developed up to now have demonstrated in the animal model (mouse) and in humans remains to be explored. In fact, in the latter the data, however encouraging, are in any case less satisfactory, suggesting there is still much to be understood about the mechanism behind the functioning of the STING pathway in humans.

What emerges from these considerations is undoubtedly a complex network of interactions involving innate and adaptive immunity, epigenetics, and the genetic predisposition of the single individual. Consequentially, the real breakthrough could be only achieved by combining the use of agonists with cell therapy, aiming at developing increasingly personalized *ad hoc* therapies for the single patient.

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