THE ROLE OF cGAS-STING PATHWAY IN VIRAL INFECTION

AN ABSTRACT

SUBMITTED ON THE TWENTY-SECOND DAY OF DECEMBER 2022

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF MEDICINE

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

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ABSTRACT

The DNA exposure in the cytosol triggers the activation of DNA sensor cyclic GMP-AMP (cGAMP) synthase (cGAS). Activated cGAS produces cGAMP, a second messenger that binds to the endoplasmic reticulum (ER) membrane protein stimulator of interferon genes (STING), resulting in STING dimerization and activation. STING then recruits the downstream signaling pathways and induces the type I interferon (IFN) expression. The cGAS-STING pathway plays a critical role in limiting DNA and RNA virus infection. However, the regulatory mechanisms of this process are not well elucidated and how cGAS-STING pathway inhibits RNA virus infection is not clear. In this study, we first analyzed cGAS protein interactome in mouse RAW264.7 macrophages and found that C1QBP is a new binding partner with cGAS. C1QBP predominantly localized in the mitochondria and leaked into the cytosol during DNA virus infection. The leaked C1QBP bound the NTase domain of cGAS and inhibited cGAS enzymatic activity in cells and in vitro. Overexpression of the cytosolic form of C1QBP inhibited cytosolic DNA-elicited innate immune responses and promoted HSV-1 infection. By contrast, deficiency of C1QBP led to elevated innate immune responses and impaired HSV-1 infection.

Second, we found that knockout of STING facilitated human coronavirus OC43 infection in H1299 cells, a lung epithelial cell line that cannot produce IFN upon DNA stimulation. We also blocked the IFNAR of primary wildtype MEFs and STING knockout MEFs using anti-IFNAR antibody and infected the cells with
HCoV-OC43, even without ISGs expression, the coronavirus infection in STING knockout MEFs increased significantly as compared with wild-type MEFs. This suggests that STING inhibits coronavirus independent of type I IFN. Viral membrane proteins, NSP4 and NSP6, are known to be involved in forming double-membrane vesicles (DMVs). By co-immunoprecipitation (co-IP) and immunofluorescence assay (IFA), we found that STING interacted and colocalized with NSP4 and NSP6. Furthermore, STING deficiency increased the DMV formation, and reconstitution of STING restored the inhibitory effect on DMV formation, suggesting that STING inhibits coronavirus replication by blocking DMV formation. Taken together, our study revealed a novel regulatory mechanism for cGAS and an IFN-independent antiviral role of STING.
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I would like to thank Dr. Shitao Li, my dissertation advisor, for tutoring me all these six years. He opened a new gate of science for me, so I can start to know how to do science and think scientifically. He contributed many hours to almost every aspect of my Ph.D. study. Sometimes I feel lost in doing science, but he can always give me good suggestions and bring me back to the right route. He also gave me so much support in my life in a new country, so I had happy six years here in the U.S., and I am going to keep it. I want to thank my dissertation committee members for their help and instructions on my project. I want to thank Dr. Xuebin Qin, who gave me suggestions on science career development; Dr. Derek Pociask, for the instructions on animal experiment design; Dr. Kislay Parvatiyar for sharing experiences on data and work presentation; Dr. Kerstin Honer zu Bentrup for the suggestions of making figures and giving a talk. I really appreciate them for serving on my committee.

I want to thank my lab mates Dr. Lingyan Wang, Dr. Yakun Wu, Wenzhuo Hao, Yiwen Sun, and Dr. Fang Hua in Li Lab. They supported me and helped in my experiments and life in New Orleans. I want to thank Dr. Lucy C. Freytag and Zylkia M. Lozano, who always patiently answered my questions and helped me in the BMS program. Finally, I want to thank my family, cousins, and friends, even though living in different places, we got each other’s back; without them, I wouldn’t
be where I am. I appreciate that I had wonderful times during my Ph.D. life and looking forward to the next stage of my life.
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The role of cGAS-STING pathway in viral infection

CHAPTER 1. Overview of the cGAS-STING pathway

1.1. General mechanisms of innate immunity

Innate immunity is the first line of host immune defense that fights invading microbial pathogens. After pathogens break through our physical barriers, such as the skin, mucosal surface, and blood-brain barrier, pathogens encounter host innate immune defenses, including non-specific immune responses and innate immune cell surveillance. The innate immune cells include macrophages, dendritic cells (DCs), natural killer (NK) cells, mast cells, neutrophils, and eosinophils. In contrast to the T and B cells that express a randomly generated repertoire of antigen recognition receptors, the innate immune cells express the germline-encoded receptors, pattern recognition receptors (PRRs), which recognize conserved molecule components expressed by pathogens, known as the pathogen-associated molecular patterns (PAMPs). The innate immune pathogen recognition model was first proposed by Charles Janeway in the 1990s. He proposed that the host has the ability to discriminate between self- and non-self-molecules, and innate immunity directs adaptive immunity, which is largely corroborated by recent studies. For example, microbial molecules, such as the structural protein components and the nucleic acid of the virus, bacteria, and fungi, can be recognized by PRRs and activate the innate immune response. Furthermore, PAMP-activated innate immune responses also initiate the adaptive immune response.
PAMPs usually are the essential components required for pathogen survival, including lipids, proteins, and nucleic acids. Generally, PAMPs have distinct molecular and cellular features that host cells do not have; thus, the innate immune cells can tell non-self from self through recognizing PAMPs by PRRs. PRRs can be divided into five types according to their protein domain homology: the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs). These receptors can be further divided into membrane receptors and non-membrane intracellular receptors. The TLRs and CLRs are membrane-bound receptors that detect the microbial pathogen ligands in the extracellular compartment and endosomes. The RLRs, NLRs, and ALRs are cytosolic receptors that detect intracellular pathogen ligands. PRR activation can initiate the downstream innate immune signaling, resulting in the production of interferons (IFNs) and proinflammatory cytokines (Fig. 1). PRR activation also induces non-transcriptional responses, including autophagy, phagocytosis, and cytokine processing. These innate immune processes are tightly controlled by the orchestrated signaling pathways that protect the host from infections and appropriate activation of adaptive immunity.
Pattern recognition receptors, including TLR, CLR, NLR, RLR, and ALR, induce innate immune responses, such as the expression of type I interferons and inflammatory cytokines.

1.2. PPRs for DNA: DNA sensors

DNA sensors are DNA-binding proteins that detect the exposed DNA and initiate innate immune responses\(^\text{21,22}\). Many DNA sensors have been reported and they can sense DNA either from invading microbial pathogens or host due to cellular stresses, such as oxidation and apoptotic processes\(^\text{23,24}\). Most of these DNA sensors activate the transcriptional regulators of interferon (IRF) and NF-kB signaling pathways\(^\text{21,25}\).

1.2.1. TLR9

The first identified DNA sensor is TLR9 which resides in the endosomes\(^\text{26}\). Human TLR9 is expressed in DCs and B cells, while mouse TLR9 is expressed in DCs, B cells, and macrophages\(^\text{26,27}\). TLR9 is important for host defense against viruses and bacteria.
by detecting their DNA. For example, TLR9 detects DNA from cytomegalovirus (CMV), herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), herpes papillomavirus (HPV), Kaposi’s sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV), and human immunodeficiency virus (HIV) \(^{28-32}\). In addition, TLR9 also detects bacterial DNA, such as *Mycobacterium tuberculosis*, *Toxoplasma*, *Plasmodium species*, and *Aspergillus fumigatus* \(^{33,34}\). TLR9 specifically recognizes the unmethylated cytosine-phosphate-guanosine (CpG) motif-containing DNA from DNA viruses and bacteria \(^{35-37}\). The CpG motifs in the mammalian cells are methylated; thus, TLR9 can differentiate self- and non-self-DNA, thereby avoiding aberrant innate immune response to self-DNA \(^{38}\). TLR9 activation leads to the production of IFN-α, IFN-λ, and other cytokines and chemokines \(^{39-41}\).

TLR9 contains a leucine-rich repeat (LRR) domain and a Toll/IL-1 receptor (TIR) domain. Like other members in the TLR family, the TIR domain of TLR9 is responsible for the recruitment of adaptor protein MyD88. At a steady state, TLR9 localizes on the endoplasmic reticulum (ER). TLR9 exits ER with the chaperone protein Unc93b1. Then, TLR9 transports through the Golgi into the endolysosome \(^{42-45}\). Once translocated to the endolysosomes, the ectodomain of TLR9 undergoes proteolitical cleavage to form a functionally competent receptor. The binding of CpG DNA induces TLR9 oligomerization, leading to recruiting MyD88 through the TIR domain \(^{36,46}\). MyD88 then interacts with the IL-1R-associated kinases (IRAks), IRAK-1 and IRAK-4, which triggers two signaling branches \(^{47}\). First, IRAK4 recruits tumor necrosis factor receptor associated-factor 6 (TRAF6) \(^{48}\) and activates the transforming growth factor-β associated kinase 1 (TAK1). TAK1 further phosphorylates the IkB kinase (IKK) complex, leading to the activation of NF-κB and induction of mRNA expression of the proinflammatory cytokines, such as IL-6, IL-12, and TNF \(^{49,50}\). Second, IRAKs recruit TRAF6 and regulatory factor-7 (IRF7),
resulting in IRF7 phosphorylation and dimerization. The IRF7 dimer translocates into the nucleus and induces the production of type I IFN \(^51\).

In addition, autophagy also plays a role in TLR9 signaling \(^52\). Binding of CpG-DNA with TLR9 results in the recruitment of autophagy component light chain 3 (LC3) and autophagy-related 5 (ATG5), which serves as an anchor for IKK\(\alpha\). The proximity of IKK\(\alpha\) with the IRF7 signaling complex results in the phosphorylation of IRF7 and activation of type I IFNs expression \(^52\). Another alternative TLR9-mediated IFN production through an IRF3/7-independent pathway has been reported in DC1 and macrophages during \(A. fumigatus\) infection, a fungal pathogen known to actively recruit TLR9 to the phagolysosome \(^53\). Induction of TLR9 with CpG-DNA can recruit MyD88 resulting in the interaction with IRF1, which promotes IRF1 nuclear translocation and the production of IFN-stimulated genes (ISGs) and inflammatory cytokines \(^54,55\).

1.2.2. IFI16

The IFN \(\gamma\)-inducible protein 16 (IFI16) is a member of the interferon-inducible human PYHIN protein family \(^56,57\). IFI16 contains two HIN200 domains at its C-terminus for DNA binding and one pyrin (PY) domain at the N-terminus for protein interaction and oligomerization \(^58\) \(^59,60\). IFI16 has multi-partite nuclear localization signal (NLS) and predominantly expresses in the nucleus \(^59,61-63\), thus IFI16 can sense viral genomic DNA in the nucleus. IFI16 was found to bind cytosolic viral DNA and physically interact with the stimulator of interferon gene (STING) in THP-1 cells \(^57,64,65\). Then, STING recruits TBK1 and consequently activates type I IFN expression \(^66\).

In addition to its role in DNA-mediated innate immune signaling, IFI16 also elicits inflammatory and apoptotic responses against foreign DNA through inflammasome activation \(^67-70\). Although inflammasome activation is thought to be restricted in the
cytoplasm, one study showed that IFI16 co-localized with KSHV genomic DNA in the nucleus of endothelial cells and formed the ASC and caspase-1-containing inflammasome. IFI16 interacts with ASC at the early stage of infection in the nucleus, however, at the late stage of infection, the mature inflammasome relocates to the cytoplasm. Moreover, the latent episomal forms of EBV or KSHV genomes elicit constitutively high caspase-1 activity and high expression of proinflammatory cytokines through IFI16 in B cells. The inflammasome activation by IFI16 is also observed during HSV-1 infection. Therefore, the IFI16 inflammasome activity might be a general response during herpesvirus infection.

1.2.3. DNA-PK

DNA-dependent protein kinase (DNA-PK) is a holoenzyme that contains the Ku70, Ku80, and the catalytic subunit DNA-PK. Its major role is DNA damage repair through nonhomologous end joining (NHEJ). In addition to its canonical role in DNA damage response, DNA-PK was also found to be a sensor for foreign DNA. Studies using affinity purification found that DNA-PK binds with DNA and mediates the IRF3-dependent innate immune response. In cells and mice lacking DNA-PK, the innate immune response against virus infection was impaired. Thus, DNA-PK plays an important role in regulating virus infection.

The subunits of DNA-PK have been shown to induce innate immune responses to dsDNA. For example, DNA-PK is recruited to the DNA through Ku80 and then initiates the IRF3-dependent immune response. It is also shown that Ku70 activates DNA-mediated type III IFN responses through IRF1 and IRF7. However, the requirement of the catalytic activity of DNA-PK is debatable. One study reported that the catalytic activity of DNA-PK that was responsible for the phosphorylation of IRF3. But another one showed that IFN production was independent of DNA-PK kinase activity. DNA-PK acts upstream
of TBK1 and IRF3, and the Ku70 could form a complex with STING during DNA transfection\textsuperscript{75}. However, whether STING is required for DNA-PK-mediated IFN responses is also debatable \textsuperscript{73,77}. Further investigation is warranted to elucidate the underlying molecular mechanisms.

1.2.4. DDX41

The DExD/H-Box helicase family contains RNA and DNA helicases involved in DNA-induced type I IFN response. The DExD/H-Box helicase family has two subgroups, the DEAH-box helicase (DHX) and the DEAD-box helicase (DDX)\textsuperscript{78,79}. The DHX9 and DHX36 are involved in detecting dsRNA and CpG-rich DNA in human cells. DHX36 induces IFN\(\alpha\) expression and IRF7 nuclear translocation, and DHX9 is associated with the production of TNF\(\alpha\), IL-6, and NF-kB activation.

Another member DDX41 was shown to sense the DNA viruses HSV-1 and AdV in myeloid DC and murine bone marrow-derived DC. DDX41 binds DNA and STING via the DEAD domain\textsuperscript{79}. DDX41 also interacts with c-di-GMP, and knockdown of DDX41 suppresses the c-di-GMP-induced innate immune response. The binding of c-di-GMP enhances the interaction between DDX41 and STING, resulting in the higher binding affinity of c-di-GMP and STING and elevated innate immune response\textsuperscript{80}.

Like other helicases, DDX41 has ATP-dependent DNA-unwinding and ATP-independent strand-annealing activity. A recent study found that DDX41 regulated cGAS-mediated DNA sensing by unwinding and annealing dsDNA and ssDNA. Knockout of DDX41 in cells led to defective cGAS-STING activation. Furthermore, patient-derived DDX41 mutant with reduced unwinding activity enhances cGAS activation\textsuperscript{81}, suggesting
that DDX41 plays an important role in DNA sensing during DNA virus infection by regulating cellular dsDNA and ssDNA homeostasis.

1.2.5. cGAS

cGAS (also known as MAB21) is a nucleotidyl transferase enzyme that belongs to the nucleotidyltransferase (NTase) family. It contains two DNA binding domains and an NTase domain. In the quiescent state, cGAS is autoinhibited; once bound to DNA, cGAS forms a 2:2 complex and initiates the conformational change at the activation site where it uses ATP and GTP to catalyze the synthesis of cGAMP. cGAMP contains two phosphodiester bonds; one is between the 3′-OH of AMP and 5′-phosphate of GMP, and the other is between the 2′-OH of GMP and 5′-phosphate of AMP. cGAMP acts as the second messenger and binds to the endoplasmic reticulum (ER)-membrane protein STING. The binding of cGAMP induces the conformational change and activation of STING. STING traffics from ER to the ER-Golgi apparatus where it recruits TBK1 and induces TBK1-mediated IRF3 phosphorylation. The phosphorylated IRF3 dimerizes and translocates into the nucleus to induce the type I IFN expression. Meanwhile, STING also activates IKK kinase, leading to the phosphorylation and degradation of IkBa in an ubiquitin-proteasome dependent way. Degradation of IkBa releases the NF-kB transcriptional factors, which activates the production of inflammatory cytokines (Fig. 2).
Fig. 2 cGAS-mediated DNA sensing pathway. cGAS detects DNA from viral pathogens or from the self in the cytosol. Upon binding with DNA, cGAS uses ATP and GTP to catalyze the second messenger cGAMP. cGAMP then binds STING and activates STING trafficking. Subsequently, STING recruits and activate TBK1, leading to the nuclear translocation of IRF3 and NF-kB to induce the expression of type I IFN and inflammatory cytokines.

1.3. Roles of cGAS-STING signaling pathway

1.3.1. Viral infection
The critical role of cGAS-STING pathway is to detect the DNA from viruses and alarm our immune system\(^9^3\). cGAS sense DNA from many DNA viruses, including the HSV-1, HSV-2, vaccinia virus (VACV), cytomegalovirus (CMV), human papillomavirus (HPV), adenoviruses and murine gammaherpesvirus 68, and retroviruses, such as HIV-1 \(^9^4-9^8\). Several studies have shown that cGAS knockout mouse cells failed to respond to DNA virus infection and cGAS deficient mice were more susceptible to DNA virus infection compared to wild-type mice \(^8^6,9^5\). Therefore, cGAS is the primary innate immune sensor that detects viral DNA.

Interestingly, the cGAS-STING pathway also plays a role in limiting RNA virus infection. Knockout of cGAS in mice increases the infection of West Nile virus (WNV), a positive sense single-stranded RNA virus \(^9^5\). Moreover, STING deficiency also leads to an increased infection of RNA viruses, including VSV, SeV, HCV \(^9^9,1^0^0\). However, the mechanisms are not well elucidated \(^1^0^1,1^0^2\).

**1.3.2. Self-DNA sensing**

The host cell DNA is usually protected from cGAS due to the physical barriers, such as the nucleus and mitochondria. However, cellular stresses caused by oxidation, viral infection, and the apoptotic process can cause host cell DNA leakage into the cytosol \(^2^3,2^4,1^0^3\). During cell death, nuclear DNA accumulates in the cytosol and activates the cGAS-STING pathway \(^1^0^4\). Meanwhile, the extracellular nuclear DNA from dead cells can also enter the cells through extracellular vesicles such as exosomes and macrovesicles \(^1^0^5\). The mitochondrial DNA (mtDNA) is another potent source of self-DNA that can activate cGAS. During the process of apoptosis and viral infection-induced mitochondria stress, mtDNA is released into the cytosol due to mitochondrial membrane permeabilization \(^1^0^6\). A recent study showed that knockout of
TFAM, an mtDNA packaging factor, resulted in the mtDNA leakage and activation of cGAS-STING signaling pathway. Moreover, both DNA and RNA viruses, such as herpes simplex virus (HSV) and dengue virus, also cause mitochondrial stress, which induces mtDNA leakage into the cytosol and activates cGAS-STING axis.

In the case of defective DNA repair, replication, and mitosis, the cytosolic chromatin or DNA structure and micronuclei can be the potent triggers of cGAS. Micronuclei comprised of chromatin fragments surrounded by the nuclear envelope-like structure such as the DNA double-stranded breaks, DNA replication or mitotic error-derived chromatin fragments, or even the non-segmented chromosomes. Micronuclei usually undergo envelope collapse due to structural defects which results in the exposure of the DNA content and activation of cGAS. During cell senescence, the cells lose the nuclear envelope integrity, which causes the senescence-associated secretory phenotype (SASP). SASP is featured by the production of various cytokines and chemokines. Studies have shown that cellular senescence-induced exposure of cytosolic chromatin can be detected by cGAS-STING and controls the SASP. Dysfunction of telomeres, such as shortening, fusion, and deprotection, causes mitotic delay and defects with dicentric chromosomes, which results in accumulation of cytosolic chromatin and micronuclei thus, activating cGAS. The activation of the alternative lengthening of telomeres (ALT) pathway can cause extrachromosomal telomere repeat DNA, which can activate cGAS. Another source of self-DNA comes from DNA damage response. It was reported that various exogenous genotoxic treatments cause DNA damage and micronuclei. The damaged DNA fragments can translocate to the cytosol and activate cGAS, whereas micronuclei membrane breakdown leads to cytosolic cGAS sequestering to the DNA.

1.3.3. Autophagy
Autophagy is an evolutionarily conserved process that is critical for cellular homeostasis. The autophagy process is modulated by a complex of machinery which targets the damaged or senescent organelles and autophagosomes to lysosomes for degradation or recycling. The autophagy process includes several steps: initiation, nucleation or phagophore formation, autophagosome formation, the fusion of autophagosome with lysosome, and degradation of contents.

In recent years, accumulating studies support a role of cGAS and STING in autophagy process. STING co-localizes with autophagy-related gene (ATG9a) and LC3-II, a marker of the autophagosome, in MEFs upon dsDNA treatment in the first place. Further studies suggest that cGAS/STING induces autophagy through either a conventional or unconventional pathway. For instance, the direct interaction of cGAS and Beclin-1 autophagy protein results in the release of Rubicon, a negative regulator of autophagy, and forms the Beclin-1 complex, thus activating the phosphatidylinositol 3-kinase (PI3K) therefore, initiating autophagy upon dsDNA sensing. cGAMP-induced STING activation initiates autophagy through a TBK1-independent but ATG5 and WIPI2-dependent way. Furthermore, DNA sensing mediated cGAS-STING activation causes p62 phosphorylation which results in the degradation of STING via autophagosome. In addition, STING can also bind directly with LC3, indicating a non-canonical way of autophagy activation.

Taken together, the cGAS-STING axis plays undoubtedly an important role in regulating autophagy. cGAS can induce STING-dependent or independent autophagy to protect the host from endogenous or exogenous threats. It might be STING itself but not its activation is required for initiating autophagy since STING-induced activation of autophagy through a TBK1- and IFN-independent way.
1.3.4. Cancer immunotherapy

Cancer immunotherapy focuses on strengthening antitumor immune response specifically targeting cancer cells. In the early 19\textsuperscript{th} century, researchers used inactivated bacteria toxins to treat sarcomas in patients \textsuperscript{127}. Currently, the groundbreaking discovery of cancer immunology leading the development of immunotherapies such as the immune checkpoint blockade \textsuperscript{128}. The programmed cell death protein 1 (PD-1) on the T cell membrane interacts with its adaptor programmed death-ligand 1 (PD-L1) to avoid the overactivation of T cells \textsuperscript{129}. Cancer cells usually induce PD-L1 expression to inhibit the activation of tumor-specific T cells, thus evading immune surveillance. However, this immune evasion can be blocked by disrupting the interaction between PD-1 and PD-L1. The Food and Drug Administration (FDA) has approved several antibodies for the treatment of tumors by blocking PD-1 or PD-L1 to prevent cancer immune tolerance \textsuperscript{130}.

Although immune checkpoint inhibitors have successfully treated many cancer patients, the overall portion that responds to the treatment is low \textsuperscript{131}. In cancer treatment, a CD8\textsuperscript{+} T cell-rich tumor environment can increase the response of immune checkpoint inhibitors. Type I IFNs have been shown to facilitate CD8\textsuperscript{+} T cell activation in cancer patients through stimulation of CD8\textalpha+ dendritic cells \textsuperscript{132-134}. Among PRRs that activate type I IFN expression, it was reported that only STING knockout mice showed impaired tumor-specific CD8\textsuperscript{+} T cell populations and enhanced tumor growth, suggesting an important role of the cGAS-STING pathway in cancer treatment \textsuperscript{135}. Besides, cGAS has been shown to be essential for the therapeutic effect of PD-L1 antibodies, since antibody-treated cGAS knockout mice have no therapeutic effect on implanted tumors \textsuperscript{136}. Thus, taking its role in activating tumor-specific T cells, the cGAS-STING pathway is a promising target for anti-tumor immunotherapy.
Over the years, many therapeutic strategies targeting cGAS-STING pathway have been tested in preclinical models\(^\text{137}\). cGAMP, the endogenous agonist of STING was shown to recruit CD8+ T cells to the tumor microenvironment and suppress the implanted tumor growth\(^\text{138}\). The combination of cGAMP and immune checkpoint inhibitors can induce a synergistic anti-tumor effect, suggesting that the cGAS-STING activation potentiates the effect of immune checkpoint inhibitors\(^\text{139}\). Although cGAMP can be synthesized directly without toxicity and side effects, the problem of transmembrane delivery of cGAMP limits its application. To address this problem, several cyclic dinucleotides (CDNs), CDN analogs, and small molecule inhibitors were developed such as the 3’5’-c-di-GMP, 3’3’-cGAMP, diABZI, MIW815, and DMXAA\(^\text{139}-\text{142}\). DMXAA is a potent activator of mouse STING. It has been reported that DMXAA significantly reduced implanted tumor growth such as non-small-cell lung cancer (NSCLC), melanoma, colon cancer, breast cancer, and glioma\(^\text{143}-\text{145}\). DMXAA-induced STING activation initiates the type I IFN response which results in the recruitment of more macrophages, neutrophils, and tumor-specific T cells to the tumor microenvironment. However, DMXAA failed to protect the mice from human breast cancer xenografts and metastatic NSCLC\(^\text{143}\).

Tumors in patients exhibit various genetic backgrounds and exploit complex immune evasion strategies, therefore combining the anti-tumor effect of cGAS with other cancer treatments might elicit better outcomes\(^\text{137}\). The radiotherapy usually cannot induce a systemic antigen-specific response, but the combination with STING agonist RRCDG can activate a two-step immune response, the T cell-independent and TNFα-dependent necrosis and the CD8+ T cell-dependent control of residual disease\(^\text{146}\). During chemotherapy, epithelial ovarian cancer (EOC) is susceptible to recurrence due to the resistance to platinum drugs, this results in decreased overall survival\(^\text{147}\).
A combination study found that tumor-bearing mice treated with carboplatin and STING agonist 2′3′-c-di-AMP showed significantly longer survival as compared with the mice treated with carboplatin alone. Further transcriptomic profiling and flow cytometry analysis revealed increased tumor-specific T cell activation and antigen presentation in STING agonist treated mice\(^{148}\), indicating the synergistic effect of STING activation on chemotherapy. Moreover, a completed phase 1 clinical trial of combining cGAS-STING agonists and anti-PD-1 antibody therapy elicited potent induction of type I IFN response and elevated response to immunotherapy in 90.9% of the patients bearing advanced metastatic solid tumors\(^{149}\). The combination of PD-1, CTLA-4, and CD47 with cGAS-STING agonists has all been shown to elicit an antitumor effect\(^{148,150}\). Taken together, the cGAS-STING pathway plays an essential role in regulating antitumor immunotherapy. The combination of cGAS-STING agonists with other cancer treatments improves the tumor immunogenicity and enhances tumor cell killing, which ultimately increases the tumor response to immunotherapy.

1.4. Structural basis for cGAS and STING activation

The main DNA binding site of cGAS is a surface groove on the back side of the catalytic domain which is opposite to the cleft of substrate binding\(^ {83-85,87}\). The interaction between cGAS and DNA molecule is mainly mediated by the electrostatic force through the positive-charged residues of cGAS and the sugar-phosphate backbone of DNA molecule, suggesting that DNA binding but not specific DNA sequence or modification plays a major role in its activation. The loop connecting strands of cGAS contain a GS (Gly212-Ser213) motif which is conserved in the NTase family\(^ {83}\). The GS motif bears the major catalytic activity and interacts with the phosphate groups in the substrate. After
binding with DNA, the GS-containing loop adopts a well-defined conformation and forms a short helix in the middle section. In addition, the GS motif locates at the N terminal of the helix that is right next to the two catalytic residues Glu225 and Asp227. The GS motif interacts with the phosphate groups of the substrate in a manner that is similar to other NTases. These findings indicate that DNA activates cGAS mainly through the stabilization of the GS-containing loop in a conformation that is optimal for interacting with the substrate. Moreover, the DNA binding with cGAS also induces conformational changes in the N-lobe of cGAS, resulting in the conformational change of the β strands and the two loops at the catalytic site. These changes were reported to facilitate the activation of cGAS enzymatic activity by promoting the access of substrate to the active site.

In addition to the first binding model of cGAS, other studies have proposed a new binding site of cGAS with DNA. Together with DNA molecule, these two DNA-binding sites of cGAS form a 2:2 cGAS-DNA complex in which each DNA binds two cGAS proteins. The two cGAS proteins also interact with each other directly. Mutations of the key residues in the new DNA binding site of cGAS result in defective cGAS enzymatic activity and type I IFN response in cells. Further studies have shown that 20 bp DNA is sufficient for cGAS binding and 2:2 complex formation; however, the full activation of cGAS requires longer DNA molecule because multiple cGAS dimers can form a ladder-like complex with long DNA molecules.

Another form of cGAS-DNA complex facilitating its activation is the liquid droplet. This process is called the DNA-induced liquid-liquid phase separation (LLPS) of cGAS. LLPS is a process that the homogenous liquid solution starts to separate into a condensed phase and a dilute phase. In cells, LLPS is responsible for the formation of membrane-less structures, such as the P bodies and stress granules. The N terminal of cGAS is
highly positively charged and disordered and binds with DNA. A recent study found that there is a third DNA binding site (site C) that is important for cGAS activation. In total, each cGAS has more than two DNA binding sites and the oligomerization of cGAS even increases the binding ability with multiple DNA molecules. Thus, the formation of a multivalent complex of cGAS and DNA results in the induction of liquid droplet. cGAS in the liquid droplet is activated more efficiently as the cGAS enzyme is concentrated.

STING contains a short N-terminal cytosolic domain, four transmembrane (TM) helices, a cytosolic ligand-binding domain (LBD), and a C-terminal tail (CTT) domain. The LBD domain forms as a constitutive dimer at steady state which generates a V-shaped ligand binding pocket capable of binding 2′,3′-cGAMP and related CDNs. Once bind with cGAMP, the STING dimer undergoes a conformational change, leading to a 180° clockwise rotation of the LBD domains in relation to the TM domains. This change releases the right-handed crossover of the connector helices and transforms the two loops into a lid structure, resulting in the closure of the ligand binding pocket. This conformational change is mainly mediated by the cGAMP-induced unwinding of connector loops between the LBD and TM domains. After the rotation, a loop that locates on the side of LBD converses to a less extended conformation and mediates the interaction between STING dimers, which results in the oligomerization of STING. The cryo-EM data showed that the cGAMP-bound STING exhibits as tetramers and the two STING dimers interact with each other by side-by-side packing. STING mutations, such as V147L, N154S, and V155M at the connector helix domain between LBD and TM, can cause autoinflammatory diseases. In the structure of apo-STING, the two connector helices are very close to each other, mutations of amino acid such as mentioned above may result in the spontaneous rotation of the LBD in relation to the TM, leading to the aberrant activation of the signaling pathway and cause inflammatory diseases. Other STING mutation sites,
such as Cys206, Arg281, and Arg284, are located close to the tetramer interface of STING. Mutations of these amino acids may cause the release of STING oligomerization inhibition and constitutive STING activation $^{161}$. A recent study showed that the Cys148 of STING can form a disulfide bond and cross-link STING dimers, which further stabilizes the formation of STING oligomer $^{162}$. Cys88 and Cys91 of STING are palmitoylated in the Golgi, which are important for STING oligomerization $^{163,164}$.

1.5. Regulation of cGAS-STING signaling pathway

1.5.1. Regulation of cGAS subcellular localizations

Although cGAS was initially found to be a cytosolic protein, accumulating studies have demonstrated that cGAS is a multi-compartment protein. It has been reported that cGAS localizes on the cell membrane in human and mouse macrophages during the rest state $^{165}$. The N terminus of cGAS is highly polarized and positively charged, which binds the negatively charged phosphatidylinositide phosphates (PIPs) of plasma membrane through electrostatic force $^{166}$. It is reported that cGAS localization on the cell membrane prevents its activation by self-DNA $^{165}$.

In 2020, several groups reported the EM structure of cGAS bound to the nucleosome, demonstrating the predominant localization of cGAS in the nucleus. Moreover, the cGAS localization in the cytosol and nucleus has also been shown to depend on cell type, cell density, and cell differentiation $^{115,167,168}$. It was reported that the cGAS 169-174 amino acid is a nuclear export signal (NES) which dictates the cytoplasm translocation of cGAS from the nucleus in a chromosome region maintenance 1 (CRM1)-dependent manner $^{169}$. However, the 169-174 amino acids of cGAS are required for the DNA binding ability of cGAS and may directly impair cGAS
A recent study showed that cGAS can rapidly enter the nucleus in a large amount during DNA damage response, which does not require the nuclear membrane rupture. Under physiological conditions, the B-lymphoid tyrosine kinase (BLK) interacts and phosphorylates cGAS in the cytoplasm which results in the retention of cGAS in the cytosol. Upon DNA damage, cGAS is dephosphorylated and translocated into the nucleus to suppress the homologous recombination (HR)-mediated DNA damage repair process. Moreover, cGAS also contains a nuclear localization sequence (NLS) that was reported to mediate its translocation into the nucleus through interacting with Importin α. Therefore, together with NES, cGAS shuttling between cytosol and the nucleus is tightly regulated. A recent study found that cGAS is tethered to the chromatin through the DNA binding sites of cGAS and the H2A-H2B in the nucleosomes. The DNA binding site mutation R255E of human cGAS significantly decreased the binding with the nucleosome core particles (NPCs), but this mutant remains in the nucleus. The other DNA binding site mutation C396A/C397A was shown to reduce cGAS nuclear localization. Besides, both the N terminal 1-160 amino acid of cGAS and the C terminal catalytic domain 161-522 amino acid of cGAS can localize in the nucleus, implying that separate nuclear localization signals in cGAS. In addition, we and others found that the deletion of the N terminal 160 amino acid of cGAS resulting its translocation to mitochondria since the deletion of N terminal 160 amino acid exposes the mitochondrial targeting sequence (MTS) within cGAS. However, under physiological conditions whether cGAS can be processed to expose the MTS and translocate to the mitochondria is not clear.

1.5.2. Post-translational modifications of cGAS

Given its critical role in regulating DNA-mediated innate immune response, cGAS activity must be tightly controlled to avoid unwanted activation. Thus, the host cells...
developed many strategies to regulate cGAS activity, including post-translational modifications.

Ubiquitination is a major post-translational modification that plays an indispensable role in regulating protein degradation, cell cycle progression, DNA damage response, endocytosis, and signal transduction. It has been reported that the tripartite motif-containing (TRIM) E3 ligase TRIM56 mediates the monoubiquitination of cGAS. The monoubiquitination of cGAS at Lys335 promotes cGAS activation by enhancing cGAS-DNA binding, dimerization, and cGAMP production. TRIM41 was also found to interact with cGAS and promote its monoubiquitination; however, the modified lysine residue remains unknown. Knockout of TRIM41 results in a defective type I IFN response in cells. cGAS also undergoes polyubiquitination. For example, the ER locating protein RING finger (RNF) containing E3 ubiquitin ligase RNF185 mediates the K27-linked ubiquitination of mcGAS on the Lys173/Lys384 residues. RNF185 overexpression increases the K27 ubiquitination of cGAS and its enzymatic activity. Another TRIM protein TRIM14 regulates cGAS activity through a distinct mechanism. Unlike other TRIM proteins, TRIM14 lacks the RING domain that confers E3 ligase activity. TRIM14 recruits the deubiquitinase USP14 to cleave the K48-linked polyubiquitin chain on Lys414 of cGAS, thus inhibiting the cGAS lysosomal degradation. Moreover, TRIM14 was also found to bind with cGAS and TBK1, promoting STAT3 phosphorylation and induction of ISG expression during *Mycobacterium tuberculosis* infection. Like TRIM14, USP27X, and USP29 were also found to mediate the cleavage of K48-linked poly-ubiquitin chains of cGAS, thus promoting cGAS-mediated antiviral innate immune response during DNA virus infection.
SUMOylation is a ubiquitination-like protein modification that regulates protein fate and function. The small ubiquitin-like modifier (SUMO) is mainly in the nucleus and regulates cell pathophysiological processes, such as DNA damage response, cancer development, and innate immunity. It has been reported that TRIM38-mediated SUMOylation of Lys217 and Lys464 prevents cGAS from K48-linked ubiquitination-directed degradation, therefore increasing cGAS protein stability. In the late stage of DNA virus infection, the deSUMOylation enzyme Sentrin/SUMO-specific protease 2 (SENP2) removes the SUMO chain from cGAS, which allows the K48-linked ubiquitination of cGAS and promotes its degradation. Moreover, SUMOylation of mouse cGAS on Lys335/Lys372/Lys382 residues inhibits cGAS binding with DNA, oligomerization, and its enzymatic activity. On the contrary, SENP7 can remove the SUMOylation from cGAS; thus, releasing the suppression of cGAS activity and restoring the cellular DNA sensing.

Glutamylation is an ATP-dependent post-translational modification of target protein, which usually regulates the microbial infection. The glutamylase is responsible for the conjugation of the glutamate chain to the γ-carboxyl groups of glutamic acid residues on the target protein. cGAS was reported to be glutamylated by tubulin tyrosine ligase-like 4 (TTLL4) and TTL6. TTLL4 catalyzes the mono-glutamylation of cGAS while TTLL6 catalyzes the poly-glutamylation of cGAS, and both modifications lead to the impaired cGAS enzymatic activity. The carboxypeptidases CCP5 and CCP6 can remove the TTLL4/6-mediated glutamylation of cGAS and restore the cGAS enzymatic activity.

Phosphorylation is an extensively studied protein modification that regulates protein functions involving cell cycle arrest, tumorigenesis, and innate immunity. Mouse cGAS was first reported to be regulated by AKT-mediated phosphorylation at
Ser291 that localizes in the C-terminal enzymatic domain of cGAS. Ser291 phosphorylation results in the inhibition of cGAS activation during viral infection. Moreover, the CDK1-cyclin B kinase was also reported to phosphorylate human cGAS at Ser305 (Ser291 in mouse cGAS) and leading to the suppression of cGAS activity in mitotic cells. By contrast, the protein phosphatase 1 (PP1) dephosphorylates cGAS upon mitotic exit and restores its DNA sensing activity. Interestingly, another phosphatase PP6 dephosphorylates mouse cGAS at Ser420, leading to inhibiting cGAS activity. In addition, the B lymphocyte kinase (BLK) was shown to phosphorylate human cGAS at Tyr215, resulting in the retention of cGAS in cytosol.

Acetylation has been well-documented as the critical regulation of the epigenetic function of gene transcription. The acetyl-transferase and deacetylase are responsible for this modification on the lysine residues of target protein. Studies have shown that hcGAS can be acetylated at Lys384/Lys394/Lys414 during a steady state, thus inhibiting cGAS activation. Aspirin was found to directly acetylate cGAS and inhibit cGAS-mediated innate immune response, which explains the use of aspirin in treating autoimmune diseases like the Aicardi–Goutieres syndrome (AGS). On the other hand, the lysine acetyltransferase KAT5 has been shown to acetylate hcGAS at Lys47/Lys56/Lys62/Lys83 residues. This modification results in the enhanced DNA binding and activation of cGAS. A proteomic study found that hcGAS Lys198, Lys285, Lys355, and Lys414 are acetylated, and the Lys414 acetylation inhibits cGAS activation, whereas the acetylation of Lys198 promotes cGAS activity. During HSV-1 infection, cGAS acetylation at Lys198 was decreased, indicating that DNA virus may exploit this regulation to benefit their infection.

In addition to the direct modifications on cGAS protein, various cGAS binding proteins from the host cells have been shown to regulate cGAS activity and the innate
immune response as well. The GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) was reported to physically bind with cGAS. G3BP1 deficiency results in decreased DNA binding and suppressed cGAS-mediated innate immune response. The binding of G3BP1 enhances the DNA binding with cGAS by facilitating the formation of cGAS oligomers, thus priming cGAS activity. The poly(rC)-binding protein 1 (PCBP1) was also shown to interact with cGAS. PCBP1 directly binds with DNA and promotes the interaction between cGAS and DNA, therefore, enhancing cGAS activation during DNA virus infection. During HIV infection, the polyglutamine binding protein 1 (PQBP1) was shown to bind with the HIV-1 DNA and interact with cGAS, facilitating the sensing of HIV-1 and cGAS-mediated antiviral innate immune response. The CCHC-type zinc-finger protein, ZCCHC3 was reported to facilitates cGAS-mediated DNA sensing. It binds with DNA molecules and serves as a bridge and enhances the binding of DNA with cGAS, thus, promoting cGAS activation. ZCCHC3 deficient mice showed increased DNA virus infection.

The cellular autophagy protein Beclin-1 directly interacts with cGAS and inhibits the cGAMP production. Furthermore, this interaction also activates the autophagy process and removes the pathogen DNA in the cytosol, thus inhibiting cGAS activation. Oligoadenylate synthetase, OASL, binds with cGAS in a DNA-independent manner, leading to non-competitive suppression of cGAMP production and decreased activation of innate immune response against virus infection. Moreover, TRIM14 was shown to bind cGAS directly and bridge the TBK1 and STAT3 to facilitate STAT3 phosphorylation, leading to decreased ISG expression. SAMHD1 was reported to mediate the clearance of cellular DNA, thus limits cGAS-mediated innate immune response.
Self-DNA of the cell can activate cGAS signaling pathway and results in aberrant activation of IFN production and autoimmune diseases. Although mainly localized in the nucleus, cGAS is kept as an inactive form by its binding proteins. The barrier-to-autointegration factor 1 (BAF), a chromatin-binding protein, dynamically competes the binding of DNA with cGAS and suppresses the formation of the cGAS-DNA complex, thus inhibiting cGAS activation \(^{203}\). Furthermore, the tightly tethering of cGAS on the H2A/H2B acidic patch residues also keeps cGAS from binding with DNA and limits its activation \(^{171}\). Except for the nuclear DNA, the mitochondrial DNA is also a potent activator of cGAS. Our recent study found that during DNA virus infection, the mitochondrial protein C1QBP leaks from the mitochondria into the cytosol and binds with cGAS, resulting in impaired cGAMP production and suppressed activation of cGAS-mediated innate immune response \(^{204}\).

Token together, cGAS activity is tightly controlled by various post-translational modifications to maintain the proper function of cGAS-mediated innate immune response and avoid autoimmune diseases. Currently, most of the studies focus on one single type of modification of cGAS. How the different modifications crosstalk with each other and regulate cGAS activity remains to be elucidated.

1.5.3. Liquid-liquid phase separation regulates cGAS activation

Liquid-liquid phase separation (LLPS) is an environment- and concentration-dependent condensation process that is driven by the solute-solute interaction. In cells, the solute usually is protein, nucleic acid, or other metabolites. During the liquid-like state, the concentrated phase can exchange components with the dilute phase. In some cases, the liquid condensates can transit to a gel-like or solid phase in which the components are highly cross-linked. During the cellular signaling process, LLPS
concentrates signaling molecules in a micrometer scale through weak interactions, so that the components can efficiently exchange with the environment, thereby promoting the enzymatic reaction within the condensates.

Recently, cGAS was reported to form a liquid phase condensate with DNA to promote innate immune signaling \(^{153}\). cGAS binds with DNA and induces the phase separation to liquid-like droplets, which function as a microreactor where the reaction components are highly concentrated, thus, promoting the production of cGAMP \(^{153}\). LLPS allows cGAS to sense the DNA in cytosol above a certain threshold to trigger a switch-like response. The binding between cGAS and DNA is potentiated by the Zn\(^+\) ions, thus promoting their phase separation at physiological salt concentrations \(^{153}\). Furthermore, studies found that the induction of cGAS phase separation facilitates cGAS enzymatic activity by preventing DNA degradation by exonuclease TREX1 \(^{205}\).

Interestingly, a recent study reported that high concentration of dsRNA can compete with dsDNA for cGAS binding and suppress cGAS activity, and low concentration of dsRNA facilitates the cGAS phase separation and cGAMP production \(^{206}\). The tumor-associated mutations G303E and K432T of cGAS disrupt the LLPS of cGAS, leading to decreased cGAMP production and immune activation \(^{155}\). Furthermore, microbial pathogens also alter LLPS of cGAS to regulate its activity. Streptavidin secreted by \textit{Streptomyces avidinii} can bind cGAS and promote the cGAS-DNA interaction, which facilitates the phase separation of cGAS and its activation \(^{207}\). The herpesvirus proteins ORF52 and VP22 can disturb the formation of cGAS-DNA condensate and suppress cGAS activity. ORF52 competes with cGAS for the binding with DNA, and DNA preferentially forms condensate with ORF52 even though ORF52 has lower binding-affinity with DNA compared with cGAS \(^{208}\), suggesting the LLPS may affect the interaction pattern of condensate components. G3BP1, an important factor that
regulates the formation of stress granules (a cytosolic phase separation structure consisting of RNA and proteins induced by cellular stress), regulates cGAS activity. Studies have shown that G3BP1 knockout cells have significantly decreased the formation of cGAS condensates and the modulation of G3BP1 on cGAS phase separation is independent of stress granule formation. The mechanism of how G3BP1 maintains cGAS phase separation and stress granules remains unclear.

Most recent studies found that STING also forms condensates with staked endoplasmic reticulum membrane during the presence of an excessive amount of cGAMP. The STING condensates constrain STING and TBK1 from activating IRF3, therefore inhibiting the activation of innate immune response. Moreover, another study reported that the PC7A, a multivalent STING agonist, induces STING condensation and initiates prolonged activation of pro-inflammatory cytokines production through direct binding with a non-competitive site distinct from cGAMP binding pocket.

Taken together, liquid-liquid phase separation plays a critical role in regulating cGAS-STING signaling and innate immune responses. LLPS can either promotes or suppress cGAS activity., which might be dependent on the interaction patterns of the component within the condensates.

1.5.4. Regulation of STING membrane trafficking

During the steady state, STING stays on the ER membrane. Once bind with CDNs such as cGAMP, STING translocates to the Golgi apparatus. Studies have shown that brefeldin A (BFA) treatment inhibits STING trafficking, which results in the impaired phosphorylation of IRF3 and production of IFN, indicating that STING ER-
Golgi translocation is important for immune signaling\textsuperscript{212}. STING ER-Golgi traffic is found to be related to the coatamer protein complex II (COPII), which is a protein complex that is responsible for encapsulating into vesicles and bud from ER\textsuperscript{213}. The COPII complex consists of Sar1 (a GTPase), Sec23, Sec24, Sec13 and Sec31. STING was shown to interact with Sar1, Sec24, Sec23B, Sec31A, and Sec13\textsuperscript{124,214}. Knockdown of Sar1A and Sar1B, the paralogs of Sar1, leads to the inhibition of STING trafficking from the ER to Golgi and downstream signaling\textsuperscript{215}. The YIP1 domain family (YIPF) proteins have been shown to associate with COPII and regulate STING trafficking. YIPF5 was shown to facilitate STING with the COPII vesicle to exit from ER. Knockdown of YIPF5 results in decreased formation of STING puncta at the perinuclear region and IFN production\textsuperscript{214}. The transmembrane emp24 domain (TMED) family protein TMED2, a single transmembrane protein that regulates ER membrane trafficking, was shown to facilitate STING exit from ER during HSV-1 infection\textsuperscript{216}. Similarly, the inactive rhomboid protein 2 (iRHOM2) was also reported to promote STING trafficking. iRhom2 can recruit the translocon-associated protein TRAPβ to the STING complex, which results in increased translocation of STING to the perinuclear region\textsuperscript{217}.

On the other hand, constitutively active STING mutants are found in patients. These mutants co-localize with the ERGIC and Golgi markers p58 and TGN38, indicating the trafficking of STING\textsuperscript{215}. These STING mutants cause severe autoinflammatory disease, termed the STING-associated vasculopathy with onset in infancy (SAVI), due to constant activation of STING signaling\textsuperscript{90}. Recently, the calcium-sensing protein, Stromal interaction molecule 1 (STIM1) was reported to play a role in triggering STING exit from ER. STIM1 serves as an anchor that keeps STING to stay on ER membrane during the steady state. Ectopic expression of STIM1 decreases the
constitutive production of IFN mediated by STING SAVI mutants. STIM1 also decreases the translocation of STING to ERGIC and Golgi. Moreover, STIM1 deficiency results in a low basal level of wild-type STING trafficking and inflammation. The interaction between STING and STIM1 can be disrupted by the binding of cGAMP, therefore triggering the STING trafficking. Other studies showed that TMEM203 can compete the binding of STING with STIM1, thus inducing an inflammatory response. TMEM203 was also shown to be upregulated in T cells from patients with systemic lupus erythematosus (SLE), suggesting a potential role of TMEM203 in regulating STING trafficking. Moreover, the calcium level has been shown to regulate STING activity since the calcium modulators such as BAPTA-AM and ionomycin block STING trafficking and IFN production. However, the mechanism by which STING activity is regulated by calcium flux remains unclear.

The STING ER exit protein (STEEP) was recently found to regulate STING traffic. After STING activation, STEEP can recruit the phosphatidylinositol 3 (PI3) kinase, VPS34 to the ER and facilitate the formation of the PI3-phosphate (PI3P) lipid region in the ER membrane. Subsequent accumulation of PI3P promotes the budding of STING-containing COPII vesicles. Deletion of STEEP in the fibroblast from SAVI patients impaired the production of IFN. In addition to the SAVI, STING was shown to involve in COPA syndrome, an autoimmune disease caused by the mutations of coatamer subunit α (COPA). The deficiency of COPA results in increased IFN production in a STING-dependent manner. In vivo study showed that mice expressing COPA patient mutant had active STING translocation to Golgi under basal conditions. Indeed, further study showed that COPA promotes STING recycling back to the ER. Knockout of COPA leads to the STING accumulation and activation in the Golgi apparatus. Moreover, additional factors such as the surfeit 4 (SURF4)
have been shown to be required for STING to contact with COPA and subsequent trafficking\textsuperscript{224}. Most recently, a research group found that adaptor protein complex 1 (AP-1) controls the termination of STING signaling. They found AP-1 can sort phosphorylated STING into the clathrin-coated transport vesicles (CCVs) and promotes its transport to endo-lysosome thus, degrades and terminates STING-mediated immune signaling\textsuperscript{225}.

1.5.5. Post-translational modifications regulate STING activation

In addition to trafficking, post-translational modification of STING is also important for regulating STING activity. After trafficking to the Golgi, STING undergoes oligomerization and recruits and binds with TBK1. TBK1 then phosphorylates the S366 of STING resulting in its activation\textsuperscript{226}. Another study showed that the C-terminal src kinase (CSK) phosphorylates STING at Y240 and Y245, leading to enhanced STING aggregation and activation of type I IFN signaling during HSV-1 infection\textsuperscript{227}. The protein phosphatase 6 catalytic subunit (PPP6C) was found to negatively regulate STING function since deficiency of PPP6C increases STING phosphorylation and IFN production thus inhibiting virus infection\textsuperscript{228}. The STING Y245 is also phosphorylated by EGFR to facilitate the STING transport to endosome\textsuperscript{229}. After the activation of STING and IRF3 at the endo-lysosome, the UNC-51-like kinase (ULK1) subsequently phosphorylates STING and inhibits its activity and IFN signaling\textsuperscript{212}.

Ubiquitination also regulates STING activity. The RING finger protein RNF115 was reported to mediate the K63-linked polyubiquitination of STING at K224, K20, and K289, which promotes the trafficking of STING from the ER to Golgi\textsuperscript{230}. TRIM56 was also reported to catalyze the K63-linked ubiquitination of STING at K150 upon DNA stimulation, resulting in enhanced STING dimerization and activation\textsuperscript{231}. Further study
found that the ubiquitin regulatory X domain-containing proteins 3b (UBXN3B) is a cofactor that facilitates TRIM56-mediated K63-polyubiquitination of STING thus promoting the STING immune signaling\(^{232}\). A proteomic study found the mitochondrial E3 ubiquitin-protein ligase 1 (MUL1) catalyzes the K63-linked ubiquitination of STING at K224, which is important for its function \(^{233}\). Another study showed that TRIM32 interacts with STING and targets STING for K63-linked ubiquitination at K20, K150, K224, and K236, thus promoting the subsequent binding with TBK1 and activation of innate immune response \(^{234}\). Moreover, the autocrine motility factor receptor (AMFR) was shown to mediate the K27-linked ubiquitination of STING through an insulin-induced gene 1 (INSIG1)-dependent manner, leading to the recruitment of TBK1 and translocation to the perinuclear region \(^{235}\).

STING is also directed to degradation through the ubiquitin-proteasome pathway. RNF5 and RNF90 target STING for K48-linked ubiquitination, which leads to STING degradation and impaired innate immune response during virus infection \(^{236,237}\). In contrast, RNF26-mediated K11-linked ubiquitination of STING at K150 inhibits RNF5-mediated K48-linked ubiquitination, which protects STING from degradation and restores the type I IFN and proinflammatory cytokines production \(^{238}\). Moreover, the TRIM29 and TRIM30\(\alpha\) were shown to mediate the K48-linked ubiquitination of STING and promote its degradation \(^{239,240}\). The death-associated protein kinase 3 (DAPK3) was shown to inhibit STING K48-linked ubiquitination and prevent its degradation. After the cGAMP induction, DAPK3 acts to promote the K63-linked STING ubiquitination mediated by LMO7 and the subsequent binding with TBK1 \(^{241}\).

SUMOylation also plays a role in regulating STING function. TRIM38 was shown to mediate the SUMOylation of STING at K338 inhibiting STING degradation and promoting the STING activation and recruitment of IRF3 \(^{185}\). Palmitoylation, a type of
lipidation, is essential for STING assembly into multimeric complexes and recruiting downstream signaling factors. Studies have revealed that palmitoylation of C88 and C91 of STING facilitates the STING-mediated innate immune responses \(^{164}\). Treatment of 2-bromopalmitate (2-BP), a palmitoylation inhibitor, abolished the IFN and proinflammatory cytokines production, resulting in the relief of autoimmune diseases such as SAVI \(^{164}\). Moreover, several nitrofuranc derivatives such as C-176, C-178, and H-151 have been found to bind STING at C91 and abolish the palmitoylation of STING, resulting in the inhibition of STING oligomerization and activation \(^{163}\).

Taken together, STING activity is tightly controlled by various post-translational modifications to ensure proper activation of STING and benefit the host during physiological processes. Further studies should explore the correlations between different PTMs to better understand the regulation mechanism of STING activity that may provide insight into STING-related immune disease therapy.

1.5.6. Regulation of STING degradation

Upon binding of STING with its ligand, STING translocates from ER to the ERGIC and Golgi. Then at the late endosomes, STING activates TBK1 and IRF3; thus, activating type I IFN production. To avoid sustained immune activation, STING traffics to lysosomes for degradation through the multivesicular body (MVB) pathway with the help of RAB7 GTPase \(^{124}\). STING degradation requires exit of ER and the function of the vacuolar ATPase complex. At the late stage of trafficking, STING-containing vesicles are sorted to Rab7-positive endo-lysosomes for degradation. STING degradation requires lysosomes but not autophagosomes \(^{242}\). Further studies found that TOLLIP directly interacts with STING and prevents its degradation during the resting state. Thus, TOLLIP acts as a stabilizer that protects STING from degradation
by IRE1α-lysosome. The Niemann–Pick type C1 (NPC1), a lysosome membrane protein, interacts with STING and recruits STING to the lysosome for degradation in human and mouse cells. Loss of NPC1 results in STING accumulation and elevated innate immune signaling. The VPS13C, a lysosome membrane protein, is also required for STING degradation. Deficiency of VPS13C leads to increased STING signaling. Furthermore, the UNC93B1 interacts with STING and facilitate the delivery of STING to the lysosome for degradation.

Furthermore, the adaptor protein complex 1 (AP-1) sorts phosphorylated STING into the clathrin-coated transport vesicles for the delivery to the endo-lysosome. Interestingly, two recent studies proposed that the endosomal sorting complexes required for transport (ESCRT) controls the degradation of STING. They found that upon activation STING undergoes UBEN2-mediated ubiquitination and ATG5/ATG16L1-dependent LC3B lipidation of STING-containing vesicles. Then ubiquitinated STING interacts with ESCRT at the late endosomes, which initiates the fusion with endo-lysosome, resulting in STING degradation. Mutations of ESCRT-I subunit UBAP1 or the knockdown of Tsg101 and Vps4, components of ESCRT, lead to the retention of STING vesicles in the cytosol and exacerbated STING signaling. During the steady state, Matteo et al. proposed that cGAS primes STING trafficking and the ESCRT serves to remove the activated STING, thus avoiding constitutive activation of innate immune responses. In addition, Yoshihiko and colleagues proposed that after the encapsulation of activated STING/TBK1 complex, STING signaling is terminated even though the STING degradation is impaired. However, the loss of lysosome degradation has been shown to cause STING accumulation and elevated innate immune signaling, thus further studies may need
to investigate the cross-talk between the known regulators and elucidate whether there are alternative pathways for STING degradation.

1.6. Viral evasion strategies targeting cGAS and STING

To antagonize the cGAS-STING pathway, most DNA viruses protect their DNA genomes in the viral capsid while in the cytosol and only expose the DNA genome in the nucleus. Studies showed that mutations altering viral capsid stability resulted in the activation of cGAS \(^{98,249}\). In addition, DNA and RNA viruses also encode proteins that target cGAS for degradation, thus suppressing immune sensing. The dengue virus (DENV) protease complex NS2B3 mediates the cleavage of cGAS during infection, thus inhibiting cGAS activation \(^{250}\). The Zika virus (ZIKV) NS1 protein stabilizes the caspase-1 protein in the cytosol, leading to enhanced cGAS cleavage by caspase-1 \(^{251}\). Moreover, the vaccinia virus (VACV) expressing F17 protein dysregulates mTOR, thus enhancing the mTOR-dependent cGAS degradation \(^{252}\).

Furthermore, HSV-1 viral protein VP22 and human cytomegalovirus (HCMV) protein pp65 bind with cGAS and inhibit cGAS-mediated type I IFN signaling \(^{253,254}\). Herpesvirus ORF52 and VP22 disrupt the cGAS-DNA LLPS to counteract antiviral signaling \(^{208}\). The Kaposi’s sarcoma-associated herpesvirus (KSHV) encodes the tegument protein ORF52 which impairs cGAS enzymatic activity by directly binding with cGAS and DNA, thus inhibiting the antiviral immune signaling \(^{255}\). Recently, HSV-1 was shown to induce the cellular DNA damage response, which results in a DNA-PK-dependent suppression of cGAS activity \(^{256}\). Moreover, HCMV UL31 and UL42 proteins disrupt the cGAS binding with DNA and inhibit the cGAS oligomerization, leading to impaired cGAS immune signaling and type I IFN production \(^{257,258}\). The cytoplasmic isoform of KSHV LANA protein directly binds with cGAS and inhibits cGAS-mediated antiviral signaling \(^{259}\).
Viruses also target STING to sabotage the signaling pathway. VACV and other poxviruses encode the nuclease called poxvirus immune nuclease (poxin) that can degrade cGAMP, thus, inhibiting cGAS-STING signaling. The DENV and ZIKV viral protease complex NS2B3 mediates the proteolytical cleavage of STING. HCMV IE86 protein facilitates the proteasome degradation of STING, leading to the block of type I IFN production. Viruses also modulate the PTM of STING to regulate its activity. The Hepatitis C virus (HCV) NS4B and the HCoV-NL63 papain-like protease (PL-pro) have been shown to inhibit STING oligomerization. The deubiquitinase activity of PL-pro is required for the suppression of STING aggregation. The human T-lymphotropic virus 1 (HTLV-1) Tax protein inhibits the K63-linked ubiquitination of STING, resulting in the disruption of STING-TBK1 binding and subsequent induction of IFN. Hepatitis B virus (HBV) polymerase interacts with STING and disrupts the K63-linked ubiquitination of STING, leading to inhibition of STING activity. The DNA virus infection-induced expression of MPN domains 1 (MYSM1) interacts with STING and removes the K63-linked ubiquitin chain of STING, leading to impaired STING activity. KSHV ORF48 interacts with PPP6C and exploits PPP6C to decrease the phosphorylation of STING, thus suppressing the immune signaling. Moreover, KSHV ORF33 interacts with STING and increases the PPM1G-mediated dephosphorylation of STING, which inhibits the innate immune signaling.

STING trafficking is also targeted by viruses to inhibit IFN induction. The HCMV tegument protein UL82 interferes with the binding of STING with iRhom2 and TRAPβ, which are required for STING trafficking. The UL42 of HCMV induces the degradation of TRAPβ, which blocks STING trafficking and suppresses the signaling pathway. KSHV vIRF1 protein inhibits the interaction between STING and TBK1, leading to impaired STING activation and IFN production. Similarly, the Avian oncogenic herpesvirus
Marek’s disease virus (MDV) expresses the oncoprotein Meq that inhibits the recruitment of TBK1 to STING, thereby inhibiting the antiviral immune response. The E7 protein of human papillomavirus (HPV) and the E1A protein of adenovirus inhibit STING-mediated signaling. HSV-1 viral protein ICP27 interacts with the STING-TBK1 complex and inhibits the recruitment of IRF3, resulting in the impaired downstream signaling.

1.7. Perspective

1.7.1. Biological significance of nuclear cGAS.

Since the discovery that cGAS predominantly localizes in the nucleus, many questions have been raised about its functional mechanism. The outstanding question is how cGAS activity is regulated within the nucleus, a DNA-rich environment. Recent structural studies revealed that nuclear cGAS is tethered to nucleosomes and kept inactive. The highly conserved residue of arginine 255 in hcGAS (mcGAS R241) acts as an anchor that tightly binds to the H2A acidic patch at E61, D90, and E92. By reconstitution of the human nucleosome core particles (NCPs) on synthetic DNA-positioning sequence, cGAS was found to interact with NCP through two tethering loops that both contain conserved amino acid residues, the hcGAS R255 (mcGAS R241) and hcGAS R236 (mcGAS R222), as well as another interacting domain, the cGAS-DNA binding site B to form a sandwich-like structure. The binding of the enzymatic domain of cGAS with the acidic patch of H2A-H2B hides the DNA binding site B of cGAS, limited access to DNA results in defective cGAS dimerization and activation. Although the DNA binding site A of cGAS is exposed in the cGAS-NCP interacting complex, mutations of site A did not change the binding affinity of cGAS with nucleosomes nor with the mitotic chromosomes. Site C in the α-Helix or KRKR loop of human cGAS might interact with DNA around the superhelical location 3 or 4 (SHL3 or SHL4) only to bridge the NCPs but not form oligomers. Disruption
of the cGAS-nucleosome acidic patch interactions resulting in the activation of cGAS-mediated innate immune response. These studies indicate that nuclear cGAS is tightly restrained on chromatin, which prevents the activation of cGAS by self-DNA.

Nuclear cGAS activity is also regulated by phosphorylation. At the mitotic phase (G2/M and M phase), cGAS is hyperphosphorylated at multiple serine and threonine residues within the N-terminus. Mutation of the 20 potential phosphorylation sites within the N terminus with phosphomimetic aspartate or glutamate (20DE) suppresses cGAS activity.

It is also interesting to know why the host keeps cGAS in an environment surrounded by DNA, which can lead to its activation and even autoimmune disorders. One possible answer is that the nucleus is a reservoir for cGAS to keep a low level of cytosolic cGAS. Thus, upon DNA stimulation, cytosolic cGAS protein levels can increase quickly without the need of the slow transcriptional process. On the other hand, DNA viruses are known to replicate in the nucleus and with their genome hidden while trafficking in the cytosol to avoid immune detection, thus cGAS might detect DNA virus infection within the nucleus. Indeed, our lab recently found that HSV-1 infection causes the release of cGAS from chromatin into the nuclear soluble component. The released nuclear soluble cGAS detects DNA stimulation and induces the production of type I IFN, which elicits antiviral activity against HSV-1 infection. Others found that HSV-1 genome DNA is packaged into nucleosomes after entry of the nucleus, some DNA viruses also encode and form nucleosome-like particles containing an acidic patch, suggesting a novel mechanism the virus may use as an immune evasion strategy.
Nuclear cGAS also plays a role in DNA repair and other processes. For example, cGAS plays a role in regulating tumor development by suppressing DNA repair. Nuclear cGAS inhibits DNA double-stranded break (DSB) repair by homologous recombination (HR), which increases the genome instability, resulting in tumorigenesis and cell death. Nuclear cGAS also interacts with PARP1 and impedes the formation of PARP1-Timeless complex, therefore inhibiting HR \(^{170}\). In addition, cGAS inhibits replication-associated DNA damage, thus suppressing DNA replication. cGAS oligomerization induces the compaction of template DNA into a high-order state, which is less accessible for strand invasion by RAD51 ssDNA filaments thus inhibiting the HR \(^{167,280}\). Moreover, cGAS was reported to regulate histone modification during RNA virus infection. Nuclear cGAS interacts with the protein arginine methyltransferase 5 (Prmt5) which mediates the symmetric demethylation of histone H3 thus promoting IRF3 binding \(^{281}\).

Taken together, cGAS in the nucleus is tightly controlled and plays roles in various cellular processes. Further study may focus on how cGAS comes off from the nucleosome tethering and elicits DNA sensing function or other functions.

1.7.2. STING: beyond interferon

Despite the well-studied role of STING signaling in mediating type I IFN production, the IFN-independent functions of STING have also been discovered in recent years. These new insights indicate that STING is not just solely a signaling adaptor that controls the IFN pathway, it acts as a hub that governs multiple cellular biological processes independent of IFN.
The deoxyribonuclease II (DNase II) is a lysosome endonuclease that is responsible for the digestion of cellular DNA from apoptotic cells. Deficiency of DNase II can cause STING-dependent and IFN-independent human polyarthritis. Studies have shown that DNase II knockout mice are embryonic lethal because of the self-DNA induced activation of IFN signaling\textsuperscript{282}. Furthermore, the DNase II and type I IFN receptor (IFNAR) double knockout mice can survive but have severe early onset of polyarthritis. Interestingly, deficiency of STING in the DNase II/ IFNAR double knockout mice rescues the phenotype of polyarthritis, implying an IFN-independent role of STING in self DNA caused polyarthritis\textsuperscript{283,284}. The STING-associated vasculopathy with onset in infancy (SAVI) is caused by the gain-of-function mutation of STING which leads to its constitutive activation. Studies found that SAVI development is independent of IFN in mouse models, the ER stress-induced T cell death plays a role in the disease onset\textsuperscript{285,286}. Moreover, clinical treatment of SAVI using JAK1/2 inhibitors was shown to have partial protection of the patients, suggesting the STING-mediated IFN independent response may play a role\textsuperscript{287}. STING has also been shown to be involved to human diseases where the role of IFN is unclear. For example, the STIM1 defect causes constitutive STING trafficking and IFN response, which are similar to the autoimmune complications in STIM1 deficient patients, however, whether IFN induces the disease or not remains to be elucidated\textsuperscript{218}. Defects in COPI-mediated STING trafficking induce STING-IFN signaling and COPA syndrome, which elicits similar clinical presentation with SAVI patients, however, the role of IFN in COPA syndrome is unclear\textsuperscript{224,288}.

In addition to the role of STING in autophagy as I discussed above in this article. STING has also been shown to mediate T-cell death. Studies have shown STING agonists can induce cell stress and lead to T cell death\textsuperscript{289}. Further studies revealed
that IRF3 and p53 might be involved in the STING-mediated T cell apoptosis. However, STING activation by agonists in myeloid cells does not induce cell death.

On the other hand, viral infection can induce STING-dependent cell death in monocytes. HTLV-1 infection of monocytes induces STING activation which then initiates the translocation of IRF3 to the mitochondria, leading to Bax-mediated apoptosis. Studies also showed that the translocation of STING to lysosome induces membrane permeabilization and lysosomal cell death, which further initiates potassium efflux and NLRP3-mediated pyroptosis. Therefore, STING-dependent cell death seems to be cell type and case specific. However, in T cell death, STING was shown to be the dominant factor. The STING N153S mutant mice were shown to suffer from T cell cytopenia, which is independent of cGAS, IRF3, and IFNAR. The N153S mutation constitutively drives STING to translocate from the ER to Golgi which disrupts the ER calcium homeostasis and induces ER stress, leading to the induction of unfolded protein response (UPR). This suggests that STING may elicit its function by regulating ER membrane homeostasis.

In recent years, accumulating studies demonstrate the IFN-independent antiviral activity of STING. RNA viruses are known to not directly activate cGAS or STING. However, STING deficiency in cells and mice leads to increased infection of RNA virus. Studies have shown that STING can inhibit virus translation initiation resulting in decreased viral and host protein production. In addition, several papers have reported that STING can protect the host against HSV-1 infection independently of IFN. They showed that the STING-S356A mice, which cannot induce IRF3 activation but retains NF-κB activation, were resistant to HSV-1 infection. Moreover, another study showed that the STING L373A and STING ΔCTT mice, which cannot recruit TBK1 and activates IRF3 and NF-κB, were susceptible to HSV-1 infection.
Since STING L373A mice can induce autophagy normally, these studies together demonstrated the IFN and autophagy-independent STING antiviral activity, and the NF-κB activation may play a role. Recently, our study found that STING inhibits human coronavirus infection independent of IFN. STING binds with the coronavirus non-structural proteins NSP4 and NSP6 and inhibited the formation of viral double-membrane vesicles (DMV). Deficiency of STING in IFNAR-blocked primary MEFs leading to increased coronavirus infection (data haven't been published). Taken together, STING is a multifunctional protein, which is not only just an IFN signaling factor, but also plays an important role in various cellular responses. Further studies are still needed to elucidate the IFN-independent activity of STING to understand its nature in regulating cellular physiology.
Significance and Innovation

The cGAS-STING pathway is critical for sensing the invasion of microbial pathogens, such as HSV-1. A recent study found that HSV-1 infection induces the escape of mtDNA into the cytosol, where it is sensed by cGAS and activates the antiviral IFN response \(^{103}\). However, how this process would benefit the HSV-1 infection is unknown. The protective mechanism to avoid mitochondria stress-induced undesirable immune response remains to be elucidated. Moreover, the cGAS-STING pathway has been shown to have antiviral activity against RNA viruses. Recent studies also revealed that STING exhibits anti-SARS-CoV-2 activity in an IFN-dependent manner \(^{298, 299}\). However, MDA5 but not STING is the sensor of SARS-CoV-2 infection. Thus, how STING elicits antiviral activity against coronavirus is not known.

To understand the cGAS regulatory mechanism and the antiviral role of the cGAS-STING pathway. We first performed affinity purification coupled with mass spectrometry (AP-MS) to find the cGAS interacting partners. Our proteomics study identified 12 cGAS interacting proteins, further experiments using RNAi found that knockdown of complement C1q binding protein (C1QBP, also known as P32, GC1qR, and HABP1) inhibited cytosolic DNA-mediated innate immune response. C1QBP have been shown to be exploited by DNA viruses to benefit their infection \(^{300-302}\). However, how C1QBP regulates cGAS-mediated innate immune response and affects DNA virus infection remains unclear. On the other hand, we also generated STING knockout cell lines using H1299 cells, a lung epithelial cell line that cannot produce IFN upon DNA stimulation. We found that knockout of STING promoted human coronavirus OC43 infection. This suggests that STING inhibits
coronavirus infection independent of IFN production. Recently, several studies found that STING activation results in the suppression of SARS-CoV-2 infection in an IFN-dependent manner. STING inhibitor reduces SARS-CoV-2-mediated inflammation in mice. However, MDA5, but not STING, is the sensor of coronavirus. Therefore, the role of STING in coronavirus infection remains to be elucidated.

Here, we propose an innovative concept that viral infection and cellular stress release C1QBP from the mitochondria matrix, and then cytosolic C1QBP inhibits viral DNA or mtDNA-mediated cGAS activation. STING interferes with the formation of ER-derived DMVs of coronavirus, thereby inhibiting viral infection. Our project is, therefore, significant because it will not only reveal a host protective mechanism against aberrant activation of innate immune responses but also elucidate a distinct viral-host interaction that may provide insight into antiviral therapeutic development.
CHAPTER 2. Leaked mitochondrial C1QBP inhibits activation of the DNA sensor cGAS.

2.1 Introduction

2.1.1 DNA virus infection-induced mitochondrial stress enhances antiviral IFN response

As we discussed in other chapters, cGAS senses cellular DNA and leads to the activation of type I IFN response. This process must be controlled tightly to avoid damage to the host. Excessive host DNA can activate the cGAS signaling pathway, leading to aberrant IFN activation and autoimmune diseases, such as Aicardi Goutieres syndrome\textsuperscript{305,306}. Therefore, cells must keep cGAS inert from host genomic DNA. Host DNA is normally restricted in the cellular compartments of the nucleus and mitochondria; however, mitochondrial DNA (mtDNA) may escape to the cytoplasm because of cellular stresses caused by infection, oxidative stress, and apoptosis\textsuperscript{23}. Recent studies showed that the leaked mtDNA is sensed by cGAS and activates IFN expression\textsuperscript{103}. DNA viruses, such as HSV-1, have been shown to trigger mitochondrial stress and release mtDNA into the cytosol, but whether this benefits the virus remains to be established\textsuperscript{307}.

2.1.2 C1QBP function

Complement C1q binding protein (C1QBP, also known as P32, GC1qR, and HABP1) was first found to bind the C1 complement complex on the cell surface. Studies showed that C1QBP is ubiquitous and abundantly expressed in the...
mitochondrial matrix and moderately expressed on the cell surface, cytosol, and nucleus. C1QBP participates in several biological processes, such as ribosome biogenesis, oxidative stress, autophagy, regulation of apoptosis, and transcriptional regulation. Studies have shown that C1QBP inhibits RNA splicing factor ASF/SF2 RNA binding and phosphorylation, thereby inhibiting RNA splicing. C1QBP has also been shown to facilitate oxidative phosphorylation. During mitochondrial stress, C1QBP regulates ULK1 stability by forming a complex with ULK1, thus controlling mitochondrial homeostasis and cell survival. Recently, C1QBP was reported to promote homologous recombination during DNA damage response.

2.1.3 The role of C1QBP in viral infection

In addition to cellular functions, C1QBP also plays a role in the host antiviral defense. C1QBP has been shown to be exploited by RNA viruses to benefit their infection. Interaction of Rubella virus capsid protein with C1QBP enhances viral infection. C1QBP can disrupt the interaction between retinoic acid-inducible gene I (RIG-I) and mitochondrial antiviral signaling protein (MAVS), thus suppressing RNA-mediated innate immune response, and benefiting viral infection. Moreover, C1QBP also facilitates DNA virus infection. Previous studies showed that adenovirus hijacks C1QBP to deliver its genome into the nucleus by interacting with viral protein V. The Epstein-Barr virus (EBV) EBNA-1 protein interacts with C1QBP and facilitates its transactivation. The pUL97 of human cytomegalovirus exploits C1QBP to promote the nuclear export of viral capsids. HSV-1 viral proteins have been shown to interact with C1QBP to facilitate the nuclear egress of viral particles. These studies suggest that C1QBP plays an important role in regulating DNA virus infection.
In this study, we first analyzed the cGAS protein complex in macrophages by proteomics and identified C1QBP as a novel cGAS interactor. We further found that C1QBP leaked from the mitochondria to the cytoplasm after HSV-1 infection. More importantly, the leaked C1QBP inhibited cGAS activation in cells and in vitro and facilitated viral infection. Collectively, we identify cytosolic C1QBP as an intrinsic regulator of the cGAS signaling pathway.

2.2 Materials and Methods

Cells

HEK293 cells (ATCC, # CRL-1573), RAW 264.7 (ATCC, # TIB-71), and Vero cells (ATCC, # CCL-81), were maintained in Dulbecco’s Modified Eagle Medium (Life Technologies, #11995-065) containing antibiotics (Life Technologies, #15140-122) and 10% fetal bovine serum (Life Technologies, #26140-079). A549 cells (ATCC, # CCL-185) were cultured in RPMI Medium 1640 (Life Technologies, #11875-093) plus 10% fetal bovine serum and 1 × MEM Non-Essential Amino Acids Solution (Life Technologies, #11140-050).

Viruses

HSV-1 KOS strain was purchased from ATCC (#VR-1493). Viral titration was performed as described in a previous study. Briefly, Vero cells were infected with a serial diluted HSV-1. After 1 h, the medium was removed and replaced by the DMEM plus 10% FBS and 1% agarose. After 3 d, cells were examined for cytopathic effects to determine TCID$_{50}$ or were fixed using the methanol–acetic acid (3:1) fixative and stained using a Coomassie blue solution to determine the multiplicity of infection (MOI).

Plasmids
Human C1QBP cDNA was synthesized and cloned into pCMV-3Tag-8 to generate C1QBP-FLAG and C1QBP-HA. Human cGAS cDNA was also cloned into pCMV-3Tag-8 to produce cGAS-FLAG and cGAS-HA. Point mutations and deletions of C1QBP and cGAS were constructed using a Q5® Site-Directed Mutagenesis Kit (New England Biolabs, # E0554S).

Antibodies

Primary antibodies: Anti-α-tubulin (CST, # 3873), anti-FLAG (Sigma, # F3165), anti-HA (Cell Signaling Technology, # 3724), anti-TBK1 (Cell Signaling Technology, # 3504S), anti-phospho-TBK1 (Ser172) (Cell Signaling Technology, # 5483S), anti-GST (Cell Signaling Technology, # 2624S), anti-His (Thermo Fisher Scientific, # MA1-21315), and anti-C1QBP (Cell Signaling Technology, # 6502S).

Secondary antibodies: Goat anti-Mouse IgG-HRP [Bethyl Laboratories, # A90-116P, WB (1:10,000)], Goat anti-Rabbit IgG-HRP [Bethyl Laboratories, # A120-201P, WB (1:10,000)], Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) [Life Technologies, # A11005, IFA (1:200)], Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) [Life Technologies, # A11034, IFA (1:200)].

Sample preparation, Western blotting, and immunoprecipitation

Western blotting and immunoprecipitation were performed as described in a previous study 73. Briefly, samples (10–15 μl) were loaded into Mini-Protean TGX Precast Gels, 15 well (Bio-Rad, # 456-103), and run in 1 × Tris/Glycine/SDS Buffer (Bio-Rad, # 161-0732) for 60 min at 140 V. Protein samples were transferred to Immun-Blot PVDF Membranes (Bio-Rad, # 162-0177) in 1 × Tris/Glycine Buffer (Bio-Rad, # 161-0734) at 70 V for 60 min. PVDF membranes were blocked in 1 × TBS buffer (Bio-Rad, # 170-6435) containing 5% Blotting-Grade Blocker (Bio-Rad, # 170-6404) for 1 h. After washing with 1 × TBS buffer
for 30 min, the membrane blot was incubated with the appropriately diluted primary antibody in antibody dilution buffer (1 × TBS, 5% BSA, 0.02% sodium azide) at 4 °C for 16 h. Then, the blot was washed three times with 1 × TBS (each time for 10 min) and incubated with secondary HRP-conjugated antibody in antibody dilution buffer (1:10000 dilution) at room temperature for 1 h. After three washes with 1 × TBS (each time for 10 min), the blot was incubated with Clarity Western ECL Substrate (Bio-Rad, # 170-5060) for 1-2 min. The membrane was removed from the substrates and then exposed to the Amersham imager 600 (GE Healthcare Life Sciences, Marlborough, MA).

For immunoprecipitation, 2% of cell lysates were saved as an input control, and the remainder was incubated with 5–10 μl of the indicated antibody plus 20 μl of Pierce Protein A/G Plus Agarose (Thermo Fisher Scientific, # 20423) or 10 μl of EZview Red Anti-FLAG M2 Affinity Gel (Sigma, # F2426). After mixing end-over-end at 4 °C overnight, the beads were washed 3 times (5 min each wash) with 500 μl of lysis buffer.

Protein purification from E. coli and pull-down assays

The C1QBP mutant dMTS was cloned into pGEX-5X-3 (GE Healthcare, # 28-9545-55) to fuse with a GST tag. cGAS was cloned into pET28b(+) (Novagen, # 69865-3) to fuse with a His tag. These constructs were transfected into BL21 (DE3) E. coli (New England Biolabs, # C2527I) and cultured in LB broth at 20 °C. IPTG (0.4 mM) was added to induce protein expression. The GST Protein Interaction Pull-Down Kit (ThermoFisher Scientific, # PI21516) was used for GST-tagged protein purification and GST pull-down assays. The His-Spin Protein Miniprep kit (Zymo Research, # P2002) was used for His-tagged protein purification.

Immunofluorescence assay.
Cells were cultured in the Lab-Tek II CC2 Chamber Slide System 4-well (Thermo Fisher Scientific, # 154917). After the indicated treatment, the cells were fixed and permeabilized in cold methanol for 10 min at -20 °C. Then, the slides were washed with 1 × PBS for 10 min and blocked with Odyssey Blocking Buffer (LI-COR Biosciences, # 927-40000) for 1 h. The slides were incubated in Odyssey Blocking Buffer with appropriately diluted primary antibodies at 4 °C for 16 h. After 3 washes (10 min per wash) with 1 × PBS, the cells were incubated with the corresponding Alexa Fluor conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. The slides were washed three times (10 min each time) with 1 × PBS and counterstained with 300 nM DAPI for 1 min, followed by washing with 1 × PBS for 1 min. After air-drying, the slides were sealed with Gold Seal Cover Glass (Electron Microscopy Sciences, # 3223) using Fluoro-gel (Electron Microscopy Sciences, # 17985-10). Images were captured and analyzed using an iRiSTM Digital Cell Imaging System (Logos Biosystems).

Real-time PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, # 74106). 500 ng of RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen, # 205311). For one real-time reaction, 10 µl of SYBR Green PCR reaction mix (Eurogentec) including 100 ng of the synthesized cDNA plus an appropriate oligonucleotide primer pair were analyzed on a 7500 Fast Real-time PCR System (Applied Biosystems). The comparative Ct method was used to determine the relative mRNA expression of genes normalized by the housekeeping gene GAPDH. The primer sequences: mouse Gapdh, forward primer 5′- GCGGCACGTCAGATCCA -3′, reverse primer 5′- CATGGCCTTCCGTGTTCTA -3′; mouse Ifnb1, forward primer 5′- CAGCTCCAAGAAAGGACGAAC -3′, reverse primer 5′- GGCAGTGTAACTCTTCTGCAT -3′; mouse Cxcl10 (IP10), forward primer 5′- CCAAGTGCTGCCGTCATTTTC -3′, reverse
primer 5’- GGCTCGCAGGGATGATTCAA -3’; mouse Ccl5 (RANTES), forward primer 5’- GCTGCTTTGCCTACCTCTCC -3’, reverse primer 5’- TCGAGTGACAAACACGACTGC-3’; HSV-1 VP16, forward primer 5’- GGACTGTATTCCAGCTTCAC -3’, reverse primer 5’- CGTCCTCGCCGCTCTAAAGTG -3’.

Plasmid transfection

HEK293 and A549 cells were transfected using Lipofectamine 3000 or Lipofectamine LTX Transfection Reagent (Life Technologies, # L3000015) according to the manufacturer’s protocol.

CRISPR/Cas9

The single guide RNA (sgRNA) targeting sequence: mouse C1bp sgRNA: 5’- GCGTGCGCGCAGGTTCCGAG -3’. The sgRNA was cloned into lentiCRISPR v2 vector 74 (Addgene). The lentiviral construct was transfected with psPAX2 and pMD2G into HEK293T cells using PEI. After 48 h, the media containing lentivirus were collected. The targeted cells were infected with the media containing the lentivirus supplemented with 10 μg/ml polybrene. Cells were selected with 10 μg/ml puromycin for 14 days. Single clones were expanded for knockout confirmation by Western blotting.

Stable Cell Line Selection

RAW264.7 cells were infected with the media containing the lentivirus supplemented with 10 μg/ml polybrene. Cells were selected with 10 μg/ml puromycin for 14 days. Cell pools were expanded for expression confirmation by Western blotting.

Purification of protein complexes and mass spectrometry
Affinity purification coupled with mass spectrometry experiments were performed as previously described 36. For protein purification, RAW264.7 cell lines stably expressing each FLAG-tagged protein were collected and lysed in 10 ml of TAP buffer 75. Cell lysates were precleared with 50 μl of protein A/G resin before the addition of 20 μl of anti-FLAG resin (Sigma, # F2426) and incubation for 16 hr at 4°C on a rotator. The resin was washed 3 times and transferred to a spin column with 40 μl of 3 X FLAG peptide for 1 hr at 4°C on a rotator. The purified complexes were loaded onto a 4-15% NuPAGE gel. The gels were stained with a SilverQuest staining kit (Invitrogen), and lanes were excised for mass spectrometry analysis by the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA).

Mass spectrometry

Samples were reconstituted in 5 - 10 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5 μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter x ~12 cm length) with a flame-drawn tip. After equilibrating the column each sample was loaded via a Famos autosampler (LC Packings, San Francisco CA) onto the column. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). As peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Velos ion-trap mass spectrometer (Thermo Fisher, San Jose, CA). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Dynamic exclusion was enabled such that ions were excluded from reanalysis for 30 s. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program, SEQUEST (Thermo Fisher, San Jose, CA). The human IPI database (Ver. 3.6) was used for searching. Precursor mass
tolerance was set to +/- 2.0 Da and MS/MS tolerance was set to 1.0 Da. A reversed-sequence database was used to set the false discovery rate at 1%. Filtering was performed using the SEQUEST primary score, Xcorr, and delta-Corr. Spectral matches were further manually examined and multiple identified peptides (≥2) per protein were required.

SAINT analysis of AP-MS data

Two biological repeats were performed for each protein complex. The resulting data are presented in Table 1. Proteins found in the control group were considered non-specific binding proteins. The SAINT algorithm (http://sourceforge.net/projects/saint-apms) was used to evaluate the MS data. The default SAINT options were low Mode = 1, min Fold = 0, and norm = 0. The SAINT scores computed for each biological replicate were averaged (AvgP) and reported as the final SAINT score. A SAINT score of AvgP ≥ 0.89 was considered a true positive BioID protein with an estimated FDR of ≤2%. Proteins with SAINT score <0.89 are considered non-specific binding proteins. We manually removed ribosomal proteins from the final HCIP list because these proteins are prone to associate with RNA-binding proteins.

In vitro cGAS enzymatic assays

Purified recombinant cGAS-His was mixed with calf thymus DNA (ctDNA), ATP, GTP, and different amounts of dMTS GST in vitro at 37 C for 1 h. the production of cGAMP was then determined by the cGAMP ELISA Kit (Cayman Chemical).

Statistical analysis
The sample size was sufficient for data analyses. Data were statistically analyzed using the software GraphPad Prism 9. Significant differences between the indicated pairs of conditions are shown by asterisks, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

2.3 Results

Proteomic analysis of cGAS protein complex in macrophages

To seek new regulators of cGAS, we performed affinity purification coupled with mass spectrometry (AP-MS) analysis of the protein interaction network of cytosolic DNA sensing pathways. We chose RAW264.7 macrophages because this cell line is much more physiologically relevant than HEK293 fibroblasts which are commonly used in AP-MS. Mouse cGAS tagged with a FLAG epitope was transfected into RAW264.7 macrophages to generate stable cell lines. After we obtained the stable cell line by puromycin selection, the cGAS protein complex was affinity purified using an anti-FLAG antibody and analyzed by mass spectrometry (Fig. 3A). The AP-MS was biologically repeated twice. To efficiently reduce false positives in AP-MS, we adopted the well-established statistical method SAINT with a database of 24 protein complexes purified from RAW264.7 cells (Table 1). Using a stringent statistical SAINT score cutoff of 0.89 (P < 0.01), we identified five high-confidence candidate interacting proteins (HCIPs), including histones and IFI202 (Fig. 3B). It is well-established that the histone-containing nucleosomes bind cGAS. IFI202 is one of the mouse homologs of human IFI16. IFI16 involves in the DNA sensing pathway and interacts with cGAS. These known interactions substantiate the high quality of our cGAS protein interaction network. Next, we performed co-immunoprecipitation (co-IP) to validate the two new cGAS interactors, C1QBP and IMPDH2. We transfected cGAS with C1QBP or IMPDH2 into HEK293 cells. Co-IP showed that cGAS interacted with C1QBP and IMPDH2 (Figs. 3C and 3D).
Fig. 3 Analysis of cGAS protein complex.

(A) Schematics of AP-MS for mapping protein interactome in RAW247.6 cells. (B) cGAS protein interaction network. Legends are indicated. (C-D) Protein interaction validation by co-IP. The indicated FLAG- and HA-tagged genes were co-transfected into HEK293 cells. After 48 hr, cells were harvested and immunoprecipitated with anti-FLAG antibody, then blotted with the indicated antibodies.

Dissecting C1QBP-cGAS protein interaction

We chose C1QBP for further investigation because of its pro-viral role in DNA virus infection. We first examined the interaction between endogenous C1QBP and cGAS. RAW264.7 macrophages were infected with HSV-1, then the cell lysates were immunoprecipitated with the anti-C1QBP antibody. As shown in Figure 4A, HSV-1
infection promoted the interaction between endogenous C1QBP and cGAS. Next, we wanted to determine which domain of C1QBP is responsible for cGAS interaction. It is well-known that the N-terminal 73 residues are the mitochondrial targeting signal (MTS) of C1QBP and cleaved after translocating to the mitochondria \(^{31}\) (Fig. 4B). The full-length C1QBP showed the same molecular weight as the MTS deletion mutant (dMTS) when expressed in mammalian cells (Figs. 4A and 4C). Furthermore, co-IP also found that the dMTS mutant interacted with cGAS (Fig. 4C). We also expressed and purified GST-tagged dMTS and His-tagged cGAS from bacteria. GST pull-down assays found that C1QBP interacted with cGAS in vitro (Fig. 4D). Next, we determined the domains responsible for cGAS-C1QBP interaction. We first created a panel of C1QBP mutants (Fig. 4E). Co-IP showed that the region of aa 74-220 in C1QBP was sufficient for cGAS binding (Fig. 4F). Overall, these data suggest that HSV-1 infection facilitates C1QBP-cGAS interaction and the aa 74-220 region of C1QBP is responsible for the interaction with cGAS.
Fig. 4 C1QBP interacts with cGAS.

(A) RAW247.6 macrophages were infected with 1 MOI of HSV-1 for 16 h. Then, the cell lysates were subjected to immunoprecipitation and immunoblotting with the indicated antibodies to detect endogenous interactions. (B) Schematics of wild-type and mutant C1QBP proteins. Mito stands for the mitochondrial localization domain. (C) HA-tagged cGAS (cGAS-HA) was co-transfected with FLAG-tagged C1QBP or the dMTS mutant into HEK293 cells. After 48 hr, cells were harvested and immunoprecipitated with anti-FLAG antibody, then blotted with the indicated antibodies. (D) Purified recombinant His-tagged
cGAS (cGAS-His) was mixed with GST or GST-tagged dMTS mutant (dMTS-GST) *in vitro* at 4 °C for 16 h. Then, the GST pull-down was performed and blotted as indicated. (E) Schematics of C1QBP truncates. (F) HA-tagged cGAS (cGAS-HA) was co-transfected with the indicated FLAG-tagged C1QBP mutants into HEK293 cells. After 48 hr, cells were harvested and immunoprecipitated with anti-FLAG antibody, then blotted with the indicated antibodies.

**HSV-1 infection causes the mitochondrial leakage of C1QBP which co-localizes with cGAS in the cytoplasm**

We examined the co-localization of cGAS and C1QBP. FLAG-tagged C1QBP or the dMTS mutant was co-transfected with HA-tagged cGAS into A549 cells. Immunofluorescence assays (IFA) found that full-length C1QBP showed exclusively mitochondrial localization whereas the dMTS mutant mainly expressed in the cytoplasm (Fig. 5A). cGAS co-localized with the dMTS mutant but not full-length C1QBP in the cytoplasm (Fig. 5B). Furthermore, we examine the subcellular localization of cGAS and C1QBP in A549 cells after HSV-1 infection. As shown in Figure 5C, HSV-1 infection caused the increased cytosolic localization of C1QBP. By contrast, transfection of DNA ligand, calf thymus DNA (ctDNA), had little effect on the mitochondrial localization of C1QBP (Fig. 5C). Further subcellular fractionation corroborated virus-induced mitochondrial leakage of C1QBP (Fig. 5D). Interestingly, the RNA virus vesicular stomatitis virus also caused C1QBP mitochondrial leakage (Fig. 5E). Taken together, our data suggest that HSV-1 infection causes the mitochondrial leakage of C1QBP and promotes the cytosolic interaction between C1QBP and cGAS.
Fig. 5 C1QBP leaks from the mitochondria during HSV-1 infection and co-localized with cGAS in the cytosol.

(A) A549 cells were transfected with FLAG-tagged C1QBP, dMTS, or cGAS. After 48 h, cells were stained with MitoTracker Red CMXRos (red), anti-FLAG (green), and DAPI nuclear stain (blue). The red and green fluorescence intensity was plotted according to
the white line in the merged image. The quantitated colocalization was determined by the Pearson correlation coefficient (Pearson r) and shown next to the fluorescence intensity plot. Thirty cells (ten cells from each experiment) were analyzed by ImageJ to quantify the colocalization between the green and red channels. The scale bar is indicated. (B) cGAS-HA was transfected with FLAG-tagged C1QBP or the dMTS mutant into A549 cells. After 48 h, cells were fixed and stained with anti-FLAG (red), anti-HA (green), and DAPI nuclear stain (blue). The red and green fluorescence intensity was plotted according to the white line in the merged image. The quantitated colocalization was determined by Pearson r and is shown next to the fluorescence intensity plot. Thirty cells (ten cells from each experiment) were analyzed by ImageJ to quantify the colocalization between the green and red channels. The scale bar is indicated. (C) A549 cells were mock infected, infected with HSV-1 for 8 h, or stimulated with ctDNA for 4 h. Cells were then stained with MitoTracker Red CMXRos (red), anti-C1QBP (green), and DAPI nuclear stain (blue). Arrows indicate the leaked cytosolic C1QBP proteins that are not localized in the mitochondria in the inset of the boxed area. The red and green fluorescence intensity was plotted according to the white line in the inset image. The quantitated colocalization was determined by Pearson r and is shown next to the fluorescence intensity plot. Thirty cells (ten cells from each experiment) were analyzed by ImageJ to quantify the colocalization between the green and red channels. The scale bar is indicated. (D) Subcellular fractionation of RAW264.7 cells infected with and without HSV-1 for 8 h. Cell lysate was blotted as indicated. (E) Subcellular fractionation of RAW264.7 cells infected with and without vesicular stomatitis virus (VSV) for 8 h. Cell lysate was blotted as indicated.

**Cytosolic C1QBP suppresses cGAS-mediated innate immune response**

We hypothesized that the leaked C1QBP inhibits cGAS activation as the majority of C1QBP localizes in the mitochondria while cGAS resides in the cytoplasm and the nucleus.
To test the hypothesis, we first examined the effect of the dMTS mutant, the leaked form of C1QBP, on cGAS-mediated ISRE reporter activity. Different doses of C1QBP and the dMTS mutant were transfected into HEK293 cells together with an ISRE reporter. As expected, the dMTS mutant, but not the full-length C1QBP (residing at the mitochondria), inhibited cGAS-induced reporter activity in a dose-dependent manner (Fig. 6A). To further examine the role of C1QBP in cGAS-mediated innate immune response, we overexpressed the dMTS mutant in RAW264.7 macrophages. The dMTS mutant impaired TBK1 phosphorylation induced by HSV-1 (Fig. 6B). Consistently, it also inhibited the mRNA expression of IFNβ, IP-10, and RANTES induced by HSV-1 (Figs. 6C-6E). Furthermore, the dMTS mutant also inhibited ctDNA-induced IFNβ expression (Fig. 6F).

**Fig. 6** Cytosolic C1QBP inhibits cGAS-mediated innate immune responses and facilitates HSV-1 infection.
(A) A total of 200 ng of cGAS was transfected with different doses (50, 100, and 200 ng) of C1QBP or the dMTS mutant together with 20 ng of pRL-SV40 (Renilla luciferase as an internal control) and 200 ng of pISRE-Luc into HEK293 cells. After 48 h, cells were collected, and the ratio of firefly luciferase to Renilla luciferase was calculated to determine the relative reporter activity. All experiments were repeated three times. The p-value was calculated by one-way ANOVA followed by Dunnett multiple comparisons test. **p < 0.01, ****p < 0.0001. (B) RAW264.7 macrophages stably transfected with vector and dMTS were infected with 1 MOI of HSV-1 for indicated times. Cell lysates were collected and blotted as indicated. Band densitometry was calculated by ImageJ. The ratio of phosphorylated TBK1 to total TBK1 in each lane was indicated. (C-E) RAW264.7 macrophages stably transfected with vector and dMTS were infected with 1 MOI of HSV-1 for indicated times. Real-time PCR was performed to determine the relative mRNA levels of IFN-β (C), IP10 (D), and RANTES (E). All experiments were biologically repeated three times. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by the Sidak multiple comparisons test. *p < 0.05, **p < 0.01. (F) RAW 264.7 macrophages stably transfected with the vector and the dMTS mutant were stimulated with 1 mg/ml ctDNA for indicated times. Real-time PCR was performed to determine the relative IFN-β mRNA levels. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by the Sidak multiple comparisons test. *p < 0.05.

**Cytosolic C1QBP increases host susceptibility to viral infection**

Next, we examined the effects of the dMTS mutant on viral infection. Wild-type RAW264.7 cells and cells expressing the dMTS mutant were infected with HSV-1 carrying a GFP (HSV-1-GFP) for 16 h. As shown in Figure 7A, the C1QBP dMTS mutant promoted viral infection activity evidenced by the increased GFP-positive cells. Furthermore,
overexpression of dMTS increased viral mRNA expression (Fig. 7B) and the production of viral particles (Fig. 7C). Interestingly, the C1QBP dMTS mutant also inhibited VACV infection (Fig. 7D). These data suggest that the leaked cytosolic C1QBP inhibits cGAS activity and facilitates DNA virus infection.

Fig. 7 Cytosolic C1QBP facilitates DNA virus infection.

(A) Wild-type and cGAS knockout (KO) RAW264.7 macrophages stably expressing vector or dMTS were infected with 5 MOI of HSV-1 GFP for 16 h. Cells expressing GFP were examined and counted under a fluorescence microscope. The relative infection was determined by the ratio of positive cells. Data represent means ± SD of three independent experiments. (>200 cells were counted in each field, and five fields were counted per experiment). The p-value was calculated by two-way ANOVA followed by the Sidak multiple comparisons test. ****p < 0.0001. (B) RAW264.7 macrophages stably transfected...
with vector and dMTS were infected with the indicated MOI of HSV-1 for 16 h. Cells were then collected for RNA extraction. The RNA levels of HSV-1 VP16 were determined by real-time PCR. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by the Sidak multiple comparisons test. *p < 0.05, **p < 0.01.

(C) RAW264.7 macrophages stably transfected with vector and dMTS were infected with 0.01 MOI of HSV-1 for the indicated days. Titers of culture supernatants containing HSV-1 were determined on Vero cells, and plaques were enumerated. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by the Sidak multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001.

(D) RAW264.7 macrophages stably transfected with vector and dMTS were infected with 1 MOI of VACV carrying a luciferase gene (VACV-Luc) for 16 h. Luciferase activities were measured to determine the relative infection activity. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by the Sidak multiple comparisons test. *p < 0.05, ****p < 0.0001. ns, not significant.

Knockout of C1QBP sensitizes cGAS-mediated innate immune responses

To examine the effect of C1QBP deficiency on cGAS-mediated innate immunity, we generated two C1QBP knockout RAW264.7 cell lines by CRISPR (Fig. 8A). We also used two cGAS knockout RAW264.7 cell lines as the controls (Fig. 8B). As predicted, ctDNA-induced IFN-β, RANTES, and IP10 mRNA expression was impaired in the cGAS knockout cell lines (Fig. 8C). However, ctDNA induced comparable mRNA expression of IFNβ, RANTES, and IP10 in C1QBP knockout cells (Fig. 8C), consistent with the finding that ctDNA transfection has no effect on C1QBP leakage. Next, we examined the effect of C1QBP on HSV-1 induced innate immune responses in wild-type, cGAS knockout cells,
and C1QBP knockout cells. Real-time PCR found that deficiency of C1QBP increased the mRNA levels of IFNβ, IP10, and RANTES induced by HSV-1, whereas ablation of cGAS abolished mRNA expression of these genes (Figs. 8D-F). Consistently, TBK1 and IRF3 phosphorylation was also enhanced in C1QBP knockout cells (Fig. 8G), suggesting that the knockout of C1QBP promotes HSV-1 infection-induced cGAS signaling. To further corroborate the role of C1QBP, we reconstituted the dMTS mutant in the C1QBP knockout macrophages (Fig. 8H). We found that the dMTS rescued the innate immune response phenotype in C1QBP knockout cells, reducing the mRNA expression of IFNβ, IP10, and RANTES induced by HSV-1 (Fig. 8I-K). Taken together, these data suggest that C1QBP deficiency increases cGAS-mediated innate immune response.
Fig. 8 C1QBP deficiency increases HSV-1-induced innate immune response.

(A) The lysates of wild-type and two C1QBP knockout (KO) RAW264.7 cell lines were blotted as indicated. (B) The lysates of wild-type and two cGAS KO RAW264.7 cell lines were blotted as indicated. (C) RAW264.7 macrophages, two cGAS KO cell lines, and two C1QBP KO cell lines were stimulated with 1 mg/ml ctDNA for 4 h. Real-time PCR was performed to determine the relative mRNA levels of IFNβ, IP10, and RANTES. All experiments were repeated three times. The p-value was calculated by two-way ANOVA.
followed by Tukey multiple comparisons test. *p < 0.05. (D-F) RAW264.7 macrophages, two cGAS KO cell lines, and two C1QBP KO cell lines were infected with 1 MOI of HSV-1 for indicated times. Real-time PCR was performed to determine the relative mRNA levels of IFNβ (D), IP10 (E), and RANTES (F). Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by Tukey multiple comparisons test. *p < 0.05. (G) C1QBP wild-type (WT) and KO cells were infected with 1 MOI of HSV-1 for indicated times. Cell lysates were collected and blotted as indicated. Band densitometry was calculated by ImageJ. The ratio of phosphorylated TBK1 to total TBK1 and phosphorylated IRF3 to total IRF3 in each lane was indicated. (H) The lysates of WT RAW264.7 macrophages, cGAS KO, and C1QBP KO reconstituted with dMTS were blotted as indicated. (I-K) WT RAW264.7 macrophages, C1QBP KO cells, and C1QBP KO cells reconstituted with dMTS were infected with 1 MOI of HSV-1 for indicated times. Real-time PCR was performed to determine the relative mRNA levels of IFNβ (I), IP10 (J), and RANTES (K). All experiments were biologically repeated three times. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by Tukey multiple comparisons test. *p < 0.05.

C1QBP deficiency impairs DNA virus infection

To examine the effects of C1QBP deficiency on viral infection, we compared viral infection in C1QBP, cGAS wild-type and knockout cells. These cells were infected with HSV-1 carrying a GFP (HSV-1-GFP) for 16 h. As shown in Figure 9A, knockout of cGAS increased HSV-1 infection, but viral infection activity in C1QBP knockout cells was much lower than in wild-type cells. C1QBP deficiency also reduced the expression of viral RNA (Fig. 9B) and the production of viral particles (Fig. 9C). Consistently, knockout of cGAS increased VACV infection (Fig. 9D). By contrast, ablation of C1QBP reduced VACV
infection (Fig. 9D). Furthermore, reconstitution of dMTS restored HSV-1 and VACV infection in C1QBP knockout cells (Figs. 9E-F). Taken together, these data suggest that C1QBP deficiency sensitizes cGAS activity and impairs infection with the HSV-1 and vaccinia DNA viruses.

**Fig. 9 Knockout of C1QBP reduces DNA virus infection.**

**(A)** RAW264.7 macrophages, two cGAS KO cell lines, and two cC1QBP KO cell lines were infected with 5 MOI of HSV-1 GFP for 16 h. Cells expressing GFP were examined and counted under a fluorescence microscope. The relative infection was determined by the ratio of positive cells. Data represent means ± SD of three independent experiments (>200 cells were counted in each field, and five fields were counted per experiment). The p-value was calculated by one-way ANOVA followed by Dunnett multiple comparisons test.
*p < 0.05, ****p < 0.0001. (B) C1QBP wild-type (WT) macrophages and two KO cell lines were infected with the indicated MOI of HSV-1 for 16 h. Cells were then collected for RNA extraction. The RNA levels of HSV-1 VP16 were determined by real-time PCR. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by Dunnett multiple comparisons test. *p < 0.05. (C) C1QBP WT macrophages and two KO cell lines were infected with 0.01 MOI of HSV-1 for the indicated days. Titers of culture supernatants containing HSV-1 were determined on Vero cells. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by Dunnett multiple comparisons test. *p < 0.05. (D) RAW264.7 macrophages, two cGAS KO cell lines, and two C1QBP KO cell lines were infected with VACV-Luc for 16 h. Luciferase activities were measured to determine the relative infection activity. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by one-way ANOVA followed by Dunnett multiple comparisons test. *p < 0.05, ****p < 0.0001. (E) WT RAW264.7 macrophages, C1QBP KO cells, and C1QBP KO cells reconstituted with dMTS were infected with 5 MOI of HSV-1 GFP for 16 h. Cells expressing GFP were examined and counted under a fluorescence microscope. The relative infection was determined by the ratio of positive cells. Data represent means ± SD of three independent experiments (>200 cells were counted in each field, and five fields were counted per experiment). The p-value was calculated by one-way ANOVA followed by Tukey multiple comparisons test. *p < 0.05, **p < 0.01. (F) WT RAW264.7 macrophages, C1QBP KO cells, and C1QBP KO cells reconstituted with dMTS were infected with VACV-Luc for 16 h. Luciferase activities were measured to determine the relative infection activity. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by one-way ANOVA followed by Tukey multiple comparisons test. ****p < 0.0001.
C1QBP interacts with cGAS NTase domain and inhibits cGAS enzymatic activity

To determine the mechanism by which C1QBP inhibits cGAS, we first examined which domain of cGAS interacted with C1QBP. We created a panel of cGAS mutants (Fig. 10A). Co-IP showed that the nucleotidyltransferase (NTase) domain of cGAS was required for the interaction with C1QBP (Fig. 10B). Furthermore, co-IP found sufficient interaction between the NTase domain and the 74-220 region of C1QBP (Fig. 10C), suggesting that C1QBP might interfere with cGAS enzymatic activity. Thus, we performed in vitro cGAS enzymatic assays. The in vitro assays found that the C1QBP dMTS mutant inhibited cGAMP production by cGAS (Fig. 10D), suggesting that C1QBP inhibits cGAS enzymatic activity.
Fig. 10 C1QBP inhibits cGAS enzymatic activity.

(A) Schematic of cGAS mutants. NTase represents the NTase domain. (B) HA-tagged cGAS (cGAS-HA) or the indicated mutant were co-transfected with FLAG-tagged C1QBP (C1QBP-FLAG) into HEK293 cells. After 48 h, cells were harvested and immunoprecipitated with anti-FLAG Ab, then blotted with the indicated Abs. (C) HA-tagged C1QBP mutant (aa 74-220) was co-transfected with the FLAG-tagged NTase domain of cGAS (NTase-FLAG) into HEK293 cells. After 48 h, cells were harvested and immunoprecipitated with anti-FLAG Ab, then blotted with the indicated Abs. (D) In vitro cGAS enzymatic assays. Purified recombinant cGAS-His (10 nM) was mixed with ctDNA, ATP, GTP, and different amounts of dMTS GST (5, 25, 50 nM) in vitro at 37°C for 1 h. The production of cGAMP was then determined by ELISA. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by one-way ANOVA followed by Dunnett multiple comparisons test. *p < 0.05, ***p < 0.001, ****p < 0.0001. (E) Model of C1QBP-mediated cGAS inhibition.

2.4 Discussion

In this study, we found C1QBP negatively regulates cGAS-mediated innate immune response. C1QBP predominantly localizes in the mitochondria; viral infection causes the release of C1QBP from the mitochondria to the cytosol. Subsequently, the leaked cytosolic C1QBP binds cGAS and inhibits cGAS activation (Fig. 10E). C1QBP is a multifunctional and multicompartmental protein. Many studies focus on the secreted C1QBP and intracellular C1QBP localized in the nucleus and mitochondria. The cytosolic C1QBP is much less studied, which might be because the significant cytoplasmic localization is only observed under some pathological conditions, such as viral infection. Our study showed that C1QBP leaked from the mitochondria during viral infection and cytosolic C1QBP...
impaired cGAS activation. We found that C1QBP interacted with the NTase domain of cGAS and inhibited cGAS activity in a dose-dependent manner in vitro, suggesting C1QBP might directly block cGAS activation sites. Interestingly, the mature form of C1QBP (i.e. the MTS deletion) is highly acidic with a calculated pl of 3.96. Thus, it is also plausible that C1QBP competes with DNA for cGAS binding. These possible mechanisms warrant further investigation in the future.

During infection, DNA viruses expose their DNA in the cytosol, which can activate the cGAS-mediated innate immune response. To evade host innate immunity, several viral proteins have been found to engage with cGAS to sabotage host defense. ORF52 of KHSV and its homologs of MHV68, RRV, and EBV have been shown to inhibit cytosolic DNA sensing by directly inhibiting cGAS enzymatic activity through a mechanism involving both cGAS binding and DNA binding. Recently, N-terminally truncated cytoplasmic isoform of KSHV latency-associated nuclear antigen (LANA) interacts with cGAS and antagonizes cGAS signaling. The UL41 and VP22 of HSV-1 abrogate type I IFN production by degrading cGAS and inhibiting cGAS enzymatic activity, respectively. In addition to viral DNA, leaked mtDNA also activates cGAS and induces host innate immune responses. For example, deficiency of the transcription factor A mitochondrial (TFAM) promotes mitochondrial stress and the leakage of mtDNA, resulting in activating cGAS and inducing a type I interferon response. DNA virus infection also triggers mitochondrial stress and the release of mtDNA to the cytosol. Interestingly, RNA virus, such as the dengue virus, also elicits a cGAS-STING response due to the release of oxidized mtDNA into the cytosol caused by viral infection. Our study showed that DNA virus infection causes mitochondrial C1QBP leak to inhibit cGAS activation, adding another layer of complexity of viral infection and host defense.
Aberrant activation of the cGAS-STING pathway causes autoimmune diseases, including Aicardi–Goutières syndrome and certain forms of lupus, like systemic lupus erythematosus. Aberrant inflammation via cGAS-STING also contributes to aging-related neurodegenerative conditions, such as Parkinson’s disease and amyotrophic lateral sclerosis (ALS). Mutations of STING cause a life-threatening autoinflammatory condition, termed STING-associated vasculopathy with onset in infancy (SAVI). Thus, the cGAS-STING pathway is not only critical in infectious diseases but also can be pathogenic in primary immune disorders when the pathway is aberrantly activated. mtDNA has been implicated to at least partially contribute to some autoimmune disorders by activating the cGAS-STING pathway. It would be interesting to know whether C1QBP is leaked during the pathogenesis and whether the leaked C1QBP provides protection by reducing mtDNA-induced IFN production. Overall, our study will not only reveal a viral strategy that evades immune surveillance but also elucidate a potential host protective mechanism that prevents the aberrant activation of innate immunity under pathological conditions.
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**Table 1.** Proteomic analysis of cGAS protein complex
CHAPTER 3. STING inhibits coronavirus infection by disrupting viral replication organelles.

3.1. Introduction

3.1.1. Coronavirus replication and DMV formation

The ongoing pandemic of coronavirus disease 2019 (COVID-19) has caused tremendous life loss and economic disruption, which overwhelmed many public health systems. The causative agent of COVID-19 is a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Together with the Middle East respiratory syndrome–related coronavirus (MERS-CoV), SARS-CoV and human coronavirus HCoV-OC43, belongs to the genus of beta-coronavirus. The HCoV-OC43 only causes moderate upper respiratory infections such as the common cold, however, MERS-CoV, SARS-CoV, and SARS-CoV-2 can cause severe lower respiratory infections with mortality rates of 30% in MERS-CoV infection \(^{327}\), 9% in SARS-CoV infection \(^{328}\), and <0.5% in SARS-CoV-2 infection \(^{329}\). Even with a low mortality rate, SARS-CoV-2 has caused more than 6 million deaths worldwide according to the WHO website. Despite many efforts that have been put into the development of antiviral strategies such as vaccines and drugs, the understanding of the host defense against HCoV infection is not fully elucidated.

The infection of human coronavirus starts with the binding of cell surface receptors, which triggers the membrane fusion upon extensive conformational change and initiates the entry process. Once get into the cell cytosol, the virus releases its genome,
which is a single-stranded positive-sense RNA with a length of around 30 kb. The viral genome ORF1a and ORF1b can be directly translated into polyproteins (pp1a and pp1b), which are cleaved to form different proteins required for genome replication complex \(^{330,331}\). The coronavirus alters the ER membrane to form a distinct replicational organelle (RO), the double-membrane vesicle (DMV), where viral RNA genomes are replicated \(^{332}\). The positive-stranded RNA serves as the template for the synthesis of negative-strand RNA and subgenomic RNA (sgRNA). Then the sgRNAs are translated to produce viral structural proteins and accessory proteins, which are inserted into the ER-Golgi compartment for viral particle assembly \(^{333}\). The viral RNA genome undergoes encapsidation and finally gets packed in newly synthesized virions that are then released into the extracellular compartment through the plasma membrane.

In the process of coronavirus replication, one of the featured steps is that the coronavirus genome replication in the DMVs. The replication intermediate dsRNA of coronavirus is a potent PAMP that can be detected by PRRs, such as RIG-I and MDA5. DMV provides a shield that may help the virus evade innate immune response \(^{334}\). DMV formation is a hallmark of all coronavirus infections. Studies have shown that the coronavirus nonstructural protein NSP3 and NSP4 colocalizes with the ER markers and the ectopic expression of NSP3, NSP4 and NSP6 is sufficient to induce the DMV formation \(^{335,336}\). Furthermore, NSP3 was shown to work together with other viral or host factors to form pores on the DMV which allows the out delivery of viral genomes and the exchange of components with cytoplasmic compartment \(^{337}\). By providing the anchor for the viral replication complex and protecting the viral genome from immune sensing, DMV serves as the primary site for viral RNA synthesis \(^{334}\). However, the mechanism of how DMV forms and whether there are other host factors that regulate the DMV formation remains unclear.
3.1.2. STING participates in DMV formation and affects coronavirus infection

Recently, several studies have reported that the artificial activation of STING by agonists inhibits SARS-CoV-2 infection due to inducing type I IFN expression\textsuperscript{298,299,338}. However, studies have shown that MDA5, but not STING, is the cognate pathogen recognition receptor for coronavirus during natural infection\textsuperscript{304}. Moreover, the block of IFNR by antibody only partially abolished the antiviral activity of STING, indicating an IFN-independent role in STING-mediated antiviral effect\textsuperscript{298}.

As an ER membrane protein, STING was recently reported to involve in the formation of viral-induced replication organelle. During the human rhinovirus (HRV) infection, STING was recruited to the replication organelle by viral 2C protein and promotes viral replication\textsuperscript{339}. Further study showed that HRV promotes STING trafficking to the replication organelle with the help of phosphatidylinositol 4-phosphate (PI4P)\textsuperscript{340}. However, whether STING is involved in coronavirus replication organelle formation is unknown. Here in our study, we found that knockout of STING facilitated HCoV-OC43 infection in H1299 cells, a lung epithelial cell line that cannot produce IFN upon DNA stimulation, suggesting that STING inhibits coronavirus infection independently of IFN. Further study showed that STING interacted and co-localized with the viral proteins NSP4 and NSP6, which are important for DMV formation. Knockout of STING significantly increased the dsRNA-positive staining by immunofluorescence assays, indicating that STING inhibits DMV formation. Therefore, our study reveals a novel, IFN-independent antiviral role of STING in coronavirus infection by perturbing the formation of viral replication organelles.

3.2 Materials and Methods

Cells
HEK293 cells (catalog no. CRL-1573; American Type Culture Collection [ATCC]), Vero cells (catalog no. CCL-81; ATCC), and primary MEFs (isolated from mouse) were maintained in DMEM (catalog no. 11995-065; Life Technologies) containing antibiotics (catalog no. 15140-122; Life Technologies) and 10% FBS (Life Technologies, catalog no. 26140-079). H1299 cells were cultured in RPMI 1640 (catalog no. 11875-093; Life Technologies) plus 10% FBS.

Viruses

HCoV-OC43 was purchased from ATCC (#VR-1558). HCoV-OC43 viral titration was performed as follows. Vero cells were infected with a serial diluted HCoV-OC43. After 1 h, the medium was removed and replaced by the DMEM plus 2% FBS. Cells were examined for cytopathic effects to determine TCID<sub>50</sub> for five days.

Plasmids

NSP4, NSP6, E, ORF7B, and ORF9B genes from SARS-CoV-2 were synthesized by GenScript and then cloned into the pCMV-3Tag-8 vector (Stratagene, #240203). Human STING cDNA was also cloned into pCMV-3Tag-8 to produce STING-FLAG. Point mutations and deletions of STING were constructed using a Q5 Site-Directed Mutagenesis Kit (catalog no. E0554S; New England Biolabs).

Antibodies

Primary antibodies: Anti α-tubulin [Cell Signaling Technology, #2144, WB (1:1,000)], anti-FLAG [Sigma, # F3165, WB (1:1,000), IFA (1:100)], anti-HA [Cell Signaling Technology, # 3724, WB (1:1,000), IFA (1:100)], anti-TBK1 [Cell Signaling Technology, # 3504S, WB (1:1,000)], anti-phospho-TBK1 (Ser172) [Cell Signaling Technology, # 5483S, WB
(1:1,000)], anti-human IRF3 [Cell Signaling Technology, # 11904S, WB (1:1,000)], anti-phospho-IRF3 (Ser386) [ABclonal, # AP0995, WB (1:1,000)], anti-IKKα [ABclonal, # A19694, WB (1:1,000)], anti-phospho-IKKα/β (Ser176/180) [Cell Signaling Technology, # 2697S, WB (1:1,000)], anti-human cGAS [Cell Signaling Technology, # 15102S, WB (1:1,000)], anti-human STING [Cell Signaling Technology, # 13647S, WB (1:1,000)], anti-mouse STING [Cell Signaling Technology, # 50494S, WB (1:1,000)], anti-phospho-STING (Ser366) [Cell Signaling Technology, # 50907S, WB (1:1,000)], anti-human MAVS [Cell Signaling Technology, # 24930S, WB (1:1,000)], anti-human MDA5 [Cell Signaling Technology, # 5321S, WB (1:1,000)], anti-IFNAR-1 (BioXCell, # BE0241), anti-HCoV-OC43 nucleocapsid protein [Sino Biological, # 40643-T62, WB (1:1,000), IFA (1:100)], anti-dsRNA [Cell Signaling Technology, # 76651, IFA (1:100)].

Secondary antibodies: Goat anti-Mouse IgG-HRP [Bethyl Laboratories, # A90-116P, WB (1:10,000)], Goat anti-Rabbit IgG-HRP [Bethyl Laboratories, # A120-201P, WB (1:10,000)], Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) [Life Technologies, # A11005, IFA (1:200)], Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) [Life Technologies, # A11034, IFA (1:200)].

Sample preparation, Western blotting, and immunoprecipitation

Approximately 1 X 10^6 cells were lysed in 500 μl of tandem affinity purification (TAP) lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 0.5% Nonidet P40, 10% glycerol, the Complete EDTA-free protease inhibitor cocktail tablets (Roche, # 11873580001)] for 30 min at 4 °C. The lysates were then centrifuged for 30 min at 15,000 rpm. Supernatants were collected and mixed with the Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific, # 39000).
Western blotting and immunoprecipitation were performed as described in a previous study (Zhao et al., 2019). Briefly, samples (10–15 μl) were loaded into Mini-Protean TGX Precast Gels, 15 well (Bio-Rad, # 456-103), and run in 1 × Tris/Glycine/SDS buffer (Bio-Rad, # 161-0732) for 35 min at 200 V. Protein samples were transferred to Immun-Blot PVDF Membranes (Bio-Rad, # 162-0177) in 1 × Tris/Glycine Buffer (Bio-Rad, # 161-0734) at 70 V for 60 min. PVDF membranes were blocked in 1 × TBS buffer (Bio-Rad, # 170-6435) containing 5% Blotting-Grade Blocker (Bio-Rad, # 170-6404) for 1 h. After washing with 1 × TBS buffer for 30 min, the membrane blot was incubated with the appropriately diluted primary antibody in antibody dilution buffer (1 × TBS, 5% BSA, 0.02% sodium azide) at 4 °C for 16 h. Then, the blot was washed three times with 1 × TBS (each time for 10 min) and incubated with secondary HRP-conjugated antibody in antibody dilution buffer (1:10,000 dilution) at room temperature for 1 h. After three washes with 1 × TBS (each time for 10 min), the blot was incubated with Clarity Western ECL Substrate (Bio-Rad, # 170-5060) for 1-2 min. The membrane was removed from the substrates and then exposed to the Amersham imager 600 (GE Healthcare Life Sciences, Marlborough, MA).

For immunoprecipitation, 2% of cell lysates were saved as an input control, and the remainder was incubated with 10 μl of EZview Red Anti-FLAG M2 Affinity Gel (Sigma, # F2426). After mixing end-over-end at 4 °C overnight, the beads were washed 3 times (5 min each wash) with 500 μl of lysis buffer. All coimmunoprecipitation (co-IP) experiments were performed by transfection into HEK293 cells.

Immunofluorescence assay

Cells were cultured in the Lab-Tek II CC2 Chamber Slide System 4-well (Thermo Fisher Scientific, # 154917). After the indicated treatment, the cells were fixed and permeabilized in cold methanol for 10 min at -20 °C. Then, the slides were washed with 1 × PBS for
10 min and blocked with Odyssey Blocking Buffer (LI-COR Biosciences, # 927-40000) for 1 h. The slides were incubated in Odyssey Blocking Buffer with appropriately diluted primary antibodies at 4 °C for 16 h. After 3 washes (10 min per wash) with 1 × PBS, the cells were incubated with the corresponding Alexa Fluor conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. The slides were washed three times (10 min each time) with 1 × PBS and counterstained with 300 nM DAPI for 1 min, followed by washing with 1 × PBS for 1 min. After air-drying, the slides were sealed with Gold Seal Cover Glass (Electron Microscopy Sciences, # 3223) using Fluoro-gel (Electron Microscopy Sciences, # 17985-10). Images were captured and analyzed using a Revolve Microscope (Discover Echo Inc.).

Real-time PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, # 74106). One µg quantity of RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen, # 205311). For one real-time reaction, 10 µl of SYBR Green PCR reaction mix (Eurogentec), including 2 µg of the synthesized cDNA plus an appropriate oligonucleotide primer pair, were analyzed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The comparative Ct method was used to determine the relative mRNA expression of genes normalized by the housekeeping gene GAPDH. The primer sequences: human GAPDH, forward primer 5’- AGGTGAAGGTCGGAGTCA-3’, reverse primer 5’-GGTCATTGATGGCAACAA-3’; human IFNβ1, forward primer 5’-TCATCCTGTCTTTGAGGCAGT -3’, reverse primer 5’- CAGCAATTTTCAGTGTCAGATCCA -3’; mouse GAPDH, forward primer 5’-CATGGGCTTTCGTTCCT -3’, reverse primer 5’- GCGGCACGTCAGATCCA -3’; mouse CXCL10 (IP10), forward primer 5’-CCAAGTGCTGCCGTATTTC -3’, reverse
primer 5'- GGCTCGCAGGGATGATTCA -3’; mouse ISG15, forward primer 5'-GGTGTCCTCGACTAACTCCATTCT -3’, reverse primer 5'-TGGAAAGGGTAAGACGTCCTCCATTTCT -3’; mouse IFIT1, forward primer 5'-CTGAGATGTCACTTCACATGGAA -3’, reverse primer 5'-GTGCAATCCCCAATGGGGTTCT -3’; HCoV-OC43 M, forward primer 5'-ATGTAGGCGATAATTGAGGACTAT -3’, reverse primer 5'-AATGTAAGATGGCGCGTATT -3’.

Plasmid transfection

HEK293 and H1299 cells were transfected using Lipofectamine 3000 (Life Technologies, # L3000015) Transfection Reagent according to the manufacturer’s protocol.

CRISPR/Cas9

The single guide RNA (sgRNA) targeting sequences: human STING sgRNA: 5'-GGGAATTTCAACGTGGCCCA -3’; human MAVS sgRNA: 5'-ACAGGGTCAGTTGTATCTAC -3’. The sgRNA was cloned into lentiCRISPR v2 vector. The lentiviral construct was transfected with psPAX2 and pMD2G into HEK293T cells using PEI. After 48 h, the media containing lentivirus were collected. The targeted cells were infected with the media containing the lentivirus supplemented with 10 μg/ml polybrene. Cells were selected with 10 μg/ml puromycin for 14 days. Single clones were expanded for knockout confirmation by Western blotting.

Statistics and Reproducibility
The sample size was sufficient for data analyses. Data were statistically analyzed using the software GraphPad Prism 9. Significant differences between the indicated pairs of conditions are shown by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

3.3 Results

The cGAS-STING pathway is silent in H1299 cells

To study the role of STING in regulating coronavirus infection, we generated STING knockout H1299 cell lines using CRISPR. To examine the integrity of RNA and DNA sensing pathways in STING wild-type and knockout H1299 cells, we stimulated these cells with polyI:C for 0, 4, 8, and 16 h. Then the cell lysate was collected and subjected to western blotting. As shown in Figure 11A, polyI:C treatment induced the phosphorylation of IRF3, TBK1, and IKK. Next, we examined IFNβ mRNA expression in these cells. Real-time PCR showed that poly(I:C) induced IFNβ mRNA expression in STING knockout H1299 cells (Fig. 11B), suggesting that the coronavirus RNA sensing pathway is intact in STING knockout cells. Unexpectedly, wild-type H1299 cells failed to respond to the DNA ligand ctDNA, indicating that the DNA sensing pathway in H1299 cells is silent (Fig. 11C).
Fig. 11 The DNA sensing pathway is inert in H1299 cells.

(A) Wildtype and STING knockout cells were treated with 1ug/ml polyI:C for the indicated times. The cell lysates were collected and blotted as indicated. (B-C) Wildtype and STING knockout cells were treated with 1ug/ml polyI:C or ctDNA for the indicated times. The total RNA was extracted, and real-time PCR was performed to determine the relative mRNA levels of IFNβ.

**STING inhibits HCoV-OC43 infection independent of IFN**

To further investigate the role of STING in coronavirus infection, we infected STING wildtype and knockout cells with HCoV-OC43 (Fig. 12A). After infection, cells were fixed and subjected to IFA with an anti-HCoV-OC43 N. As shown in Figure 12B, STING knockout cells were more susceptible to coronavirus infection (Fig. 12B). Similarly, deficiency of STING led to a higher expression of viral M mRNA (Fig. 12C) and N protein (Fig. 12D). Next, we reconstituted STING-FLAG in the STING knockout cell lines and found that reconstitution of STING rescued the antiviral activity in H1299 cells (Fig. 12E).
Furthermore, viral titration also showed increased viral particle production in STING knockout H1299 cells (Fig. 12F).

To examine the RNA and DNA signaling pathways in STING knockout cells, we infected wild-type and STING knockout H1299 cells with OC43 and found that the expression of MDA5, MAVS, and TBK1 was unaffected (Fig. 12G). Furthermore, STING deficiency had little effect on TBK1 activation. HCoV OC43 infection did not activate STING (Fig. 12G), which is consistent with a recent study. To further rule out the effect of IFN response, we generated MAVS single knockout and MAVS/STING double knockout H1299 cell lines and infected them with OC43. We found that STING knockout further promoted coronavirus infection in the MAVS deficient cells (Fig. 12H). Moreover, overexpression of STING-FLAG in MAVS knockout cells inhibited HCoV OC43 infection (Fig. 12I). Taken together, these data indicate that STING inhibits HCoV-OC43 infection and the inhibition is independent of IFN.
Fig. 12 STING-mediated anti-coronavirus activity is independent of IFN.

(A) The lysates of wildtype and two STING knockout (KO) H1299 cell lines were blotted as indicated. (B) H1299 and two STING KO cell lines were infected with 0.1 MOI of HCoV-OC43 for 16 h. Cells were fixed and stained with anti-N antibody, the positive cells were examined and counted using a fluorescence microscope. The relative infection was determined by the ratio of positive cells. Data represent means ± SD of three independent experiments (>200 cells were counted in each field, and five fields were counted per experiment). The p-value was calculated by one-way ANOVA followed by Dunnett multiple comparisons test. **p < 0.01, ***p < 0.001. (C) H1299 and two STING knockout cell lines were infected with 0.1 MOI of HCoV-OC43 for the indicated times. Cells were then collected for RNA extraction. The RNA levels of HCoV-OC43 M were determined by real-time PCR. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by Dunnett multiple comparisons test. *p < 0.05. (D) H1299 and two STING KO cell lines were infected with 0.1 MOI of HCoV-OC43 for the indicated times. Cell lysates were collected and blotted as indicated. (E) STING KO cells and KO cells reconstituted with STING-FLAG were infected with 0.1 MOI of HCoV-OC43 for the indicated times. Cell lysates were collected and blotted as indicated. (F) H1299 cells and two STING KO cell lines were infected with 0.1 MOI of HCoV-OC43 for the indicated times. Titers of culture supernatants containing HCoV-OC43 were determined using Vero cells. Data represent means ± SD of three independent experiments. All experiments were repeated three times. The p-value was calculated by two-way ANOVA followed by Dunnett multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001. (G) STING wild type and KO cells were infected with 0.1 MOI of HCoV-OC43 for indicated times. Cell lysates were collected and blotted as indicated. (H) The H1299 cells, MAVS KO cells and MAVS/STING double knockout (DKO) cells were infected with 0.1 MOI of HCoV-OC43 for the indicated times. Cell lysates were collected and blotted as indicated. (I) H1299 MAVS KO cells were
transfected with the indicated constructs and 24 h after transfection, cells were infected with 0.1 MOI of HCoV-OC43 for the indicated times. Cell lysates were collected and blotted as indicated.

The CTD and DI domains of STING are required for anti-HCoV activity

To examine which domain of STING is responsible for its anti-coronavirus activity, we generated a serial of STING mutants, including deletion and point mutation (Fig. 13A). We transfected these mutants into H1299 cells and then infected the cells with OC43. Western blotting showed that deletion of the C-terminal domain (CTD) and dimerization domain (DI) failed to inhibited coronavirus infection, suggesting that the CTD and DI domains of STING are critical for antiviral activity (Fig. 13B).

![Fig. 13 Domains of STING required for anti-HCoV activity.](image)

(A) Schematic of STING truncation mutants. (B) H1299 cells were transfected with the indicated STING mutants, and 24 h after transfection the cells were infected with HCoV-OC43 for 8 h. Cell lysates were collected and blotted as indicated.
**STING interacts and colocalizes with coronavirus viral proteins**

A proteomic study reported that coronavirus viral proteins interact with STING \(^{341}\). To validate the data, we performed co-IP between STING and coronavirus viral proteins and found that STING interacted with NSP4 and NSP6 (Fig. 14A). To determine the domains of STING that are responsible for the binding with NSP4 and NSP6, we co-transfected STING mutants with NSP4 and NSP6 in HEK293 cells. As shown in Figure 14B, the deletion of the CTD domain of STING dramatically disrupted the interaction with NSP4. Deletion of N-terminal 140 amino acids significantly decreased the interaction between STING and NSP6 (Fig. 14C). Thus, NSP4 mainly interacts with the CTD domain, whereas the NSP6 mainly interacts with the N-terminal transmembrane domain of STING. Next, we co-transfected STING with NSP4 or NSP6 and the IFA showed that STING colocalized with NSP4 and NSP6 in the cytosol (Fig. 14D). These data suggest that STING interacts and co-localizes with coronavirus NSP4 and NSP6.
Fig. 14 STING interacts and colocalizes with NSP4 and NSP6.

(A) STING-HA was co-transfected with the indicated FLAG-tagged viral proteins into HEK293 cells. After 48 h, cells were harvested and immunoprecipitated with anti-FLAG antibody (Ab), then blotted with the indicated Abs. (B-C) FLAG-tagged NSP4 and NSP6 were co-transfected with the indicated HA-tagged STING mutants. After 48 h, cells were harvested and immunoprecipitated with anti-FLAG antibody, then blotted with the
indicated antibodies. (D) STING-FLAG was co-transfected with NSP4-HA or NSP6-HA into H1299 cells. After 48 h, cells were fixed and stained with anti-FLAG (red), anti-HA (green), and DAPI nuclear stain (blue).

**STING inhibits DMV formation induced by HCoV-OC43**

Since STING interacts with coronavirus DMV formation proteins NSP4 and NSP6, we speculate that STING might impair coronavirus infection by inhibiting DMV formation. In this regard, we infected STING wild-type and knockout H1299 cells with HCoV-OC43 and stained the cells with anti-dsRNA, a DMV marker. IFA showed that STING knockout significantly increases the formation of DMV (Fig. 15). Next, we reconstituted FLAG-tagged STING in the STING knockout H1299 cells and found that reconstitution of STING restored the antiviral activity by reducing the DMV formation (Fig. 15). These data suggest that STING inhibits coronavirus-induced DMV formation.
Fig. 15 STING inhibits DMV formation during HCoV-OC43 infection

H1299 cells, two STING KO cell lines, and STING KO reconstituted with STING-FLAG cells were infected with 0.1 MOI HCoV-OC43 for 24 h. Cells were stained with anti-dsRNA (red) and DAPI nuclear stain (blue).

STING inhibits HCoV-OC43 infection in primary cells independent of IFN

To determine whether STING also elicits anti-coronavirus infection in primary cells, we isolated primary MEFs from the wild-type and STING knockout mice. Cells were blocked with anti-IFNAR to neutralize the IFN signaling response and then were infected with HCoV-OC43 for different times. As shown in Figures 16A-C, the IFNAR antibody blocked the IP10, ISG15, and IFIT1 mRNA expression induced by OC43 infection. During HCoV-
OC43 infection in the wildtype MEFs, IFNAR blockade increased the virus infectivity (Fig. 16D). However, in the STING-/- cells, IFNAR blockade did not affect the HCoV-OC43 infection (Fig. 16E). Even without ISGs expression, STING knockout MEFs still have significantly higher virus infection compared with wild-type MEFs (Fig. 16F). These data indicate that STING inhibits HCoV-OC43 infection in primary cells, which is independent of IFN.

Fig. 16 STING inhibits HCoV-OC43 infection in primary MEFs.

(A-C) Wildtype MEFs were blocked with anti-IFNAR-1 antibody for 4 h, then cells were infected with HCoV-OC43 for the indicated times, and the mRNA expression of IP10 (A), ISG15 (B) and IFIT1 (C) was checked by real-time PCR. (D-F) The wildtype and STING KO MEFs were blocked with anti-IFNAR-1 antibody for 4 h, then cells were infected with HCoV-OC43 for the indicated times, and the RNA levels of HCoV-OC43 M were determined by real-time PCR. The p-value was calculated by two-way ANOVA followed by Dunnett multiple comparisons test. ****p < 0.0001.
3.4 Discussion

In this study, we demonstrate that STING inhibits coronavirus infection by disrupting viral replication organelle formation and this process is independent of IFN signaling. Firstly, we found that the cGAS-STING pathway in the human lung epithelial cell line H1299 is silent since it cannot respond to ctDNA treatment. Consistently, a recent study reported that HCoV-OC43 infection did not activate STING-dependent IFN response. Thus, knockout of STING should not alter coronavirus infection-induced innate immune response. Secondly, we found that knockout of STING in H1299 cells led to a dramatic increase in HCoV-OC43 infection and reconstitution of STING inhibits viral infection in STING knockout cells. These data suggest that STING inhibits coronavirus infection independent of IFN. Thirdly, STING interacts and colocalizes with coronavirus viral proteins NSP4 and NSP6, which are important for virus-induced DMV formation. Indeed, the immunofluorescence assay showed that STING deficiency dramatically enhances the dsRNA staining, whereas the reconstitution of STING abolishes the enhancement. Finally, neutralization of IFNAR in primary MEFs has little effect on the STING-mediated antiviral activity. Taken together, we reveal a novel role of STING in coronavirus infection. STING interacts with coronavirus proteins NSP4 and NSP6 and suppresses the formation of DMV, thereby, inhibiting coronavirus infection.

Recent studies have demonstrated multiple IFN-independent roles of STING in regulating host cell responses, which extensively expands the biological functions of STING. STING was shown to elicit IFN-independent antiviral activity to both DNA and RNA virus infection. STING S356A knock-in mice that cannot induce type I IFN are still resistant to HSV-1 infection. Furthermore, the block of IFNAR in mice only partially abolished the STING-mediated anti-coronavirus infection, suggesting an IFN-independent antiviral activity of STING. Recently, ER membrane proteins have been reported to be involved in
coronavirus-induced DMV formation, thus modulating virus infection \textsuperscript{342}. Similarly, STING was shown to be recruited to the virus-induced replication organelle (RO) to regulate RO biogenesis \textsuperscript{339,340}. These studies together suggest a potential role of STING in regulating RO-mediated virus infection. In our study, we found that STING inhibited coronavirus infection and STING deficiency led to a significant increase in virus-induced DMV formation.

Despite the ongoing pandemic caused by SARS-CoV-2, the understanding of the basic processes of viral infection is limited. Our study provides new insight into the host regulating mechanism of virus infection, which may contribute to novel anti-coronavirus therapeutic strategy development.
CHAPTER 4. Summary and Future Perspective

As an emerging innate immune defense against virus infection, the cGAS-STING pathway has been widely studied and targeted for drug development. The activity of cGAS-STING must be tightly controlled so that the signaling pathway can elicit a protective effect instead of causing damage, such as autoimmune diseases, to the host. Thus, the regulation of cGAS-STING is critical for understanding the function and nature of innate immune signaling. To seek for cGAS regulator, we identified that C1QBP is a negative regulator of cGAS that controls cGAS activity and promotes DNA virus infection. Although C1QBP predominantly localizes in the mitochondria matrix, we found that during HSV-1 infection C1QBP is released into the cytosolic compartment and binds with cGAS, which in turn inhibits cGAS enzymatic activity and facilitates DNA virus infection. Studies have shown that HSV-1 infection can induce mitochondria stress, which primes the cGAS-mediated antiviral immune response. However, why the virus induces IFN response that does no good to its infection? Our study demonstrated that C1QBP plays a counter-act role against cGAS activation. DNA virus-induced mitochondria stress releases C1QBP into the cytosol and then C1QBP binds and suppresses cGAS activation, thereby limiting IFN production. This may explain why many DNA viruses exploit C1QBP to benefit their infection. However, in this study, we have not examined whether C1QBP deficiency leads to impaired viral infection in mice because the C1QBP knockout mice are embryonic lethal. Moreover, the mechanism by which C1QBP is released from mitochondria is unknown. cGAS has recently been shown to localize in the nucleus and tethered on nucleosomes through the acidic patch. Similarly, C1QBP is also a highly acidic protein and is reported
to be involved in homologous recombination in the nucleus\textsuperscript{313}, thus, it is interesting to know whether C1QBP binds and inhibits cGAS in the nucleus and regulates nuclear cGAS activity through a competing mechanism.

It is well known that the cGAS-STING pathway elicits an antiviral role through the induction of IFN. However, many RNA viruses which do not activate cGAS-STING were shown to be inhibited by this signaling pathway\textsuperscript{101,295,343}. During our study of the innate immune response, we found the lung epithelial cancer cell line H1299 cannot respond to DNA stimulation, although the cells expressing cGAS and STING (Fig. 9C). Interestingly, the infection of HCoV-OC43 in STING knockout H1299 cells significantly increased (Fig. 10). HCoV-OC43 infection is inhibited by STING even though HCoV-OC43 does not activate STING and the H1299 cells have silent STING signaling, suggesting that STING-mediated coronavirus inhibition is independent of IFN. We further revealed that STING interacts with coronavirus DMV formation proteins NSP4 and NSP6\textsuperscript{341}. Thus, we sought to determine whether STING regulates the coronavirus-induced DMV formation. As we expected, knockout of STING significantly increased the DMV formation, as shown by the increased dsRNA, a DMV marker, staining. Taken together, we propose that STING inhibits coronavirus infection by disrupting DMV formation.

The future studies of this project will focus on resolving the role of STING in regulating DMV formation by transmission electron microscopy (TEM). We will reconstitute the STING knockout cells with STING mutants to determine whether the interaction of STING with viral proteins is required for DMV biogenesis. To study the role of STING in controlling coronavirus infection \textit{in vivo}, we will generate STING/IFNAR double knockout mice and examine whether STING elicits antiviral activity in the mouse model. The CTT domain of STING is known to be critical to induce IFN and inflammatory response but is not responsible for the anti-coronavirus infection; thus in the future, we will use CTT knock-in
mice to determine whether CTT-mice can inhibit coronavirus infection without inducing inflammatory response and tissue damage.
Appendix A

Publications


Wu, Y., **Song, K.** *, Hao, W., Li, J., Wang, L., & Li, S. (2022). Nuclear soluble cGAS senses double-stranded DNA virus infection. Communications biology, 5(1), 433. (Featured Article) [*co-first author]


Honors and Awards

The ASV Student Travel Award, American Society for Virology 2022

The AAI Trainee Abstract Award, The American Association of Immunologists 2021
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