Cyclic Dimeric Guanosine Monophosphate: Activation and Inhibition of Innate Immune Response

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\section*{Abstract}
Cyclic dimeric guanosine monophosphate (c-di-GMP) is a universally conserved second messenger that contributes to the pathogenicity of numerous bacterial species. In recent years, growing evidence has shown that bacterial extracellular c-di-GMP can interact with the innate immune system and regulate host immune responses. This review summarizes our current understanding on the dual roles of bacterial c-di-GMP in pathogen-host interaction: activation of the antibacterial innate immune response through the cytosolic surveillance pathway and inhibition of innate immune defense for iron restriction.

\section*{Introduction}
Innate immunity is an essential line of defense against pathogenic infection. It uses various pattern recognition receptors (PRRs) to sense the pathogen-associated molecular patterns and activate immune responses \cite{1}. For example, cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase, an intracellular sensor of the cytosolic surveillance pathway, can detect cytosolic pathogen-derived DNA and activate antibacterial immune responses \cite{2, 3}. By contrast, pathogens usually utilize their specific structures to avoid the sensing of immune receptors and/or secreted molecules and block the immune signal transductions for survival \cite{4–6}.

Bis-(3\textsuperscript{'},5\textsuperscript{'})-cyclic dimeric guanosine monophosphate (3\textsuperscript{'},5\textsuperscript{'}-cyclic diguanylic acid, cyclic di-GMP, c-di-GMP) is a small molecule that was first discovered as an allosteric activator of the cellulose synthase of \textit{Acetobacter xylinum} in 1987 \cite{7}. Intracellular c-di-GMP can be synthesized and broken down by diguanylate cyclases and phosphodiesterases. Interestingly, these c-di-GMP metabolic enzymes usually contain various receptor domains, which suggest the potential roles of c-di-GMP in the regulation of bacterial response to environments. c-di-GMP plays its roles by binding to and regulating functions of various downstream effectors, including enzymes, transcriptional factors, and riboswitches. It is now recognized as a multifunctional intracellular small-molecule second messenger that regulates a wide range of cell processes, such as biofilm formation and motility. Of note, c-di-GMP...
contributes to the pathogenicity of numerous pathogens [8–13].

c-di-GMP is ubiquitous in bacteria but not in mammals. However, increasing evidence suggests that c-di-GMP can be secreted by bacteria and interacts with the host immune system. In this review, we summarize our understanding on the dual interactions of c-di-GMP (activation and inhibition) with the human innate immune system at the molecular level.

Activation of Innate Immune Responses by c-di-GMP

In an earlier study in 2007, Karaolis et al. [14] found that treatment with c-di-GMP (200 nmol) significantly attenuated the infection of Staphylococcus aureus in a mouse model and reduced the bacterial survival (> 10,000-fold; p < 0.001). However, c-di-GMP unexpectedly exerted no apparent inhibitory or bactericidal effect on S. aureus in vitro. This finding implies that c-di-GMP may have a biological effect on the in vivo environment and the host immune responses. Hypothesis testing demonstrated that c-di-GMP is not only an important microbial signaling molecule but also a modulator of the host immune responses [14]. It is found that c-di-GMP can activate the innate immune response of multiple cell lines, including dendritic cells (line D2SC), macrophages (line RAW 264.7, BMDCs), and monocytes (line THP-1). Intraperitoneal injection of mice with c-di-GMP activated monocyte and granulocyte recruitment. Subcutaneous administration of c-di-GMP prior to intratracheal challenge with Klebsiella pneumoniae stimulates protective immunity against infection. Therefore, c-di-GMP can activate systemic immune response.

Meanwhile, the innate immune system utilizes cell membrane receptors and cytosolic receptors as PRRs to sense the pathogen-associated molecular patterns and activate location-specific immune responses. Interestingly, McWhirter et al. [15] found that c-di-GMP can activate the innate immune response of multiple cell lines, including dendritic cells (line D2SC), macrophages (line RAW 264.7, BMDCs), and monocytes (line THP-1). Intraperitoneal injection of mice with c-di-GMP activated monocyte and granulocyte recruitment. Subcutaneous administration of c-di-GMP prior to intratracheal challenge with Klebsiella pneumoniae stimulates protective immunity against infection. Therefore, c-di-GMP can activate systemic immune response.

STING Protein

STING (also known as MYPS, MITA, ERIS, or TMEM173), a protein localized on the endoplasmic reticulum membrane, is a membrane adaptor protein linking the upstream cytosolic detection of microbial nucleic acids with downstream cytokine production. The stimulation of cells by double-stranded DNA induces the recruitment of the protein kinase TBK1 and the transcription factor IRF3 to STING. The proximity of TBK1 and IRF3 facilitates the phosphorylation of IRF3, leading to its dimerization and translocation to the nucleus to activate IFN-β gene transcription [16] (Fig. 1).

c-di-GMP stimulates the production of type I IFN in a STING-dependent manner [17]. By using an in vitro ultraviolet radiation cross-linking assay, Burdette et al. [18] showed that the adaptor protein STING itself is a direct binding protein of c-di-GMP. Structural studies suggested that c-di-GMP binds to STING at its dimer surface [19–21].

DDX41 Protein

DDX41 has been characterized as another direct sensor of c-di-GMP. Cells treated with DDX41-specific shRNA achieved a much lower induction of IFN-β in response to c-di-GMP. c-di-GMP directly binds to the DEAD box domain of DDX41. Strikingly, the binding affinity of c-di-GMP for DDX41 is much greater than that of c-di-GMP for STING. More interestingly, the interaction of c-di-GMP with STING was substantially enhanced in the presence of DDX41. It is proposed that, upon detecting and binding with c-di-GMP, DDX41 promotes its physical interaction with STING, leading to an increase in the binding affinity of STING for c-di-GMP, which ultimately drives downstream signaling events [22, 23] (Fig. 1). Nevertheless, the evidence for DDX41 as the receptor of c-di-GMP is not as strong as STING; therefore, the exact involvement of DDX41 in this process remains to be further clarified.
Inhibition of Innate Immune Defense by c-di-GMP

Recently, by using an inverse docking method, we identified the human LCN2 protein as a candidate target of c-di-GMP. Subsequent analysis indicated that c-di-GMP plays a novel role in the iron seesaw battle between bacteria and host [24].

First Round: Host Iron Restriction

Nutritional immunity, an innate immune defensive strategy, inhibits pathogenic infection by limiting the access of pathogens to essential nutrition in the host environment. Iron is an essential trace element for almost all life, and the cell uses rigorous regulation for iron homeostasis and decreases the concentration of free iron in a host environment to a very low level [25, 26].

Second Round: Bacterial Small-Molecule Siderophore

Siderophores (Greek: “iron carrier”) are small, high-affinity iron-chelating compounds secreted by microorganisms that transport iron across cell membranes. Siderophores are among the strongest soluble Fe^{3+} binding agents known. They are secreted into the extracellular space where they chelate free iron. Then, they are transported into the intracellular space, and iron is released to the bacterial cytoplasm (Fig. 2, indicated by blue arrows) [27–28].

Third Round: Human Siderocalin/LCN2 Protein

Bacteria use siderophores to enrich iron from the host environment for survival. However, the innate immune system also develops a specific defensive strategy to target bacterial siderophores. Lipocalin-2 (LCN2), also known as a neutrophil gelatinase-associated lipocalin, is an innate immune protein with antimicrobial activity. It can
specifically bind to iron-containing siderophores and disrupt bacterial siderophore-mediated iron transport, which leads to the inhibition of bacterial growth (Fig. 2, indicated by red arrow) [29–32]. By contrast, given the high affinity between iron and siderophore, isolating iron-free siderophores for analysis may be difficult. Therefore, the binding of LCN2 with iron-free siderophores has not been addressed clearly to date.

**Fourth Round: Bacterial Small-Molecule c-di-GMP**

Recently, our group found that LCN2 is a new target of c-di-GMP. Both ultraviolet cross-linking and isothermal titration calorimetry analysis confirmed the binding of c-di-GMP with LCN2 protein (Kₐ of 1.63 ± 0.05 μM). Modeling of the c-di-GMP and LCN2 complex indicated that c-di-GMP targets the siderophore binding site of LCN2. Multiple amino acid residues involved in siderophore binding also participate in c-di-GMP binding. This result indicates the potential competitive inhibition of c-di-GMP to LCN2. *Mycobacterium tuberculosis* is an intracellular pathogen that acquires iron from the host environment. *M. tuberculosis* also uses siderophores to enrich iron, and its siderophores are sensitive to LCN2 [29, 30]. Therefore, the interaction of c-di-GMP with LCN2 can interfere with the LCN2-mediated antibacterial activity.

Our in vitro assay shows that c-di-GMP can rescue the growth of *Escherichia coli* and *M. tuberculosis* H37Ra from LCN2-mediated inhibition [24]. Compared with the secretion of LCN2-resistant siderophores, this result represents a novel molecular mechanism of bacterial response to LCN2-mediated iron restriction by the cooperation of c-di-GMP and bacterial siderophores (Fig. 2, indicated by green arrows). This result suggests that extracellular c-di-GMP can be a weapon that facilitates bacterial survival. Interestingly, c-di-AMP cannot bind to LCN2, which suggests that the interaction between c-di-GMP and LCN2 is highly specific. Many pathogens can produce LCN2-sensitive siderophores and also produce c-di-GMP. Therefore, c-di-GMP may mediate LCN2-mediated antibacterial activity in other pathogens.

**Weapon or Encumbrance**

To date, three direct immune proteins targeted by c-di-GMP, namely, STING [18], DDX41 [22], and LCN2 [24], have been reported. However, the interaction between c-di-GMP with the host immune proteins leads to two intuitively opposite consequences for bacteria. On the one hand, STING and DDX41 sense c-di-GMP and activate the immune response to eliminate bacteria. This indicates that c-di-GMP is as an encumbrance for bacterial escape from the immune system. On the other hand, c-di-GMP targets LCN2 and inhibits its antimicrobial activity. This indicates that c-di-GMP can be a weapon for bacterial survival in the host environment. Therefore, the roles of extracellular c-di-GMP in pathogens, as a weapon or encumbrance, cannot be simply defined.
We prefer to consider extracellular c-di-GMP mainly as a weapon for bacterial survival. In other words, bacteria generally benefit from extracellular c-di-GMP. This speculation is based on two considerations. First, even if c-di-GMP cannot be sensed by the immune system, other components, such as c-di-AMP and DNA, of bacteria can be sensed by the immune system and activate immune responses to eliminate bacteria [33–35]. Therefore, the negative effects of secreted c-di-GMP for bacteria are masked and very limited to a certain extent. Second, if the activation of the immune response is the main effect of extracellular c-di-GMP, the secretion of c-di-GMP should be lost during long-term co-evolution of bacteria and host. Given this consideration, the c-di-GMP-induced exposure of the pathogen to the immune system may allow bacteria to obtain the essential nutrients such as iron.

**Secretion of c-di-GMP**

One of the issues that needs to be elucidated is the secretion of c-di-GMP. To play its extracellular roles, c-di-GMP needs to be released to the bacterial extracellular space. In *Listeria monocytogenes*, a similar cyclic dinucleotide, c-di-AMP, can be secreted by multidrug efflux pumps MdrM, which is a nonspecific transporter [36, 37]. However, the secretion pathways of c-di-GMP are largely known.

Our previous bioinformatic prediction and bacterial two-hybrid assays characterized a physical interaction between *M. tuberculosis* sole diguanylate cyclase Rv1354c with a group of ABC transporters [38]. Recently, by using liquid chromatography and mass spectrometry, we determined the concentrations of c-di-GMP in bacterial culture supernatants to be 1.042 ± 0.12 mM for *E. coli* cells and 0.568 ± 0.06 mM for *M. tuberculosis* [24]. In addition, c-di-GMP was found to regulate type III and type VI secretion systems in *Pseudomonas aeruginosa* [39, 40]. Therefore, growing evidence suggests that bacteria can secrete c-di-GMP. However, whether these transporters or secretion systems contribute to c-di-GMP secretion remains unclear.

**Comparison with c-di-AMP**

c-di-AMP is another bacterial cyclic dinucleotide second messenger and is structurally similar to c-di-GMP. Like c-di-GMP, c-di-AMP also regulates intracellular cell processes by binding to various downstream effectors [41, 42]. In addition, both of them can be secreted by bacteria and interact with the host immune system. They can be sensed by the same PRRs, including DDX41 and STING, and activate the type I IFN response [43]. However, some differences exist between these two molecules. Recently, bacteria have been found to degrade extracellular cyclic dinucleotides by extracellular phosphodiesterase for immune evasion. Moreover, c-di-AMP seems to be the main target of extracellular phosphodiesterase [44–46]. Besides interacting with DDX41 and STING to facilitate type I IFNs, c-di-AMP can be sensed by a more specific ER adaptor protein and activate NF-κB antibacterial response pathways [47, 48]. In addition, c-di-GMP, but not c-di-AMP, binds to and inhibits the antibacterial activity of the LCN2 protein. Taken together, extracellular c-di-AMP generally plays negative roles for bacterial survival, and bacteria degrade extracellular c-di-AMP for immune evasion. By contrast, c-di-GMP plays positive and negative roles for bacterial survival in the host.

**Conclusions and Future Prospects**

It has been about 30 years since the first discovery of c-di-GMP in 1987. As its intracellular roles in bacteria have been widely studied, the extracellular roles of c-di-GMP have also been uncovered during the last 10 years. Interestingly, c-di-GMP exhibits dual roles in the interface of pathogen-host interaction. On the one hand, c-di-GMP binds to DDX41 and STING and activates the host antibacterial immune response. On the other hand, it binds to LCN2 and inhibits the antibacterial activity to promote bacterial survival. Strikingly, many bacterial pathogens, including *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Yersinia*, *Listeria*, and *Mycobacteria*, can produce c-di-GMP [11, 13, 49]. Given that it is ubiquitous in bacteria but not in mammals, c-di-GMP signaling has become a very attractive target for antibacterial drug development.

Identifying direct binding proteins of c-di-GMP in host cells is essential for deeply understanding the roles of c-di-GMP in the interface of pathogen-host interaction. Therefore, it is important to further systematically investigate potential binding proteins of c-di-GMP in the host using computational or proteomic methods in the future. In bacteria, besides proteins, c-di-GMP can also regulate the cell process by targeting RNA riboswitches. Therefore, whether a similar regulation exists in the host environment remains to be elucidated. Moreover, the mechanisms by which c-di-GMP binds to DDX41 and LCN2 and regulates their functions require further investigation by cocrystallization studies.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

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