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Review

Cyclic Dinucleotides and the Innate Immune Response

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Cyclic dinucleotides (CDNs) have been previously recognized as important secondary signaling molecules in bacteria and, more recently, in mammalian cells. In the former case, they represent secondary messengers affecting numerous responses of the prokaryotic cell, whereas in the latter, they act as agonists of the innate immune response. Remarkable new discoveries have linked these two patterns of utilization of CDNs as secondary messengers and have revealed unexpected influences they likely had on shaping human genetic variation. This Review summarizes these recent insights and provides a perspective on future unanswered questions in this exciting field.

Cyclic di-GMP as a Second Messenger in Gram-Negative Bacteria

Cyclic dinucleotides (CDNs) were originally described more than 25 years ago as activators of bacterial cellulose synthase (Ross et al., 1987). These investigators defined cyclic di-GMP (cdG) as two GMP molecules linked in a heterocyclic configuration via two 3′-5′ phosphodiester bonds. However, only in 1998 did Tal and colleagues suggest that cdG may be important for other processes in bacteria (Tal et al., 1998). Since then, cdG has been implicated in central bacterial processes, including, but not limited to, virulence, stress survival, motility, antibiotic production, metabolism, biofilm formation, and differentiation (reviewed in detail in Römling et al. [2013]). In Gram-negative bacteria, cdG is now accepted as a universal bacterial secondary messenger in that genes encoding enzymes involved in synthesis and degradation of this CDN are recognizable in the genomes of all corresponding bacterial species.

The cytosolic level of cdG in bacterial cells is tightly controlled by the dual activity of diguanylate cyclases (DGCs) with GGDEF domains and phosphodiesterases (PDEs) with EAL or HD-GYP domains. The large number of enzymes that are involved in cdG-related pathways (for example, *Vibrio cholerae* encodes 72 DGC and PDE proteins) suggests tremendous complexity of cdG-mediated signaling in bacteria. Intriguingly, analyses show that genomes carry many genes encoding multidomain proteins that encode both DGC and PDE domains. However, in the majority of these cases, only one domain is active while the other one is involved in protein-protein or protein-RNA interactions. Even more diverse is the number of receptors that have been implicated in cdG sensing in ways that alter cellular activity by modifying transcription, translation, or protein activity (Römling et al., 2013). Thus far, we know that bacteria can sense their environment and respond by modulation of cdG levels to stimuli that include O_2 and NO levels, light flux, redox state, and the stage of the cell cycle (Mills et al., 2011). However, our knowledge of the regulatory networks in which cdG (or other CDNs, see below) are involved is far from complete, and further research on environmental stresses, cdG-binding effectors, and the pathways that CDNs control is still needed. Newly developed methods such as fluorescence resonance energy transfer (FRET)-based biosensors that allow the monitoring of cdG concentrations within single bacterial cells (Christen et al., 2010), differential radial capillary action of ligand assay (DRaCALA) that allows rapid and high-throughput measuring of protein-CDN interactions (Roelofs et al., 2011), and development of similar tools for other CDNs will eventually lead to significant advances in the field.

Cyclic di-AMP in Gram-Positive Bacteria

In addition to the role of cdG as a central signaling molecule in Gram-negative bacteria, recent discoveries suggest that cyclic di-AMP (cdA) plays an important role, particularly in many Gram-positive bacteria. The diadenylate cyclase (DAC, DUF147) domain is found in almost 2,000 hypothetical proteins encoded by numerous bacterial species, and many of these DACs are fused to domains of unknown functions (Corrigan and Gründling, 2013), suggesting that signaling pathways for cdA are probably as complex and widespread as for cdG. The best-characterized DAC enzymes are from *Bacillus subtilis*: DisA and CdaS (YojJ) are required for sporulation, whereas another DAC CdaA (YbbP) is involved in cell wall biosynthesis (Mehne et al., 2013; Oppenheimer-Shaanan et al., 2011; Witte et al., 2008). Supporting evidence for the role of cdA in cell wall homeostasis comes from a study by Luo and Helmann (2012) that showed that overproduction of cdA phosphodiesterase *gdpP* (*yybT*) sensitizes *B. subtilis* to ampicillin, similar to a strain in which the gene encoding a cdA synthetase is deleted. A comparable antibiotic susceptibility phenotype was observed in *Staphylococcus aureus* and *Listeria monocytogenes* (Corrigan et al., 2011; Witte et al., 2012). In *Mycobacterium smegmatis*, overexpression of a DisA homolog was linked to changes in cell morphology and motility (Zhang and He, 2013).

Although the functions of cdA are far from being understood, observations that deletion of cdA synthetase are lethal in

L. monocytogenes, *Streptococcus pneumonia*, *S. aureus*, and *B. subtilis* suggest that cdA controls central cellular pathways in many Gram-positive organisms. Corrigan and colleagues suggested that essentiality of the cdA oligonucleotide might be due to a cumulative effect on cdA receptors (Corrigan et al., 2013). Their recent work identified a potassium transporter, KtrA, a cation/proton antiporter, CpaA, a PII-like signal transduction protein A, PstA, and a sensor histidine kinase, KdpD, as specific receptors for cdA in *S. aureus* (Corrigan et al., 2013). It is possible that simultaneous inactivation of these and possibly other unidentified proteins under cdA control can, in sum, have a lethal effect on a cell that is experiencing depletion of cdA.

Cyclic AMP-GMP in *V. cholerae*

A new type of CDN called cyclic AMP-GMP (cAMP-GMP) was recently identified by Mekalanos and colleagues as a signaling molecule in *Vibrio cholerae* that is involved in virulence (Davies et al., 2012). This hybrid molecule is synthesized by the enzyme dinucleotide cyclase or DncV, a protein that shows similarity to eukaryotic 2′-5′ oligo-adenylate synthetase (OAS1). However, evidence suggests that DncV produces a CDN composed of AMP and GMP linked by two 3′-5′ phosphodiester bonds (Ablasser et al., 2013; Davies et al., 2012; Diner et al., 2013). Predicted orthologs of DncV are also recognizable in many other Gram-negative and -positive bacterial species (Davies et al., 2012). Interestingly, in addition to its function as synthase for cAMP-GMP, DncV can also synthesize cdA and cdG, thus raising a possibility that one enzyme can control the pool size of multiple CDNs in the bacterial cell. Thus, some CDN-binding effectors (and CDN degradation enzymes) might have specific or overlapping specificities toward cdA, cdG, and cAMP-GMP. Overexpression of DncV inhibits the chemotactic response of *V. cholerae*, a phenotype that has been linked to hyperinfectivity by Camilli and colleagues (Butler et al., 2006). Because chemotaxis is not altered by overexpression of a cdG synthetase in *V. cholerae*, these data suggest that different CDNs could alter different regulatory networks within the same cell. These networks are likely to be more complex in the light of the possibility that there might be spatially and temporally distinct pools of CDNs controlling any given phenotype (Lindenberg et al., 2013).

Recognition of Pathogens by Host Innate Immune Receptors

Microbial organisms express molecules that can be chemically differentiated from those produced by host cells and, thus, can be targeted for recognition by receptors of the innate immune system. The term pathogen-associated molecular patterns (PAMPs) has been coined to define such microbial signaling agonists, and the receptors that recognize these agonists are referred to as pattern recognition receptors (PRRs) (Iwasaki and Medzhitov, 2010). Innate immune agonists include lipid A derived from lipopolysaccharides, peptides encoded within bacterial flagellin, peptidoglycan fragments, lipopeptides, microbial polysaccharides, and certain types of nucleic acids, among others molecules (Takeuchi and Akira, 2010). In some cases, host molecules can be recognized as "PAMP like" if they are present in an abnormal subcellular context (e.g., the presence of DNA in the cytosol rather than in the nucleus) (Barber, 2011a; Vance et al., 2009). This strategy for innate immune recognition can be rationalized by the host's need to detect the genetic products of pathogen replication within the cytosol, as well as the cellular disruptions caused by intracellular pathogens. Accordingly, host cells express a spectrum of PRRs in plasma membrane, vacuolar membranes, and cytosol in order to detect threats that have different anatomical and subcellular locations during pathogenesis (Palm and Medzhitov, 2009). The recognition of PAMPs by PRRs activates host cell signal transduction cascades that drive production of interferons (IFNs) and other proinflammatory cytokines, as well as antipathogen effector molecules, thereby triggering protective cell biological changes (e.g., inflammasome activation, apoptosis, and autophagy) needed to limit pathogen growth within infected cells (Baxt et al., 2013). The innate immune response also stimulates many elements of the adaptive immune system in order to marshal a specific counterattack on the detected microbial threat. Thus, PAMPs have been recognized for some time as potent adjuvants in the context of vaccines and immunotherapeutics (Duthie et al., 2011). The presence of CDNs in virtually all known bacteria and the fact that eukaryotes were not previously known to rely on these secondary messenger molecules suggested that CDNs might be PAMPs. Early evidence for this concept was provided by the observation that cdA and cdG had adjuvant and immunomodulatory activity in mice (Ebensen et al., 2011; Karaolis et al., 2007) and that STING was the innate immune receptor of bacterial CDNs (see below) (Burdette et al., 2011).

PRR Activation by Innate Immune Agonists

Several different types of PRRs are now recognized. In brief, Toll-like receptors (TLRs) play major roles in the recognition of PAMPs within the extracellular milieu and endosomal lumen (Kawai and Akira, 2011). TLR activation occurs after ligand (agonist) binding and leads to the recruitment of the adaptor molecules MyD88 and TRIF and subsequent activation of a kinase-driven signaling pathway, culminating in activation of NF-κB and IRF3/7 and thereby host gene expression (Dev et al., 2011). *N*ucleotide-binding domain *l*eucine-rich *r*epeat-containing proteins (NLRs) are PRRs that reside in the cytosol and most often are activated by bacterial products such as peptidoglycan fragments and peptides derived from flagellin, the type III secretion system rod components, toxins, bacterial, and viral double-stranded DNA (dsDNA) (Franchi et al., 2012). Besides NLRs, additional cytosolic innate receptors exist, including RNA helicases of the retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), which typically recognize cytosolic viral dsRNA and then recruit the adaptor IFN-β promoter stimulator 1 (IPS-1; also called MAVS, VISA, and Cardif), leading to phosphorylation of the transcription factors IRF-3 and IRF7 and expression of Type I IFN genes (Loo and Gale, 2011). Another non-NLR is absent in melanoma 2 (AIM2), an interferon-inducible protein that can bind dsDNA in the cytosol and induce autocleavage of caspase-1 and thereby inflammasome activation (Barber, 2011b). C-type lectin receptors (CLRs) such as Dectin-1 bind fungal cell wall components and trigger activation of NF-κB (Takeuchi and Akira, 2010). Given that PRRs can detect PAMPs associated with all pathogenic microorganisms, it seems likely that even

Table 1. Examples of Bacterial and Viral Proinflammatory Response Modifications

Organism	Effector protein	Mechanism	Target	Reference
Bacillus anthracis	lethal factor	metalloprotease	MAPK kinase	(Duesbery et al., 1998)
Chlamydia trachomatis	ChlaDub1, 2	DUB, deneddylase	IκBα	(Le Negrate et al., 2008b)
	CT441	protease	RelA	(Lad et al., 2007)
Pathogenic *E. coli*; *B. pseudomallei*	Cif; CHBP	deaminase	ubiquitin and ubiquitin-like protein NEDD8	(Cui et al., 2010; Jubelin et al., 2010)
Pathogenic *E. coli*	NleC	protease	RelA	(Yen et al., 2010)
Yersinia spp.	YopJ	acetyltransferase	RICK, TAK1	(Meinzer et al., 2012)
	YopM	inhibitor	caspase-1	(LaRock and Cookson, 2012)
Legionella pneumophila	LegK1	Ser/Thr-kinase	IκBα, p100	(Ge et al., 2009)
Salmonella Typhimurium	AvrA	acetyltransferase	IKKα/β	(Jones et al., 2008)
	SseL	DUB	IκBα	(Le Negrate et al., 2008a)
	SspH1	E3 ligase	PKN1	(Ashida et al., 2010; Haraga and Miller, 2006)
Shigella spp.	IpaH9.8	E3 ligase	NEMO	(Ashida et al., 2010)
	OspF	phosphothreonine lyase	MAPK kinases	(Li et al., 2007)
	OspG	Ser/Thr-kinase	UbcH5 (E2)	(Kim et al., 2005)
HSV-1	ICP27		IκBα	(Kim et al., 2008)
	ICP0	E3 ubiquitin ligase	IFI16	(Orzalli et al., 2012)
Influenza A	NS1		TRIM25 and Riplet	(Rajsbaum et al., 2012)
Vaccinia virus	B14R		IKKβ	(Chen et al., 2008)
	CP77		SCF complex, p65	(Chang et al., 2009)
	N1L		TBK1	(DiPerna et al., 2004)
	K1L		IKK	(Shisler and Jin, 2004)

prions might be detected by some element of the innate immune system because of the unusual β-amyloid structures they can form and the cellular disruptions that these polymers cause (Bradford and Mabbott, 2012; Sheedy et al., 2013).

Despite the presence of multiple PRRs in various cell compartments, both bacteria and viruses have developed mechanisms that allow them to avoid intracellular killing and subvert induction of the innate immunity. These include downregulation or modification of innate immune agonists such as flagellin (Gründling et al., 2004) or lipid A (Trent et al., 2006). In the case of bacteria, effectors delivered by type III and IV systems have also been shown to modify many different cellular pathways, including those involved in innate immune responses (Baxt et al., 2013; Galán, 2009). In some cases, bacterial effectors are similar in function to eukaryotic proteins that are involved in central cellular processes such as phosphorylation, ubiquitination, and Rho family GTPase signaling. In other cases, bacterial effectors are capable of targeting host proteins by modifying their function by dephosphorylation, acetylation, AMPylation, and N-myristoylation (Hicks and Galán, 2013). Among the myriad effects on host cells, bacterial effectors trigger blocks in signal transduction, avoidance of autophagy, interference with proinflammatory responses, and modifications of vesicular trafficking (Baxt et al., 2013) (Table 1). Similarly, viruses can suppress host IFN induction by various mechanisms, including, for example, inhibition of RIG-I signaling by blocking its ubiquitination by the TRIM25 ubiquitin E3 ligase (Rajsbaum et al., 2012) (Table 1). Thus, innate immune signaling is clearly a host response that pathogenic microorganisms have learned to manipulate through effector protein action as well as alterations in the production of innate immune agonists, including CDNs (see below).

Activation of the Type I Interferon Response by STING

Among the downstream effectors of the signaling cascade driven by activation of PRRs are specific cytokines and chemokines, including the type I interferons (IFN-α/β). These secreted cytokines can be produced in response to either viral or bacterial intracellular infections through signaling pathways that have only recently been revealed in molecular detail. For example, cytosolic DNA induces a type I IFN response that is dependent on a protein called STING (also known as ERIS, MITA, MPYS, and TMEM173), an endoplasmic reticulum (ER)-localized transmembrane protein (Ishikawa and Barber, 2008). The STING-dependent, type I IFN response appears to be critical to limit the replication of DNA viruses such as HSV-1 (Ishikawa and Barber, 2008). Although there is evidence that STING may directly recognize dsDNA with low affinity (K_d > 200 μM) (Abe et al., 2013), this is unlikely to be a critical property of STING in light of new information (see below). However, the pathway to type I IFN production after STING activation seems quite clear (Burdette et al., 2011). After a DNA recognition signal is received within the cytosol, STING is "activated," and this corresponds with its relocation to discrete foci in the cell cytoplasm. These cell biological changes correlate with recruitment of the kinases TBK1 and IKK, which in turn activate IRF3, STAT6, and NF-κB, resulting in type I IFN induction (Cavlar et al., 2012) (Figure 1). The biochemical connection between cytosolic DNA recognition and activation of STING has been elegantly revealed by the recent work of

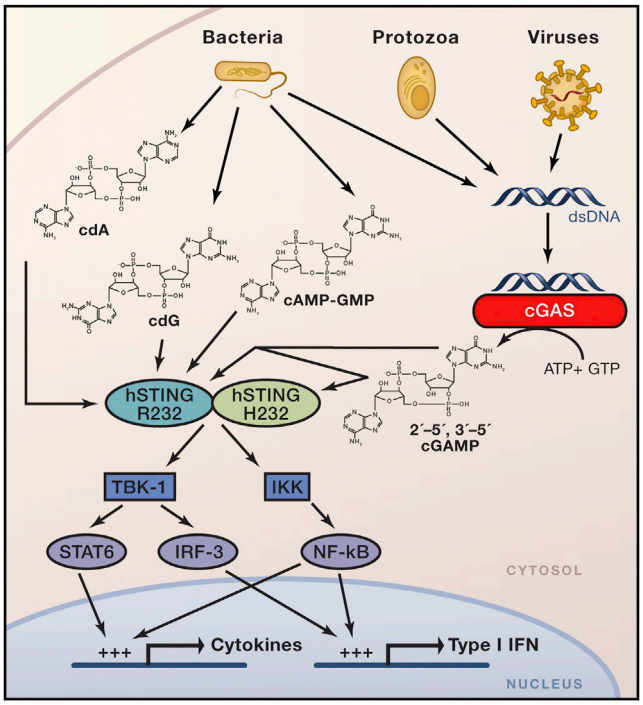

Figure 1. Overview of STING-Dependent Interferon Induction
Infection of eukaryotic cells with viruses, protozoa, and bacteria leads to accumulation of extracellular DNA that signals the presence of pathogens to the cellular immune system. Cytosolic dsDNA is recognized by cGAS in a sequence-independent manner. cGAS induces production of 2′-5′, 3′-5′cGAMP to stimulate STING. At least some STING alleles (such as R232) can additionally be activated by CDNs produced by bacteria that include cdA, cdG, and 3′-5′, 3′-5′cGAMP (cAMP-GMP), whereas other STING alleles (such as H232) are not responsive to bacterial CDNs. Binding of STING to an activating ligand induces conformational change in STING, resulting in formation of a "closed form" in which ligand is tightly bound to the binding pocket. Following relocation of STING to discrete foci in the cell cytosol, activated STING recruits TBK1 and IKK kinases, which in turn activate IRF-3, STAT6, and NF-κB. Upon translocation of activated transcriptional factors to the nucleus, they bind to corresponding promoters, thus resulting in induction of type I IFN and cytokines.

Chen and colleagues (Sun et al., 2013; Wu et al., 2013) (see below).

The generation of STING mutant mice (Goldenticket or Gt mice) has greatly facilitated the identification of other STING-dependent pathways for induction of type I IFNs, implicating this molecule in the sensing of PAMPs produced by intracellular bacterial pathogens (Jin et al., 2011a; Sauer et al., 2011). STING was eventually shown to directly bind to cdG, and STING mutants defective in such binding lose their ability to induce the production of type I IFNs in response to bacterial CDNs (Burdette et al., 2011). Consistent with this discovery, there are now several lines of evidence suggesting that bacterial CDNs can gain access to the host cell cytosol during infection. For example, the cytosolic intracellular pathogen *L. monocytogenes* utilizes an efflux pump to secrete cdA directly into the host cytosol, where it triggers a STING-dependent production of type I IFNs in mouse cells (Woodward et al., 2010). Mutants of *L. monocytogenes* that are confined to the vacuolar lumen do not induce production of IFN-β, suggesting that this pathogen must escape the endosome to deliver cdA to the cytosol. More recently, it was shown that concentration of cdA in cytosol of *Chlamydia*-infected mouse macrophages is greatly increased compared to uninfected cells and that this correlated with STING-dependent activation of the type I IFN response (Barker et al., 2013). Thus, evidence is accumulating that cytosolic bacterial pathogens can modulate innate immune responses through the CDNs they produce. However, given that some human STING alleles do not respond to bacterial CDNs (see below), more detailed studies are needed to fully understand the bacterial CDN, STING-dependent IFN response.

Synthesis of cGAMP by the Mammalian DNA Sensor cGAS Provides the Missing Link between DNA Sensing and STING Activation

The role of STING as the common downstream regulator of the type I IFN response induced by both cytoplasmic DNA and CDNs has been recently explained through the discovery by Chen and colleagues of the enzyme cyclic GMP-AMP synthase or cGAS (also known as C6orf150 and MB21D1) and its ability to synthesize a CDN ligand that activated STING (Sun et al., 2013; Wu et al., 2013). In brief, Chen and colleagues showed that, in the presence of dsDNA, cGAS selectively catalyzed the synthesis of cAMP-GMP (which they termed cGAMP) and that, after STING binds this endogenous CDN, it activates the type I IFN response (Sun et al., 2013; Wu et al., 2013). Like most major advances, the discovery of cGAS gave rise to a new series of questions. How is cGAS activated by recognition of only dsDNA in a sequence-independent fashion? Given that cGAS belongs to the same nucleotidyltransferase (NTase) family as DncV, is it making exactly the same hybrid CDN as the bacterial enzyme? If so, how could this conclusion be reconciled with the existence of a mutant STING allele (H231) that was not responsive to cdG and cdA but still able to sense dsDNA? Do all naturally occurring STING alleles recognize all CDNs with equal affinity? Recently, a series of seven papers have been published that answer many of these questions (Ablasser et al., 2013; Civril et al., 2013; Diner et al., 2013; Gao et al., 2013a, 2013b; Kranzusch et al., 2013; Zhang et al., 2013).

Crystal structures of the nucleotidyl transferase (NT) domain of porcine cGAS (residues 135–497) (Civril et al., 2013), mouse cGAS (147–507) (Gao et al., 2013a), and human cGAS (residues 157–522) (Kranzusch et al., 2013) have been solved with or without dsDNA ligand and nucleotide substrates. cGAS is a 60 kDa protein that consists of protease-sensitive, unstructured, and poorly conserved ~150 amino-acid-long N-terminal domain and a protease-resistant, conserved NTase C-terminal domain. cGAS dinucleotide synthase activity is dependent on the presence of dsDNA, Mg^{2+} or Mn^{2+}, ATP, and GTP. The crystal structures revealed that the catalytic NTase domain fold shows high similarity to an RNA sensor 2′-5′ oligo-adenylate synthetase 1 (OAS1) (Donovan et al., 2013). cGAS interacts with the sugar-phosphate backbone along the minor groove of DNA, thus determining its specificity toward dsDNA in a sequence-independent manner. In comparison to other NTase-containing enzymes, vertebrate cGAS contains a zinc-ribbon domain with an atypical $H(X_5)CC(X_6)C$ motif that is essential for a metal coordination and interaction with the major groove of DNA, suggesting that it functions as a molecular "ruler" that determines specificity of cGAS

toward dsDNA (Civril et al., 2013; Kranzusch et al., 2013). Binding of DNA to cGAS induces structural changes that result in rearrangement of catalytic residues in the NTase active site and enzyme activation. Given the similarity in the NTase fold of OAS1 and cGAS and the fact that both enzymes make secondary messenger molecules in response to detected nucleic acids, it seems likely that cGAS and OAS constitute a novel evolutionary conserved group of PRRs, the OAS-like second messenger receptors (OLRs), that evolved to detect the nucleic acids of cytosolic pathogens (Kranzusch et al., 2013).

Discovery of the Second "Missing Link" Corresponding to a Unique Bond in Mammalian cGAMP

Further characterization of cGAS showed that the enzyme produces a CDN that has a very rare in nature 2′-5′ phosphodiester linkage between GMP and AMP followed by a 3′-5′ return linkage from AMP to GMP (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013a; Kranzusch et al., 2013; Zhang et al., 2013). This CDN isomer (designated here 2′-5′, 3-5′cGAMP for simplicity) is different from all characterized bacterial CDNs, including cAMP-GMP synthesized by DncV, which is apparently a 3′-5′, 3′-5′ isomer (Ablasser et al., 2013; Davies et al., 2012; Diner et al., 2013). Thus, the phosphodiester linkages of the cGAS product are different in structure than the initially proposed 3′-5′, 3′-5′cGAMP isomer (Wu et al., 2013). The cGAS product has been confirmed to be the 2′-5′, 3′-5′cGAMP isomer by reverse-phase high-performance liquid chromatography (HPLC) analysis (Ablasser et al., 2013; Gao et al., 2013a; Zhang et al., 2013), high-resolution tandem mass spectrometry (MS/MS) spectra of cGAS product (Ablasser et al., 2013; Zhang et al., 2013), and nuclease digestions (Ablasser et al., 2013; Diner et al., 2013) using a chemically synthesized cGAMP isomers as a gold standard. NMR analysis (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013a; Zhang et al., 2013) and CD spectrum (Zhang et al., 2013) also indicated that the cGAS product had 2′-5′ as well as 3′-5′ phosphodiester bonds. Furthermore, crystallization of cGAS in the presence of ATP and GMP clearly showed that a linear oligonucleotide corresponding to pppG(2′-5′)pA was bound to its catalytic pocket, suggesting that ring closure occurs by formation of a phosphodiester bond between the 5′ phosphate of G and the 3′ OH of A (Gao et al., 2013a). Thus, combined evidence from several groups has confirmed that cGAS produces the 2′-5′, 3′-5′cGAMP isomer.

It remains a mystery why cGAS has evolved to make the 2′-5′, 3′-5′cGAMP. It is possible that the 2′-5′ phosphodiester linkage promotes a greater stability to cGAMP product, thus allowing stronger and more prolonged signal amplification (Gao et al., 2013a). Furthermore, production of 2′-5′, 3′-5′cGAMP instead of 3′-5′, 3′-5′cGAMP might be a defense mechanism of eukaryotic cells that allows them to avoid subversion of innate immune response by bacteria because bacterial cells might not be able to degrade 2′-5′ phosphodiester linkages. While cGAMP phosphodiesterases are currently unknown both in bacteria and eukaryotes, determining how 2′-5′, 3′-5′cGAMP turnover occurs in eukaryotic cells is a particularly exciting challenge in the field. It would be interesting if such enzymes (both in prokaryotes and eukaryotes) have substrate specificity that could differentiate 3′-5′, 3′-5′ from 2′-5′, 3′-5′ linkages and if they are strictly specific toward cGAMP molecules or also recognize other CDNs such as cdG and cdA. Such mammalian phosphodiesterases would be potentially interesting targets for altering CDN homeostasis in the context of immunity and inflammation. However, as discussed below, variation in the responsiveness of naturally occurring alleles of STING to bacterial CDNs, which have exclusively 3′-5′ phosphodiester bonds, may provide clues to the evolutionary selection that drove cGAS to make a distinctly different CDN.

The Product Specificity of the cGAS Enzyme and the Response Selectivity of STING Variants and Mutants

Several groups have now confirmed that 2′-5′, 3′-5′cGAMP is a strong inducer of the type I IFN response in a STING-dependent manner (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013b; Zhang et al., 2013). Isothermal titration calorimetry (ITC) experiments showed that 2′-5′, 3′-5′cGAMP binds to human STING (hSTING), and this reaction is endothermic compared to the 3′-5′, 3′-5′cdG binding reaction that is exothermic; these results suggest that STING undergoes structural rearrangements exclusively upon binding to 2′-5′, 3′-5′cGAMP (Zhang et al., 2013). However, the controversy over STING preference toward 2′-5′, 3′-5′cGAMP or bacterial 3′-5′, 3′-5′cGAMP still remains. For example, (Zhang et al., 2013) concluded that hSTING (carrying the more common R232 allele) has much higher affinity for 2′-5′, 3′-5′cGAMP than 3′-5′, 3′-5′CDNs based on titrating different CDNs into the hSTING-3′-5′, 3′-5′cdG complex. In their hands, the affinity of 2′-5′, 3′-5′cGAMP for STING was almost 300-fold higher than the affinity seen with 3′-5′, 3′-5′cdG and 3′-5′, 3′-5′cGAMP. In contrast, Patel and his group did ITC experiments and directly measured binding of mSTING R231 and A231 alleles and hSTING H232 and R232 alleles (position 231 in mSTING is identical to 232 in hSTING) to cGAMP isomers (Gao et al., 2013a). They were able to show that all alleles of STING bind to 2′-5′, 3′-5′ and 3′-5′, 3′-5′cGAMP with similar affinity and observed only a 2-fold difference between 2′-5′, 3′-5′cGAMP and 3′-5′, 3′-5′cGAMP in STING activation. Although 2′-5′, 3′-5′cGAMP was 10- to 20-fold more potent at inducing an interferon response in cells than 3′-5′, 3′-5′cdG, this variation could reflect different efficiencies of cellular uptake of these two different CDNs because digitonin was used to permeabilize cells. These binding experiments correlate well with the in vitro data of Zhang and colleagues showing that both 2′-5′, 3′-5′cGAMP and 3′-5′, 3′-5′cGAMP can induce a strong IFN signaling response in the mouse cell line L929 carrying the R231 allele (Zhang et al., 2013), similar to what is seen with the hSTING R232 allele (Diner et al., 2013). Curiously, neither Diner et al. (2013) nor Ablasser et al. (2013) observed activation of the hSTING H232 allele by the DncV product (presumably 3′-5′, 3′-5′cGAMP), although this hSTING allele does respond to the 2′-5′, 3′-5′cGAMP cGAS product. The different experimental approaches and STING alleles used by various investigators make it difficult to draw consensus conclusions regarding a correlation between binding affinity and signaling strength for different CDNs and their isomers. In contrast, new STING structural studies have advanced our understanding in this area quite dramatically.

Crystal structures of both mouse STING (mSTING) and hSTING R232 (residues 139–379) revealed that 2′-5′, 3′-5′ and

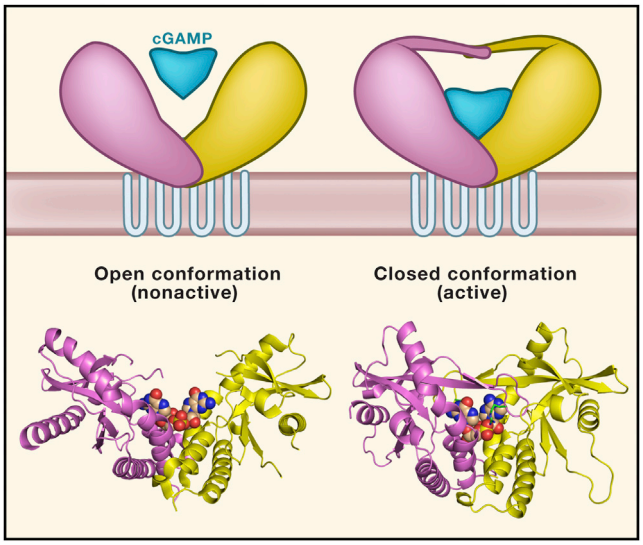

Figure 2. Model of STING Binding to CDNs
In its "open" form, STING does not fully encapsulate cdG and possibly other bacterial 3′-5′, 3′-5′CDNs. STING structure is more flexible; partially disoriented loops are covering the binding pocket. Binding of 2′-5′, 3′-5′cGAMP to STING happens at a deeper pocket compared to cdG and results in formation of the "closed" form. STING it its closed form is more compact, and the binding pocket is covered by a four-stranded β sheet cap. RCSB Protein Data Bank coordinates: cdG-hSTING H232 complex (4EF4) and 2′-5′, 3′-5′cGAMP-hSTING H232 complex (4LOH).

3′-5′, 3′-5′cGAMP induce a "closed" conformation, whereas 3′-5′, 3′-5′cdG is bound in a more "open" STING conformation (Figure 2) (Gao et al., 2013b; Zhang et al., 2013). In the "closed" conformation, the binding pocket for cGAMP is slightly deeper and allows coordination of 2′-5′, 3′-5′ and 3′-5′, 3′-5′cGAMP isomers via extensive hydrophobic and polar interactions with STING residues. Furthermore, crystal structures of mSTING R231 and hSTING H232 bound to 2′-5′, 3′-5′cGAMP were found to be identical, thus confirming that both of these STING alleles bind this CDN in a "closed" conformation. In contrast, cdG only induced the "open" conformation in hSTING H232, perhaps explaining why this CDN fails to significantly activate this human STING variant (Ablasser et al., 2013; Burdette et al., 2011; Diner et al., 2013; Gao et al., 2013b). Thus, both mSTING and R232 and H232 alleles of hSTING are responsive to 2′-5′, 3′-5′cGAMP, but only mSTING and the R232 allele of hSTING respond to 3′-5′, 3′-5′cdG and 3′-5′, 3′-5′cGAMP (Burdette et al., 2011; Zhang et al., 2013). Although the closed conformation was observed earlier for hSTING solved with cdG (Huang et al., 2012), this may be a rare conformation for the 3′-5′, 3′-5′ CDN complex that depends more on crystallization conditions. The hSTING H232 allele binds 2′-5′, 3′-5′cGAMP with slightly lower affinity than the hSTING R232 allele, but the signaling response selectivity for the 2′-5′ bond by hSTING H232 defines a clear difference between it and hSTING R232 (Diner et al., 2013; Gao et al., 2013b). Furthermore, because mSTING (R231) binds 3′-5′, 3′-5′CDNs in a nearly identical closed conformation (Gao et al., 2013b), the responsiveness of these two STING alleles to the bacterial CDNs connects the closed conformation (rather than binding affinity per se) with downstream signaling that induces the type I IFN response. Crystal structures of hSTING H232 allele with 3′-5′, 3′-5′ CDNs (which do not fully activate this protein) should provide a definitive answer to this hypothesis. Together, these data suggest a view of how various alleles of STING might function in innate immune recognition, depending on the source of the CDN signal (Figure 1). It is also worth noting that different alleles of STING might also be activated in some cells by binding to the DExD/H-box helicase DDX41, which has been implicated in binding of DNA and triggering a type I IFN response (Parvatiyar et al., 2012; Zhang et al., 2011), but the role of cGAS and 2′-5′, 3′-5′cGAMP in this alternative pathway for STING activation has not yet been carefully addressed. Furthermore, a recent extensive biochemical analysis has detected numerous proteins that may contribute to the innate immune response against cytosolic or foreign nuclear DNA (Lee et al., 2013), but these host proteins have not been evaluated for the role of cGAS, CDNs, or STING in their observed biochemical interactions. Thus, there is still much to learn about how cGAS and STING integrate their activity with multiple host responses to foreign or mislocalized DNA.

Impact of Allele Variations in STING on Human Fitness

Variations in the hSTING locus have been documented by Jin et al. (2011b). Approximately 18% of humans in two large cohorts, totaling over 1,000 individuals, were found to be heterozygous for the H232 allele, with the more prevalent allele being R232 (thus, it should be considered a wild-type allele). Given that the H232 allele is nonresponsive to 3′-5′, 3′-5′CDNs, it will be interesting to know whether this allele is dominant (that is, whether heterodimers with the R232 allele produce STING that is nonresponsive to cdG). Given that certain alleles of human STING (e.g., H232) have likely lost the specific ability to respond to bacterial CDNs while retaining their ability to respond to the human endogenous messenger 2′-5′, 3′-5′cGAMP produced by cGAS, it is tempting to speculate that there was a strong selective pressure to lose responsiveness to bacterial CDNs during human evolution (Diner et al., 2013). The need to preserve a robust innate immune response to various viruses, including the smallpox virus and retroviruses, provides a reasonable explanation for retaining STING responsiveness to the endogenous 2′-5′, 3′-5′cGAMP produced by cGAS (Gao et al., 2013c). However, understanding the selective pressure to "blind" some hSTING alleles to bacterial CDNs is more challenging (Monroe et al., 2009). The frequency and penetrance for immune dysfunction of such hSTING alleles in the global human population and their geographic distribution may provide clues to the nature of the selective process. One can envision at least two types of selective pressure that may have been driving this human STING genetic variation: (1) bacterial CDN-driven susceptibility or enhanced pathology associated with bacterial diseases of high prevalence in certain human populations and (2) bacterial CDN-driven alterations of nutritional or metabolic states that significantly affected human mortality.

Selective pressure under hypothesis 1 would include exposure to bacterial pathogens whose replication (and thus virulence) was enhanced by a type I IFN response. This could occur by the known ability of type I IFNs to polarize the adaptive

immune response as well as cause increased pathology by driving excessive levels of inflammation. Bacterial invasive diseases with high-inflammatory symptoms such as tuberculosis, bubonic plague, shigellosis, meningitis, enteric fever, and pneumonias might be considered as selective drivers under hypothesis 1 if their causative agents expose host cells to bacterial CDNs during infections. *Mycobacterium tuberculosis* specifically induces a type I IFN response by releasing bacterial DNA into the host cell cytosol after permeabilization of a phagosomal membrane via the activity of its virulence-associated ESX secretion system (Manzanillo et al., 2012). The bacterial CDNs produced by *M. tuberculosis* apparently do not contribute to this response (Manzanillo et al., 2012), and thus this pathogen may have evolved this new way to activate STING by stimulating production of the endogenous cGAS synthesized 2′-5′, 3′-5′cGAMP. Mice defective in type I IFN signaling are considerably more resistant to *M. tuberculosis* (Manzanillo et al., 2012), and induction of type I IFNs exacerbates pulmonary tuberculosis in an IFN-α/β receptor-dependent fashion (Antonelli et al., 2010). Thus, one could easily imagine that the human host would be under selective pressure to lower its steady-state level of STING activation in order to counteract this pathogen's immune modulation strategy. As noted earlier, other pathogens such as *L. monocytogenes* actively secrete CDNs into the cytosol of cultured cells and directly activate the type I IFN response by a STING-dependent pathway (Woodward et al., 2010). However, the level of STING activation in different host tissues is largely unknown both before and after bacterial infections. Understanding whether susceptibility to replication or host injury by bacterial pathogens can be altered in mice by replacement of mSTING with the hSTING H232 allele could reveal how some bacteria may have utilized the host response to bacterial CDNs to enhance their own replication or fitness within the human host.

Selective pressure under hypothesis 2 might include any STING-driven process that interferes with nutrition and thus increases infant or child mortality through secondary effects that could include susceptibility to severe diarrheal disease. In this regard, recent data suggest that mutations in innate immune pathways are enriched in patients that appear more susceptible to cholera (Karlsson et al., 2013). Inflammatory states in the gut are known to decrease nutrient uptake; however, little is known about the role of STING in contributing to mucosal inflammatory states. The large quantity of bacterial mass typically contained within the human intestine might be a source of bacterial CDNs that somehow find their way into host cells and produce significant STING activation within the local intestinal epithelium. Because the gut microbiome is also thought to play a role in metabolic syndromes such as obesity and diabetes (Gross, 2013), it is possible that alterations in the response CDNs and other PAMPs derived from commensal bacterial cells could be the selected outcome of human evolution that was seeking nutritional fitness in the face of famine. The fact that mutations in receptors associated with the innate immune system have been linked to colitis and other inflammatory conditions (Cario, 2010) underlines how little we know about the selective forces that drove mutational changes in humans that were challenged with pathogens as well as the need for immune homeostasis when stimulated by a complex intestinal milieu.

In conclusion, the newly recognized cGAS-STING-type I IFN pathway for innate immune responses to cytosolic DNA will likely lead to exciting new investigations on the role of bacterial CDNs in altering host susceptibility to pathogens as well as inflammatory conditions linked to the human microbiota or disease states that lead to leakage of nuclear DNA into the host cell cytosol. Targeting both cGAS and STING with either activating or inhibitory drugs may be a promising new strategy for treatment of chronic infection, inflammatory states, and other diseases in which immunomodulation of the type I IFN pathway might show therapeutic benefit.

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