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An emerging role for calcium signalling in innate and autoimmunity via the cGAS-STING axis

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ABSTRACT

Type I interferons are effector cytokines essential for the regulation of the innate immunity. A key effector of the type I interferon response that is dysregulated in autoimmunity and cancer is the cGAS-STING signalling axis. Recent work suggests that calcium and associated signalling proteins can regulate both cGAS-STING and autoimmunity. How calcium regulates STING activation is complex and involves both stimulatory and inhibitory mechanisms. One of these is calmodulin-mediated signalling that is necessary for STING activation. The alterations in calcium flux that occur during STING activation can also regulate autophagy, which in turn plays a role in innate immunity through the clearance of intracellular pathogens. Also connected to calcium signalling pathways is the cGAS inhibitor TREX1, a cytoplasmic exonuclease linked to several autoimmune diseases including systemic lupus erythematosus (SLE). In this review, we summarize these and other findings that indicate a regulatory role for calcium signalling in innate and autoimmunity through the cGAS-STING pathway.

1. Introduction

Host-pathogen interactions evolved from the pool of diverse organisms that comprised early life in the form of viruses, bacteria, and archaea. Through the evolution of CRISPR/Cas9, bacteria and archaea both sense and defend against invading viral nucleic acids [1]. As eukaryotic multicellular organisms evolved, viral pathogens evolved with them, requiring similar foreign DNA and RNA sensing mechanisms in these metazoans. In 2006, two groups reported that mammalian cells can detect double-stranded DNA during viral infection and then respond by producing type I interferons (IFNs) to defend against potential viral pathogens [2,3]. However, IFN-stimulating DNA does not need to be foreign, and can arise from "self-DNA", including genomic DNA following DNA damage and mitochondrial DNA (mtDNA) leakage from impaired mitochondria [4,5]. The type I IFN response mainly relies on IFNα and IFNβ proteins which are two dominant effector cytokines that are responsible for the host immune response against viral infections [6]. These cytokines are also involved in regulating other aspects of the immune system, such as the host response to bacterial infections and inflammasome activation [7]. Type I IFNs are associated with human disease even in the absence of pathogens. Dysregulation of the IFN response leads to a variety of autoimmune disorders such as psoriasis, Aicardi-Goutières syndrome, Sjögren syndrome, rheumatoid arthritis, myositis, systemic sclerosis, and systemic lupus erythematosus (SLE) [8,9]. Moreover, the IFN response affects cancer progression by influencing the tumour microenvironment, with IFN-associated genes showing altered expression in malignant cells [10,11]. As a result, a better understanding the mechanisms governing the IFN response is critical for the development of targeted therapeutic approaches to infectious and autoimmune diseases as well as a variety of cancers.

To initiate the type I IFN response during viral infections, the sensor protein cyclic GMP-AMP synthase (cGAS) binds to cytoplasmic DNA to then produce the second messenger cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP or cGAMP) [12]. The second messenger cGAMP then binds to and activates the stimulator of interferon genes (STING) protein located on the endoplasmic reticulum. STING is the key adaptor protein that is required to initiate the type I IFN response [13,14]. However, prolonged IFN response through STING can result in pathogenic inflammation and damage to organs. For example, in SLE, the most common form of lupus, cGAS (and other DNA sensors) are hyperactivated by cytosolic DNA, which in turn leads to an overly robust type I IFN response and harmful inflammation [15]. To prevent this form of pathogenic inflammation, metazoans have evolved regulatory machinery that controls the cGAS-STING axis. One key

Abbreviations: STING, stimulator of IFN genes; TREX1/2, three prime repair exonuclease 1/2; SLE, systemic lupus erythematosus; cGAS, cyclic GMP-AMP synthase; IFN, interferon; Ca^{2+}, calcium

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regulator protein upstream of the cGAS-STING axis is TREX1, an exonuclease that acts to supress the type I IFN response by degrading cytosolic DNA [16]. However, there is also growing evidence that other regulatory mechanisms exist; thus, regulation of the entire pathway is more complicated then first thought. One emerging mechanism of regulation is through calcium (Ca^{2+}) levels and, hence, Ca^{2+} signalling pathways [17–22].

Eukaryotic cells use the import and export of Ca^{2+} at organelles to mediate a variety of cellular processes, including exocytosis, cell motility, apoptosis and nerve cell transmission [23]. Collectively, this process is known as Ca^{2+} signalling within the cell. Most often, Ca^{2+} signalling orchestrates the regulation of cellular processes through a central cytoplasmic protein known as calmodulin, which undergoes conformational rearrangements by binding to Ca^{2+} ions [24]. For example, the actions of calmodulin can trigger a number of kinase pathways through the calmodulin-dependent kinase family. Ca^{2+} signalling has been examined in the context of innate immunity, where it influences both macrophage activation and survival [25,26]. It is also involved in adaptive immunity, where it regulates T-cell growth and maturation [27]. The type I IFN response is connected to intracellular Ca^{2+} levels and can be supressed in the presence of Ca^{2+} chelators [28]. Previously, it was thought that this was a consequence of global effects from Ca^{2+} quenching, but there is now evidence suggesting that changes in cytosolic Ca^{2+} is an essential and key regulator of the cGAS/STING pathway. Here, we review the cGAS/STING pathway and the recent findings that highlight how the pathway is regulated by Ca^{2+} levels and related signalling proteins to modulate innate and autoimmunity.

2. TREX1 and cGAS in STING activation: a brake and acceleration pedal

Until recently, the identity of the key cytosolic DNA sensor proteins in innate immunity were the subject of intense debate. The list of potential proteins included cGAS, DAI, MRE11, PQBP1, DDX41 and Aim2-like receptors [29,30]. In evaluating these potential DNA sensors, two key criteria were essential for their function: (1) sensing the DNA; and (2) triggering an IFN response by relaying DNA binding to STING activation. It is now understood that cGAS is the essential and primary cytosolic double-stranded DNA sensor for mammalian cells (Fig. 1) [14]. This cytosolic DNA can be generated during viral infections through reverse transcription of retrovirus RNA [12], and from the aberrant release of viral DNA to the cytoplasm during infection; for example, during herpes simplex virus type 1 (HSV1) infection [31]. cGAS also detects micronuclei (a product of genomic instability) and single-stranded DNA produced in response to DNA damage by the combined actions of BLM and EXO1 (Fig. 1) [4,32]. This has implications for cancer progression as BLM and EXO1 generate free single stranded DNA (which leaks to the cytoplasm) after DNA damage from radiation or chemotherapy. Once these cytosolic DNA substrates are detected by cGAS, the protein produces cGAMP which binds STING (Fig. 1) [14]. After the cyclic dinucleotide binds STING, this activates STING signalling, which through a series of molecular events (outlined in detail below) the cell produces IFNs.

STING can be found in the tree of life more than 600 million years ago [33]. Orthologs of STING and cGAS are found in choanoflagellate *Monosiga brevicollis* which lacks the key proteins involved in the IFN response (i.e., NF-κB). This suggests that the cGAS-STING signalling predates IFN based immunity. Human STING localizes to a number of cellular compartments including the Golgi, acidified endolysosomes, endoplasmic reticulum (ER), ER-Golgi intermediate compartment (or perinuclear vesicles), and the mitochondria-associated ER membranes [13,34–36]. Intriguingly, these mitochondria-associated ER membranes also represent contact sites between the ER and mitochondria, where Ca^{2+} is exchanged through channels like voltage-dependent anion channel 1 (VDAC1) and mitochondrial Ca^{2+} uniporter (MCU) [37]. STING plays the mediator role in the type I IFN response and its activation causes interacting partners to activate the NF-κB pathway (Fig. 1) [38,39]. After STING recognizes and binds cGAMP, STING is trafficked from the ER to perinuclear vesicles [34]. During this process, STING interacts with the kinase TBK1 and migrates to perinuclear vesicles [40]. After this occurs, STING functions as a scaffold for TBK1 and IRF3 assembly to trigger IRF3 phosphorylation and dimerization promoting its nuclear translocation (Fig. 1) [40,41]. STING also activates the IKK complex triggering phosphorylation of the IkB family of inhibitors, in turn promoting their degradation via the ubiquitin-proteasome system [41]. Ultimately, this releases the transcription factor NF-κB to translocate across the nuclear membrane, where it functions with IRF3 and others factors to induce interferon and inflammatory cytokine expression (e.g. TNF, IL-1b and IL-6) [13]. Alternative mechanisms of STING activation play a role during bacteria or RNA virus infection. This occurs through different sensor proteins (i.e., RIG-I-like receptors) or direct binding of STING to cyclic dinucleotides produced by bacteria (e.g. cyclic di-AMP produced by *Listeria monocytogenes*) [42–44]. Thus, STING activation is critical for controlling the type I IFN response and occurs through multiple innate immune pathways in host-pathogen defense.

Since the presence of cytosolic DNA initiates the type I IFN response, mammalian cells have evolved mechanisms to target these DNA substrates, and thus supress cGAS-STING (Fig. 1). Both double stranded and single stranded DNAs are degraded by a cytosolic exonuclease, three-prime repair exonuclease 1 (TREX1) [4,16,45]. TREX1 belongs to a family of exonucleases known as the DEDDh family or DnaQ-like exonuclease family. Loss of function mutations in TREX1 also cause a variety of other autoimmune disorders, including SLE, Aicardi-Goutieres syndrome, Familial Chilbain Lupus, Retinal vasculopathy, Cerebral Leukodystrophy [46]. In SLE, the overactive cGAS sensing occurs as a result of accumulating cellular cytoplasmic DNA and this is observed in Trex1 deficient mice (*Trex1*$^{-/-}$) (Fig. 1) [47]. This overactive IFN response in *Trex1*$^{-/-}$ mouse embryonic fibroblasts was abolished in the absence of murine cGAS [48]. These data support a model whereby TREX1 regulates the cGAS-STING axis by targeting cytosolic DNA for degradation. Therefore, at its core, the DNA-activated type I IFN response is composed of three key proteins that regulate (TREX1, the brake), sense (cGAS, the accelerator), and mediate (STING, the effector of) innate immune signalling.

3. Growing evidence that SLE pathology is coupled to Ca^{2+} homeostasis

3.1. Ca^{2+} signalling is affected in SLE

In SLE, there are changes to both the innate and adaptive immune system from cytosolic DNA accumulation which fuels the autoimmune response. Sustained type I IFN production (in the absence of pathogenic stimuli), which is observed in SLE, leads to the activation of myeloid dendritic cells that promote autoimmunity by presenting nucleic acid containing autoantigens such as cellular DNA found in apoptotic bodies [15,49]. With the high expression of costimulatory molecules from the activated myeloid dendritic cells, this leads to the expansion of autoreactive lymphocytes (including T-cells and B-cells). $CD4^+$ T-cells which are now autoreactive, assist in the differentiation of $CD8^+$ T-cells and autoreactive plasma cells from B-cells. This “overactive” IFN response from autoimmune lymphocytes causes further apoptotic body overload and eventually tissue damage. Therefore, elucidation of the mechanisms regulating IFN production and secretion is key for our understanding of autoimmune disorders like SLE.

Suppressing the cGAS-STING pathway in a SLE disease models may show success in ameliorating the overactive IFN response since genes involved in the pathway are altered in patients [50,51]. SLE patients tend to have higher serum Ca^{2+} levels and often experience Ca^{2+} imbalances that lead to episodes of hypocalcaemia [52,53]. These

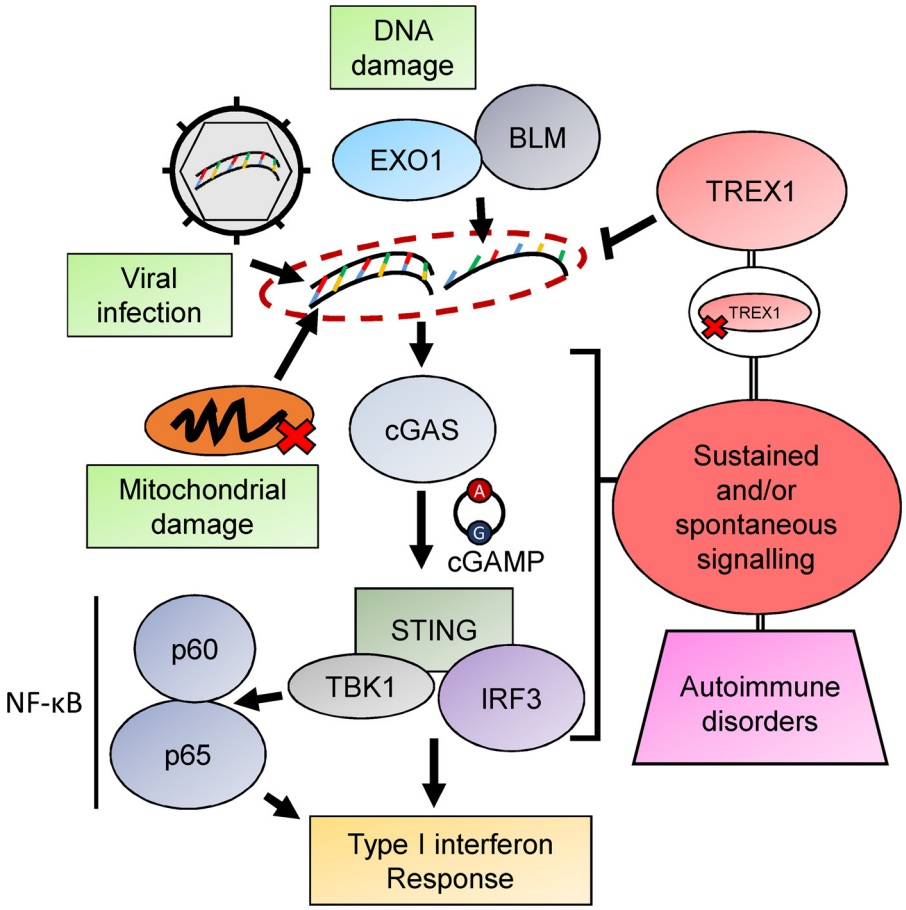

Fig. 1. General overview of the cGAS-STING signalling pathway. In the cGAS-STING signalling pathway, cytosolic DNA accumulates in cells that experience DNA damage, viral infection and damage to their mitochondria. This DNA is sensed by cGAS and results in the production of cGAMP which binds STING. When STING binds cGAMP, it is activated and this causes STING to function as a scaffold for IRF3 and TBK1 assembly, ultimately leading to a type I interferon response mediated by the translocation of NF-κB and IRF3 (dimer) transcription factors to the nucleus. The TREX1 exonuclease suppresses cGAS-STING signalling by degrading cytoplasmic DNA. Thus, the loss of TREX1 leads to a sustained/spontaneous type I interferon response.

alterations reduce bone mineral density in SLE patients and this was shown to be reversible in a clinical trial where patients were supplemented with Ca^{2+} [52]. Relationships between Ca^{2+} levels and SLE extends to the aberrant inflammatory pathways observed in SLE pathology. Treatment with dipyridamole, which is both an inhibitor of cGMP phosphodiesterase and a Ca^{2+} channel blocker, reduces the cytokine production in SLE patient derived T-cells [54]. This clinical finding between Ca^{2+} and IFN production in SLE patients indicates that the two may share common molecular pathways.

Kyttaris et al., also demonstrated that the elevated levels of cytokine production in SLE occurs through Ca^{2+} signalling pathways [54]. Dipyridamole inhibits calcineurin, a calmodulin-dependent phosphatase, which normally functions synergistically (but targeting different substrates) with another calmodulin-dependent kinase known as Ca^{2+}/calmodulin-dependent protein kinase IV (CAMKIV) to initiate cytokine production [55]. Activated CAMKIV promotes transcriptional changes that reduce the production of IL-2, a negative regulator/suppressor of autoimmunity. CAMKIV is often overexpressed in lupus nephritis, a manifestation of SLE that describes the inflammation of the kidneys [56]. When murine CAMKIV is absent, this reduces lupus nephritis associated symptoms in mice as a result of normalization of IL-2 levels. Recent work has shown that targeted delivery of CAMKIV inhibitors (i.e., KN-93) to the $CD4^+$ T-cells restores kidney function in lupus-prone mice [57]. Thus, there is growing evidence to suggest that altered Ca^{2+} signalling in SLE influences both innate and adaptative immune pathways; and as such, interventions that modulate Ca^{2+} signalling represent a potentially promising therapeutic strategy for SLE patients.

3.2. STING activation requires Ca^{2+} signalling

DNA-dependent activator of IFN-regulatory factors (DAI) functions as a secondary cytoplasmic DNA sensor to cGAS [30]. DAI undergoes dimerization and oligomerization after interacting with self-DNA (i.e., derived from apoptotic bodies, genome instability and mitochondrial damage) in the cytoplasm to initiate the DAI-STING axis [19]. Blocking DAI function, similar to cGAS, ameliorates SLE in murine models and suggests that DAI also has a significant role in sensing and initiating the signal cascade that promotes inflammation through STING. As we will discuss below, the parallels between these two cytoplasmic sensors extends to their regulation by Ca^{2+} signalling and points to potentially overlapping mechanisms underlying SLE pathology.

Zhang et al., demonstrated that inhibition of Ca^{2+} signalling during DAI-mediated activation of macrophages dampened the autoimmune response (Fig. 2) [19]. This was done by quenching cytosolic Ca^{2+} using BAPTA-AM (a Ca^{2+} chelator) and targeting mitochondrial Ca^{2+} export using CGP37157 (inhibits the mitochondrial sodium- Ca^{2+} pump). Reductions in cytosolic Ca^{2+} and the mitochondrial export of Ca^{2+} reduced the activation of NF-κB and IRF3, and suggests that increases in cytoplasmic Ca^{2+} from DAI-STING signalling are required for the autoimmune response. Similar results were seen in another study looking at STING activation from bacterial infections, where BAPTA-AM treatment inhibited the IFN response [58]. It is possible that the activation of NF-κB and IRF3 down stream of STING activation is regulated by calmodulin. This hypothesis is supported by the finding that the α isoform of Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) is phosphorylated on Thr-286 in macrophages stimulated with self-DNA (this was dependent on a DNA sensor like DAI) (Fig. 2) [19]. Further support for a Ca^{2+}/calmodulin-mediated signalling pathway comes from observation that the type I IFN response can be inhibited by 70–80% by treating monocytes with W-7, a potent calmodulin inhibitor [58]. These findings bridge previous studies regarding SLE patient T-cells and suggest interconnected Ca^{2+} signalling

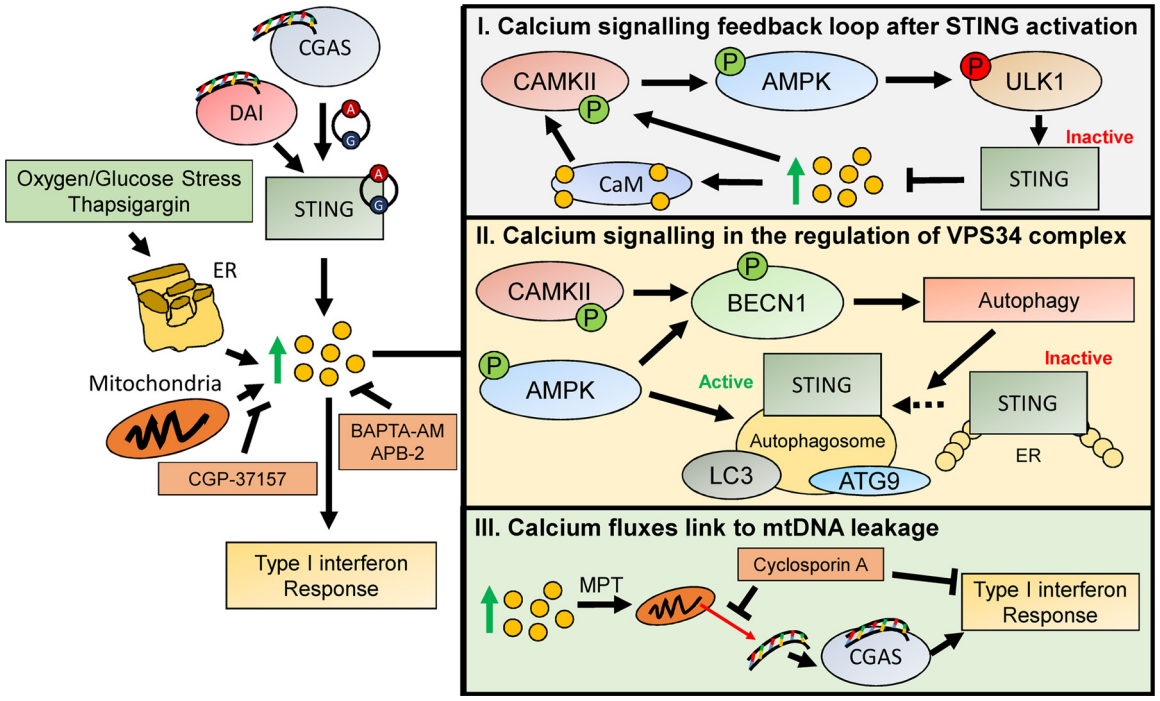

Fig. 2. The role of Ca^{2+} signalling in the CGAS-STING axis. STING activation requires an increase in intracellular Ca^{2+} from the endoplasmic reticulum (ER) and mitochondria. Evidence for this comes from a variety of inhibitors of cytosolic Ca^{2+} flux (orange boxes) inhibiting the STING-mediated interferon (IFN) response. Whereas, stimulants of cytosolic Ca^{2+} flux (green box) promote the STING-mediated IFN response. These changes in cytosolic Ca^{2+} influence a number of described signalling pathways involved in STING activation (I-III). (I) The actions of calmodulin (CaM) lead to a signalling cascade culminating in the phosphorylation of CAMKII and then AMPK, which are required for STING activation, to inhibit ULK1, which is in an inhibitor of STING. (II) Autophagy induction is required for STING activation. Both CaMKII and AMPK target BECN1 within the VPS34 complex to promote its phosphorylation and the consequent induction of autophagy. (III) Increases in cytosolic Ca^{2+} may also be involved in a feedback loop for STING activation through mtDNA. The mitochondrial permeability transition (MPT) is required for the type I IFN response via STING, which does not occur when mammalian cells are treated with cyclosporin A (inhibitor of MPT). The changes in cytosolic Ca^{2+} cause a MPT, which leads to the leakage of mtDNA into the cytoplasm activating cGAS and ultimately STING.

pathways modulate innate and adaptive immune responses through IFN production.

4. Ca^{2+} regulation of STING activation and potential STING-mediated mechanisms

4.1. STING has a Ca^{2+} binding site involved in its regulation

STING exists as a homodimer, which forms a binding site as a dimer for the cyclic dinucleotides involved in its activation [59]. Similar to the structures of other cyclic dinucleotide binding proteins that bind Ca^{2+} ions [60], two Ca^{2+} ions are shared between the two STING monomers as they form a dimer. Specifically, the Glu316 and Asp320 side chains from one monomer coordinate Ca^{2+} with the side chain of Asp205 and carbonyl group of Ala318 from the other STING monomer, allowing the coordination with two Ca^{2+} ions and two water molecules in an octahedral structure [59]. These Ca^{2+} ions appear to be critical for STING functions, as when the corresponding amino acids in murine STING (Glu315 and Asp204) are altered, the resulting STING mutant cannot bind cyclic dinucleotides and triggers a spontaneous IFN response [59] (Fig. 3A). These data indicate that the Ca^{2+} ions are critical in preventing aberrant STING activation in the absence of pathogenic stimuli.

4.2. Changes in cytosolic Ca^{2+} facilitate STING activation after pathogen exposure

Exposure to pathogens such as bacteria, DNA or RNA viruses causes the activation of STING, and the translocation of the protein from the ER to cytoplasmic vesicles [34,43]. For example, human cytomegalovirus (HCMV, a DNA virus) causes the activation of STING through a cGAS-dependent pathway [18]. What is perplexing is that viral DNA is not always required. For example, mammalian cells exposed to virus-like particles derived from HSV1, which do not contain the viral capsid or genome, can still elicit a STING-dependent type I IFN response [61]. This data implies that additional DNA-independent mechanisms are at play. One of these alternate mechanisms of virus sensing may involve changes in cellular Ca^{2+} as an early signalling event, observed in both HSV1 and HCMV infection [62,63]. Supporting this hypothesis, the virus-like particles used to activate STING were sufficient to cause increased cytosolic Ca^{2+}. This Ca^{2+} flux relied on signalling through the PLC-γ–PI3 kinase pathway and involves ER Ca^{2+} stores. When this cytosolic Ca^{2+} increase is inhibited by a Ca^{2+} channel blocker (APB-2), STING translocation to cytoplasmic vesicles is reduced (Fig. 2) [18]. Similar changes in intracellular Ca^{2+} were also observed during bacterial infections (i.e., *L. monocytogenes* infection), which can also induce a STING-dependent IFN response [17,43]. In this model, the relationship between Ca^{2+} levels and STING is also tightly regulated, where both dramatic increases of cytosolic Ca^{2+} (from high concentrations of ionomycin) or reductions in cytosolic Ca^{2+} resulted in reduced STING activation [17]. Therefore, transient Ca^{2+} increases upon exposure to both viral and bacterial pathogens to facilitate STING activation, even in the absence of pathogen DNA.

4.3. STING and STIM1 connect Ca^{2+} homeostasis to type I interferon signalling

STIM1 (stromal interaction molecule 1) is a transmembrane ER Ca^{2+} sensor which senses Ca^{2+} in the ER lumen through its N-terminus EF-hand domain [64]. It maintains ER Ca^{2+} levels by binding to Ca^{2+} channel ORAI1 at ER-plasma membrane sites when ER Ca^{2+} pools are depleted. This leads to the opening of Ca^{2+} release–activated Ca^{2+}

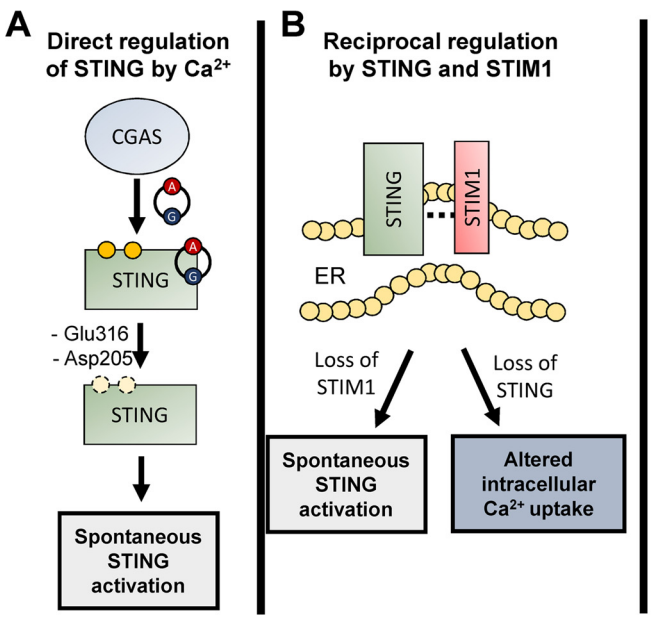

Fig. 3. STING is regulated by Ca^{2+} and STIM1 but may also reciprocally regulate intracellular Ca^{2+}. (A) The STING dimer binds calcium ions which can regulate its activation. Glu316 and Asp205 of human STING are involved in the binding of calcium and when the corresponding amino acids in murine STING are altered (dotted circles), this causes the spontaneous activation of STING. (B) STING and STIM1, a regulator of store-operated Ca^{2+} entry, interact with one another. This interaction anchors the two proteins to the endoplasmic reticulum (ER) and influences their activation in their associated processes. As a consequence, by regulating each other, the STING and STIM1 connect the pathways of type I interferon signalling and Ca^{2+} regulation. (C) STING physically interacts with a number of calcium channels which are involved in regulating intracellular Ca^{2+}. These include ATP2A2(SERCA2), ATP2B1, VDAC1 and VDAC3, suggesting that STING may regulate Ca^{2+} flux within the cell through these interactions during its activation.

channel to cause Ca^{2+} influx from the extracellular space into the cell, a process referred to as store-operated Ca^{2+} entry. Recent work has determined that STIM1 physically interacts with STING; binding mediated through interaction with STING via the EF-hand domain of STIM1, which results in inhibition of both ligand and ligand-independent STING activation (Fig. 3B) [22]. In this manner, STIM1 regulates STING by anchoring it to the ER, which inhibits its translocation to the Golgi and thus STING-mediated signalling under resting/unstimulated conditions. As a consequence, the loss of STIM1, but not ORAI1, leads to both a spontaneous IFN response under normal conditions as well as enhanced IFN signalling during herpes virus infection in mouse macrophages [22].

Similar to how STIM1 is involved in STING regulation, there is also evidence showing that the relationship is reciprocal (Fig. 3B) [22]. In certain cell types (mouse embryonic fibroblasts and human Jurkat T cells but not human THP1 monocytes) the loss of STING leads to elevated Ca^{2+} influx. With the absence of STING, STIM1 became enriched at endoplasmic reticulum-plasma membrane sites and this likely led to the dysregulated induction of store-operated Ca^{2+} entry. Thus, it is possible that the STIM1-STING binding under resting conditions reciprocally regulates the pool of STIM1 protein to maintain Ca^{2+} homeostasis. This also suggests that during STING activation, STIM1 may be free to translocate and interact with ORAI1, resulting in a transient cytosolic Ca^{2+} influx. Future work studying this relationship will be important in furthering our understanding of the interconnecting pathways linking innate immune signalling and Ca^{2+} homeostasis.

4.4. Evidence for alternative mechanisms of STING-mediated Ca^{2+} flux

Research into the role of STING in mediating changes in cytosolic Ca^{2+} through Ca^{2+} exporting from the ER is still underway. An interaction between STING and ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2 (ATP2A2, also known as SERCA2) was identified in a study looking at STING protein interactors using a mass-spectrometry approach (Fig. 3C) [65]. Supporting a possible functional connection between SERCA Ca^{2+} pumps, cytosolic Ca^{2+} and STING is the finding that thapsigargin, which is a well-known inhibitor of SERCA isozymes (i.e. SERCA1/ATP2A1, SERC2/ATP2A2 and SERC3/ATP2A3), can increase cytoplasmic Ca^{2+}, triggering STING-dependent IRF3 phosphorylation which is a requirement for type I IFN induction [20]. Further support for STING as a mediator of changes in cytoplasmic Ca^{2+}, come from a study of the function of STING during *Chlamydia trachomatis* infection [66]. *Chlamydia trachomatis* is a pathogen which induces mammalian host cell death through a STING-dependent but type I IFN-independent mechanism that relies on increases in intracellular Ca^{2+}. It was shown that this cell death mechanism requires an influx of intracellular Ca^{2+} and that SERCA inhibition by two pharmacological inhibitors (i.e., thapsigargin and cyclopiazonic acid), prevented STING-dependent cell death from occurring. This work suggests that STING maintains an interaction with SERCA2 to manage Ca^{2+} fluxes and supports the potential for STING to be involved in mediating intracellular Ca^{2+} influx to promote the type I IFN response.

As mentioned, thapsigargin is an established inhibitor of SERCA activity and its treatment leads to ER stress [20]. Intriguingly, it was shown that under conditions where cytosolic Ca^{2+} was elevated, but ER stress was absent, IRF3 phosphorylation did not occur (Fig. 2) [67]. Rather, ER stress needed to coincide with the Ca^{2+} mobilization for downstream pathways of STING to be activated. Furthermore, induction of the innate immune response following ER stress-induced IRF3 phosphorylation can occur after inhibition of N-linked glycosylation by tunicamycin, as well as growth of cells in oxygen/glucose deprivation conditions (Fig. 2) [67]. Although IRF3 phosphorylation after tunicamycin treatment was STING-independent, it was dependent on STING during oxygen/glucose deprivation. Consistent with this finding, a transcriptomics study found that STING is upregulated during oxygen/glucose deprivation conditions [68]. Oxygen/glucose deprivation conditions cause cytoplasmic Ca^{2+} increases through the activity of ryanodine type Ca^{2+} channels [69]. This raises the possibility that STING influences the activity of these channels, in addition to a number of other Ca^{2+} channels that have been shown to interact with STING including SERCA2/ATP2A2 and plasma membrane Ca^{2+}transporter ATP2B1 (Fig. 3C) [65].Therefore, induction of the type I IFN response by STING through Ca^{2+} flux relies on ER stress and multiple Ca^{2+} channels may play a role in this process.

5. Mitochondrial dynamics involved in the relationship between STING and Ca^{2+}

The discussed work and other findings support that STING contributes to the rise in intracellular Ca^{2+} through the mobilization of the ER Ca^{2+} pool. However, STING localizes to mitochondria-associated ER membranes where it likely affects mitochondrial Ca^{2+} pools as well [17]. STING has interacting partners that are key channels at these ER-

mitochondria contact sites including two VDAC family channels, VDAC1 and VDAC3, which facilitate Ca^{2+} uptake by mitochondria (Fig. 3C) [65,70]. It is possible that STING uses this interaction to influence mitochondrial Ca^{2+} homeostasis in a similar manner to its interaction with SERCA2/ATP2A2 to regulate ER Ca^{2+} pools.

Mitochondria act as mediators for the normal STING-mediated IFN response through Ca^{2+} exchange. The mitochondrial Ca^{2+} pool was shown to be essential for the STING response by treatment with CGP-37157, an inhibitor of the mitochondrial sodium-Ca^{2+} pump (Fig. 2) [19,71]. The mitochondrial sodium-Ca^{2+} pump is key in the export of Ca^{2+} out of the mitochondria, and thus, this appears to be a required step for the STING-mediated IFN response. Mitochondrial fission is also linked to the regulation of STING activation. Treatment with high concentrations of ionomycin leads to saturating levels of cytosolic Ca^{2+} and this inhibits the STING-mediated IFN response [17]. However, the loss of DRP1, a protein involved in mitochondrial fission, prevents the ionomycin-induced inhibition of STING. Mitochondrial fission plays a role in Ca^{2+} relays between mitochondria and maintains a labile pool of Ca^{2+} by distributing mitochondria uniformly throughout the cell [72]. Alterations in these relays have been shown to influence apoptotic processes and it is possible that similar changes are being observed with respect to STING signalling.

These findings that mitochondrial Ca^{2+} pools and mitochondrial fission influence the STING-mediated IFN response suggest that STING signalling may intersect with these processes to respond to mtDNA stress (Fig. 2). When mtDNA packaging proteins are absent and mtDNA leaks into the cytoplasm, the IFN response is induced via the cGAS-STING axis [5,73,74]. Since mtDNA stress is exaggerated in the presence of HSV1 infection, this causes the induction of a type I IFN response. Mechanistically, the hyperfusion of mitochondria was responsible for the mtDNA stress and consequent leakage. However, mtDNA release into the cytoplasm could be fueled by the mitochondrial-permeability transition (MPT), which may also occur in response to transient increases of cytosolic Ca^{2+}during STING activation [75,76]. The aforementioned study by Zhang et al., demonstrated that by using cyclosporin A, an inhibitor of MPT, the STING-mediated IFN response could be abolished in macrophages [19]. However, there is still debate as to whether or not this is true, since conflicting reports show that agonist-mediated activation of STING requires an intact mitochondrial membrane potential for the IFN response to occur in mouse macrophages [77]. Thus, additional work is required to identify whether or not the intracellular Ca^{2+} changes associated with STING activation can positively feedback to cGAS sensing through mtDNA leakage.

6. AMPK activation by STING links autophagy to the IFN response and relies on Ca^{2+} signalling

6.1. AMPK activity is required for STING activation

STING activation requiring an increase in cytosolic Ca^{2+} was also identified in a study where peritoneal mouse macrophages were exposed to the synthetic agonist specific to mouse STING, 5, 6-dimethyl xanthenone-4-acetic acid (DMXAA) [21]. Consistent with the other discussed findings, treatment with BAPTA-AM prevented the STING-mediated IFN response. Prantner et al., 2017 suggested that BAPTA-AM treatment likely impairs Ca^{2+} signalling required to activate AMPK. A feedback loop also appears to exist between STING activation and AMPK (Fig. 2). Short term increases in cytosolic Ca^{2+} increase AMPK activity through the actions of CAMKII, which is activated with STING activation [78]. When AMPK is absent, the addition of DMXAA did not induce an IFN response [21]. This is likely due to the actions of ULK1/ATG1, which can inhibit STING by phosphorylation in the absence of AMPK —a kinase that is also a regulator of ULK1 (Fig. 1) [79]. Under ideal conditions after STING activation, a feedback loop is initiated where AMPK phosphorylates ULK1 (at Ser555), which in turn inhibits the ULK1-dependent phosphorylation and activation of STING. Moreover, AMPK has a role in maintaining mitochondrial dynamics within the cell [80]. Active AMPK promotes DRP1 localization at mitochondria and promotes their fission, which ultimately will feedback to influence Ca^{2+} homeostasis in the context of the cGAS-STING axis. Thus, through these mechanisms, AMPK can regulate the cGAS-STING axis.

6.2. Ca^{2+} signalling promotes autophagy which is required for STING activation

Autophagy is a cellular mechanism involved in maintaining cell and tissue homeostasis by facilitating the degradation of damaged or senescent organelles, misfolded proteins and infectious agents [81]. Accumulating evidence continues to build a complex connection between autophagy and various autoimmune diseases like SLE, psoriasis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis [48]. Four identified roles for autophagy in immune responses include intracellular pathogen removal, lymphocyte development, the secretion of cytokines, and pro-inflammatory signalling [82–84]. This includes an innate immune function for autophagy in the removal of intracellular pathogens in dendritic cells. Furthermore, blocking autophagy in murine macrophages inhibits the activated lymphocyte-derived DNA-induced type I IFN response [85].

Given the role of cGAS-STING in the type I IFN response, it seems likely that there would be cross-talk between pathways regulating both this axis and autophagy. This is indeed the case, as proteins involved in both STING activation and autophagy are regulated by Ca^{2+} signalling. ATG9A (involved in autophagosome biogenesis) regulates the translocation of activated STING (Fig. 2) [36,86]. STING is found at both ATG9-positive and LC3-positive puncta (LC3 is key in autophagosome biogenesis [81]) in mouse embryonic fibroblasts, after the cells are stimulated with double stranded DNA [36]. Calmodulin can also activate autophagy through its regulation of VPS34, an interactor of ATG9 [87]. After cytosolic Ca^{2+} increases, CAMKII, which is activated by the STING pathway, phosphorylates Beclin 1 (BECN1) at Ser90. This phosphorylation event promotes the ubiquitination of BECN1 and thus, activates the VPS34 complex [88]. Finally, AMPK can also act as a co-regulator of the VPS34 complex by initiating autophagy via phosphorylating BECN1 [89]. Thus, AMPK and CAMKII represent two independent mechanisms by which Ca^{2+}-calmodulin-dependent signalling induces autophagy to promote STING activation.

7. TREX1 is implicated in Ca^{2+} signalling

7.1. Evidence for co-regulation of intracellular Ca^{2+} levels and TREX1 via miR-103

MicroRNAs influence many signalling processes in the cell, including the regulation of intracellular Ca^{2+} stores within the ER, mitochondria and cytosol [90,91]. Recent work has revealed the importance of miRNA-103 in the cGAS-STING axis because of its role in regulating TREX1 [92]. Inhibition of TREX1 mRNA translation by miRNA-103 leads to the induction of the IFN response in a cGAS-STING-dependent manner. Prior to the work identifying miRNA-103 regulation of TREX1, miRNA-103 was shown to regulate the L-type Ca^{2+} channel CACNA1C, where reductions in the expression of miRNA-103 promoted CACNA1C translation [93,94]. Interestingly, murine *CACNA1C* expression is increased in *Trex1*$^{-/-}$ mouse embryonic fibroblasts [95]. Since CACNA1C regulates cytosolic Ca^{2+} and mitochondrial Ca^{2+} pools, miR-103 likely influences the STING pathway through regulation of both CACNA1C and TREX1 protein translation.

7.2. Loss of TREX1 induces transcriptional changes in genes encoding Ca^{2+} signalling proteins

Although the cGAS-STING axis has been examined in the context of

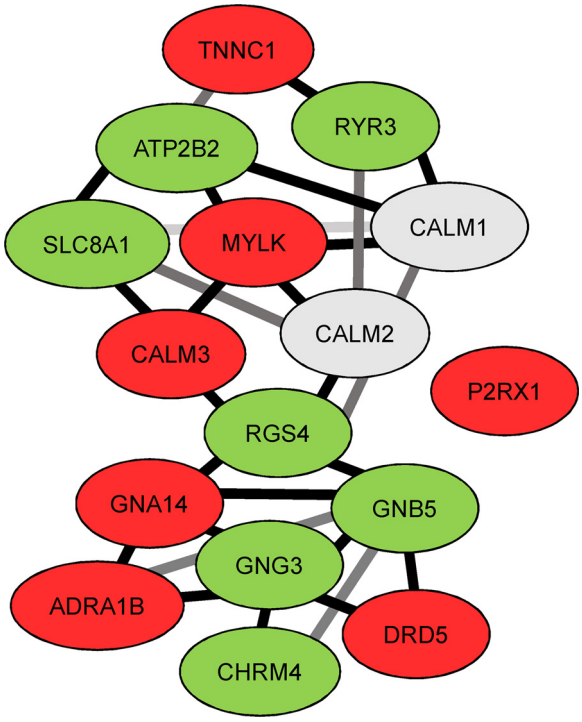

Fig. 4. Loss of Trex1 affects Ca^{2+} regulation and signalling pathways. STRING (v10.5) database generated network showing the physical and genetic interactions between the identified differentially expressed genes (i.e., enriched genes belonging to Ca^{2+} regulation and signalling pathways) affected in murine B-cells in Trex1 deficiency. Colours for genes indicate the type of alteration in transcript levels (green = up-regulated; red = down-regulated). Connecting lines between genes represent genetic or protein interactions.

Ca^{2+} signalling, it is not clear to what extent TREX1 may also impact Ca^{2+} signalling pathways. We examined the publicly available RNA-sequencing data from splenic B-cells obtained from 41-week old *Trex1*-deficient mice compared to wild type B-cells to identify any links to Ca^{2+} signalling that were not previously reported [96]. Analyzing this dataset for enrichment through the BioJupies tool (which employs KEGG and WikiPathways annotation databases, https://amp.pharm.mssm.edu/biojupies/) revealed that Ca^{2+} signalling and regulation pathways are enriched in the absence of Trex1 [97]. Using the KEGG database for annotation resulted in the identification of enriched downregulated genes related to Ca^{2+} signalling pathways (murine *Gna14*, *Tnnc1*, *P2rx1*, *Adra1b*, *Mylk*, and *Drd5*). Whereas, using the WikiPathways database for annotation identified an enrichment of upregulated genes related to Ca^{2+} regulation in cardiac cells (murine *Rgs4*, *Gng3*, *Chrm4*, *Serca2/Atp2b2*, *Gnb5*, *Ryr3*, and *Slc8a1*). These transcriptional changes in Ca^{2+} signalling and regulation pathways suggest that they are connected to Trex1 function or regulation.

We then generated a network of the interactions between the proteins encoded by the genes identified in our analyses using STRING (v10.5), which forms protein-protein associations by acquiring data across various organisms, and using data from high-throughput screens, database, literature mining, and predicted from genomic context analysis [98]. By using STRING, it was revealed that these Ca^{2+}-related proteins functionally relate to one another, with calmodulin forming a key part of this network (Fig. 4). CALM3 was one of the identified calmodulin paralogs and it appears to be closely associated with TREX1. Supporting the potential co-regulation of TREX1 and CALM3, there was a ~20% reduction in murine *Calm3* expression in *Trex1*$^{-/-}$ mouse embryonic fibroblasts [95]. Intriguingly, *CALM3* also shows altered expression in the myeloid cells collected from SLE patients, a disease linked to TREX1 dysfunction [99]. Further examination of how *CALM3* is related to TREX1 may provide new insights into how TREX1 is regulated.

A protein similar in structure to CALM3, the related EF-hand protein calmodulin-like 6 (CALML6) protein has also recently been proposed as a negative regulator of the cGAS-STING axis [100]. CALML6 could attenuate the type I IFN response both in vivo and in vitro, and in contrast to TREX1, it appears to function downstream of STING. Mechanistically this occurs by CALML6 binding to the phosphorylated serine-rich portion of IRF3 to inhibit its dimerization and nuclear translocation. Thus, IRF3 regulation by Ca^{2+} signalling proteins like CALML6 provides yet another mechanism of regulating cGAS-STING signalling and raises the possibility that other calmodulin and calmodulin-like proteins may also modulate this innate immune pathway.

8. Conclusion

Ca^{2+} signalling regulates many of the cellular processes within the cell. There is growing evidence that the cGAS-STING axis is one of the cellular pathways governed by Ca^{2+} signalling, and dysregulation of both this axis and Ca^{2+} homeostasis are implicated in a host of autoimmune disease including systemic lupus erythematosus (SLE). Ca^{2+} fluxes derived from mitochondrial and ER Ca^{2+} pools, and key proteins in Ca^{2+} signalling such as calmodulin, CAMKII and CAMKIV, all function to regulate the cGAS-STING axis; with autophagy and AMPK being a major target of the Ca^{2+} signalling. Furthermore, the absence of Trex1 in mice alters the transcription of Ca^{2+} signalling networks, and loss of function of TREX1 in humans can cause a variety of other autoimmune disorders, including SLE, Aicardi-Goutieres syndrome, and Familial Chilbain Lupus. These recent findings point towards Ca^{2+} being a critical regulator of the cGAS-STING pathway and autoimmunity. Future work exploiting these Ca^{2+} signalling pathways may not only lead to more therapeutic targets for autoimmune disease, but also a better understanding of how these pathways regulate the type I interferon response in microbial infections, viral disease and oncogenesis.

Conflicts of interest

The authors have declared no conflicts of interest.

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