Drew University

College of Liberal Arts

CYTOKINE RESPONSES GENERATED BY cGAMP AND OTHER CDNS DEPEND ON DELIVERY METHOD

A Thesis in Biochemistry and Molecular Biology

By

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Abstract:

Cyclic GMP-AMP synthase (cGAS) is an important protein in pattern recognition pathways involving viral DNA. Activated cGAS produces the small molecule 2'3'-cyclic GMP-AMP (cGAMP) as a second messenger. cGAMP will normally bind to its downstream receptor STING and induce an interferon (IFN) response. In order to understand cGAMP signaling, THP-1 cells were stimulated with cGAMP with or without viral DNA. Two methods were used to deliver cGAMP to the cells: lipid transfection or addition straight to the media. Analyses of the cells' responses were completed via qPCR to measure either *cGAS* or innate immune products *IFNb*, *ISG56*, and *IL-1b*. It was found that cGAMP delivered to the media repressed expression of *cGAS*. Untransfected cGAMP also led to repression of *ISG56*, while lipid-delivered cGAMP led to increased expression of *ISG56*. It was hypothesized that this activity of cGAMP was dependent on location of cyclic dinucleotide (CDN) exposure, inside or outside the cell, which may due to cGAMP's structural similarities to bacterial quorum sensing molecules. Location-dependent activity of cGAMP was compared to location-dependent activity of other CDNs: c-di-GMP, c-di-AMP, and c-di-UMP. Preliminary results indicate a trend that CDNs delivered to the media had repressive effects on *ISG56*. However, this pattern was reversed when *IL6*, a bacterial immune product, was measured. The data suggest that there is an extracellular receptor that senses CDNs in addition to the currently known intracellular receptor.

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Common Acronyms and Abbreviations:

PRR – Pattern Recognition Receptor

PAMP – Pathogen-Associated Molecular Pattern

DAMP – Damage-Associated Molecular Pattern

IFN – Interferon

ISG – Interferon-Stimulated Gene

IFNAR – Interferon α/β Receptor

IRF – Interferon Regulatory Factor

IL – Interleukin

NF-κB – Nuclear Factor κ-Light Chain Enhancer of Activated B Cells

cGAMP – Cyclic Guanosine Monophosphate-Adenosine Monophosphate

cGAS – Cyclic Guanosine Monophosphate-Adenosine Monophosphate Synthase

STING – Stimulator of Interferon Genes

RECON – Reductase Controlling NF-κB

c-di-AMP – Cyclic Dimeric Adenosine Monophosphate

c-di-GMP – Cyclic Dimeric Guanosine Monophosphate

c-di-UMP – Cyclic Dimeric Uridine Monophosphate

CDN – Cyclic Dinucleotide

PMA – Phorbol Myristate Acetate

LPS - Lipopolysaccharide

Lipo – Lipofectamine 2000

RPL37a – Ribosomal Protein L37a

Introduction:

Background:

The immune system is a system of organs, tissues, cells, and proteins that are broadly responsible for preventing infection of the host and for clearing diseased or damaged cells. This system of defenses can be broken into two categories: adaptive immunity and innate immunity (Parham, 2015). Adaptive immunity consists of slow-acting, but very specific, immune reactions that are designed to target and clear a specific infection or disease state and retain some memory of the pathogen. For example, B cells will produce antibodies that target a specific antigen on a certain pathogen, leading to control of only that pathogen, and also producing memory B cells to induce a faster response upon re-infection. Conversely, innate immunity is a series of fast-acting, non-specific immune reactions meant to prevent infection altogether or to control early replication of pathogens (Parham, 2015). One important class of proteins in innate immunity are pattern recognition receptors (PRRs).

PRRs bind to a particular non-self pathogen-associated molecular pattern (PAMP) during infection in order to induce a signaling cascade resulting in the transcription of a messenger molecule called a cytokine. PAMPs tend to be structures present on foreign microbes that are clearly distinguishable from host molecules (Parham, 2015), allowing for PRRs to respond only in the presence of the microbe. A good PAMP will be a structure that does not readily mutate and is used by a broad variety of microorganisms. This is because PRRs are encoded in the germline and so cannot alter their structure easily to accommodate changing PAMPs (Parham, 2015), and because a conserved PAMP will allow the host to respond to many microbes no matter how the microbes may

mutate. In addition to PAMPs, some PRRs respond to danger-associated molecular patterns (DAMPs), which are self molecules that are only present during periods of stress (Martinon et al., 2002). These DAMPs could be normal host structures present in an unusual location, such as DNA in the cytosol, or structures only made in response to stress, such as heat shock proteins. No matter what they sense, PRRs work to start the innate immune response quickly following infection.

This early innate immune response can be characterized by inflammation and the production of pro-inflammatory cytokines (Parham, 2015). This response is critical for slowing the spread of infection and recruiting other immune cell types to the site of the damage. One broad class of inflammatory cytokine is the interferons (IFNs). IFNs are characterized by their activation of the antiviral state, which is a broad immune response activated in order to prevent spread of a viral infection (Levy et al., 2001). Type I IFNs, consisting of IFNα and IFNβ, are under the transcriptional control of IFN regulatory factor 3 (IRF3) and are vital in inducing the antiviral state in cells (Mogensen, 2009). The IFN made by an infected cell in response to infection will then be secreted. The IFN will bind to the IFN α/β receptor (IFNAR) in either an autocrine or paracrine fashion (Parham, 2015). This binding event will result in the transcription and translation of several IFN-stimulated genes (ISGs). Some PRRs can activate IRF7, which directly controls the transcription of some ISGs without the need for IFN production (Barber, 2011). These ISGs have many functions, but all work to slow the replication of a virus in infected cells or to prevent infection of healthy cells (Mogensen, 2009). For example, some ISGs may inhibit translation, and because viruses rely on host translational machinery to replicate, this will slow viral spread. This physiological response may not

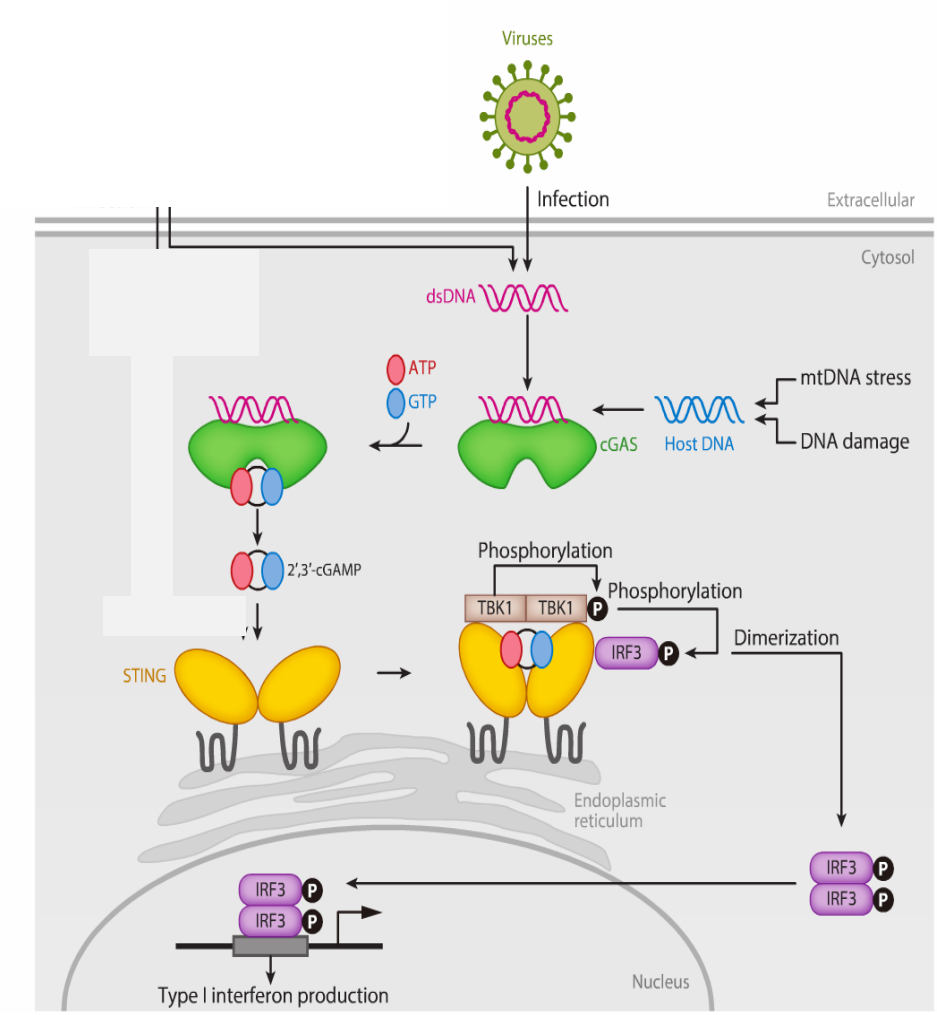
always be helpful, as prolonged translational shutdown could lead to pathology or susceptibility to other infections, so other responses counteract the IFN response.

Another class of pro-inflammatory cytokines are those under the transcriptional control of NF-κB, such as IL-6 and IL-1. These cytokines are controlled separately from IFNs and function mainly to increase clearance of extracellular pathogens such as bacteria (Mogensen, 2009). This increase in bacterial clearance can result from recruitment of phagocytic immune cells like macrophages, or by increasing the speed at which phagocytosis occurs. Both IFNs and IL-6 and IL-1 are necessary to combat infections, but each cytokine class functions in certain types of infection and are regulated by different PRRs.

Due to its importance during viral infection, one PRR that is of particular interest is cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS). cGAS is a cytosolic PRR that binds to DNA in order to induce an immune response (Wu et al., 2013). Cytoplasmic DNA sensors like cGAS exist because DNA in the cytoplasm is not normal for a cell, so cytoplasmic DNA can act as a PAMP or DAMP. While cytoplasmic DNA can come from many places, one common source is the genome of an actively replicating cytoplasmic virus, and it is in this way that cGAS becomes an important PRR for detecting viral infection (Sun et al., 2012). cGAS will normally bind to the B-form of dsDNA using a zinc thumb motif, and this binding occurs with the phosphodiester backbone of the DNA, so the binding does not require sequence specificity (Civril et al., 2013). However, cGAS can bind to ssDNA in a sequence-specific manner if there are repetitive tracts of guanine that form a Y-shaped double-stranded secondary structure (Herzner et al., 2015). The binding partner polyglutamine

binding protein 1 (PQBP1) has also been shown to be necessary for the production of IFN via cGAS signaling and it is hypothesized to help cGAS bind the DNA (Yoh et al., 2015). This could be due to the low affinity of cGAS for DNA.

When cGAS and its partners bind DNA, this induces production of the second messenger cGAMP (Wu et al., 2013), which is a type of cyclic dinucleotide (CDN). cGAMP made by cGAS is composed of an AMP and a GMP cyclized with a 2'-5' linkage and a 3'-5' linkage (Schaap, 2013). Once cGAMP is produced, it will bind to stimulator of IFN genes (STING) (Sun et al., 2013), an endoplasmic reticulum (ER) resident protein with four transmembrane domains (Chen et al., 2016). Many studies have delivered cGAMP to cells in order activate STING and induce IFN. STING is a vital pathway member for some PRRs that induce IFN, but STING is not required for production of other classes of cytokines (Ishikawa et al., 2008). When cGAMP binds to STING, STING dimerizes and traffics to the ER-Golgi intermediate complex (ERGIC), and this also causes STING to expose its C-terminal tail (CTT) (Chen et al., 2016). TANK-binding kinase 1 (TBK1) will phosphorylate the CTT, and this will recruit IFN regulatory factor 3 (IRF3). Once IRF3 and TBK1 co-localize, TBK1 will phosphorylate IRF3 and IRF3 will form a homodimer (Chen et al., 2016). Dimerized IRF3 can then enter the nucleus and act as a transcription factor for Type I IFNs. A simplified cGAS signaling cascade can be seen in Figure 1.



<u>Figure 1:</u> Simplified cGAS-STING Signaling Cascade: This pathway demonstrates how cGAS can sense DNA and produce cGAMP as a result of that binding. This cGAMP is used to activate dimerized STING. The STING acts to recruit TBK1 and IRF3. TBK1 will phosphorylate another TBK1, and this active TBK-1 can phosphorylate IRF3. p-IRF3 can then enter the nucleus and act as a transcription factor for IFN, thus starting an antiviral immune response. Image adapted from Kato et al., 2017

However, STING can also bind to molecules other than cGAMP; cGAMP is a type of cyclic dinucleotide (CDN) made by human cells, but other CDNs are made by bacteria and these bacterial CDNs are also able to bind STING. Cyclic dimeric guanine monophosphate (c-di-GMP) is a CDN used by many different bacteria as a secreted

quorum sensing molecule to determine the size of the surrounding bacterial population (Pesavento et al., 2009). In this sense, c-di-GMP can control motility, biofilm formation, virulence, and cell cycle progression of many bacteria (Pesavento et al., 2009). Cyclic dimeric adenine monophosphate (c-di-AMP) is another CDN, used primarily by Gram positive bacteria (Moretti et al., 2017). This secreted CDN is used by the intracellular pathogen *Listeria monocytogenes* and can influence cell growth, virulence, and cell wall maintenance (Schaap et al., 2013). Both of these bacterial CDNs have one crucial difference from eukaryotic cGAMP: the bacterial CDNs contain only 3'-5' linkages while eukaryotic cGAMP contains one 3'-5' linkage and one 2'-5' linkage (Schaap et al., 2013; Sun et al., 2013). This division between eukaryotic and prokaryotic CDNs is further demonstrated by the fact that some bacteria also use cGAMP for signaling, but this bacterial cGAMP contains only 3'-5' linkages, differentiating it from eukaryotic cGAMP (Davies et al., 2012). Structures of all of these CDNs, including the synthetic cyclic dimeric uridine monophosphate (c-di-UMP), are shown in Figure 2. Due to structural similarities between the bacterial CDNs and eukaryotic cGAMP, there may be overlap in the immune responses to any individual CDN.

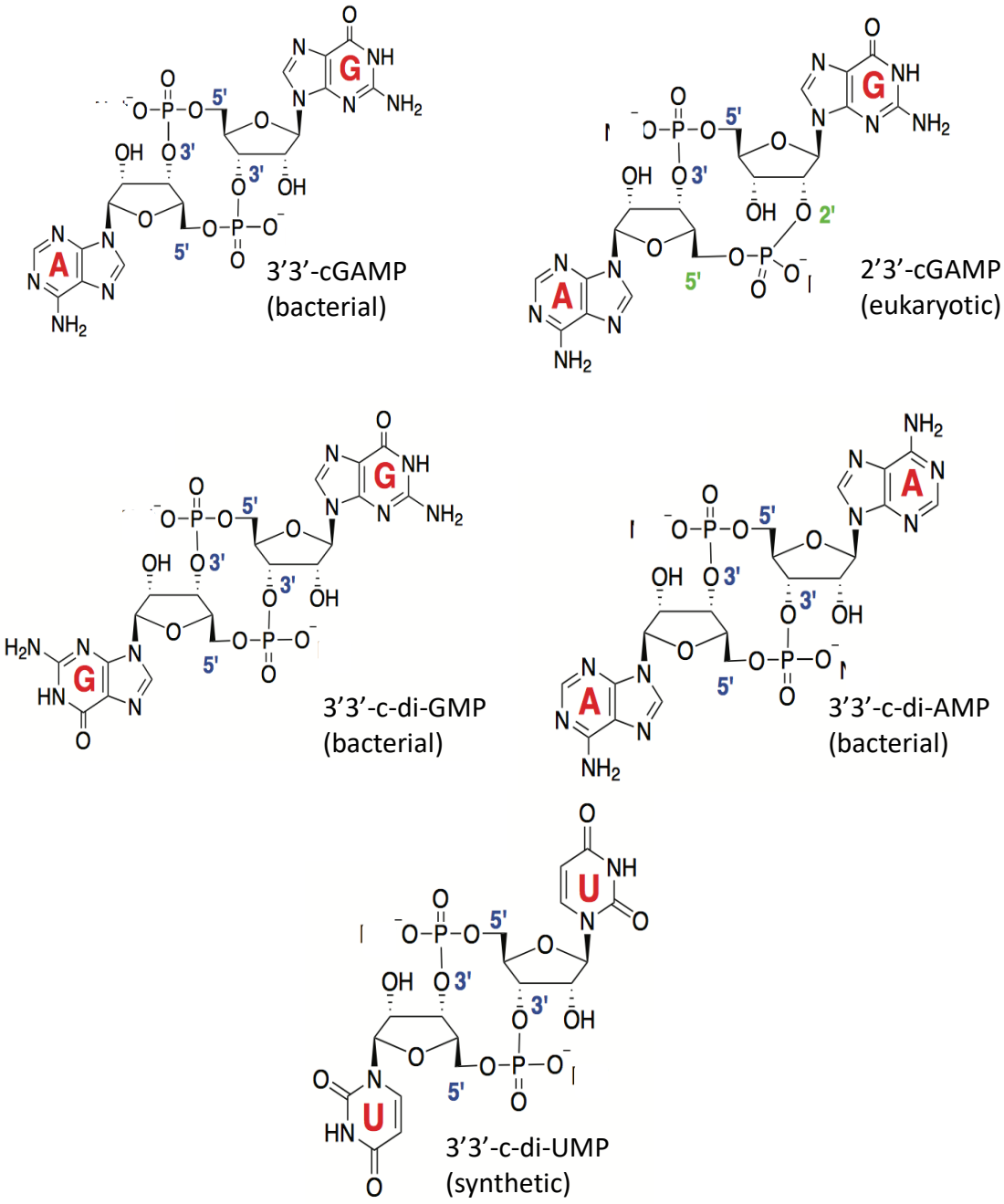


Figure 2: Structures of various CDNs: The structures of CDNs vary either by the phosphodiester backbone linkages or by the nucleotide residues, yet all contain some level of homology to one another. All images taken from Material Data Safety Sheets from Invivogen.

While many different CDNs can bind and activate STING, the patterns of binding differ depending on the structure of the CDN. STING has been shown to bind

radiolabeled c-di-GMP, and added c-di-AMP can compete for this binding (Burdette et al., 2011). CDN binding occurs at the interface of the STING dimer, and this binding involves direct and solvent-mediated hydrogen bonds with STING as well as hydrophobic ring-stacking interactions (Shu et al., 2012). Both c-di-AMP and cGAMP bind in this same fashion, but synthetic c-di-UMP cannot as its pyrimidine rings cannot participate in the ring-stacking interactions (Yin et al., 2012). This demonstrates that the structural similarities between the natural CDNs allows for overlap in the immune responses that they generate; STING is not specific for any one CDN.

However, there are still differences in the binding patterns of the CDNs and STING. Arginine residue 231 of human STING is vital for binding any of the bacterial CDNs (Ablasser et al., 2013). R231 is necessary for a solvent mediated hydrogen bond to form between STING and the 3'-5' backbone of the bacterial CDNs. Since eukaryotic cGAMP contains a 2'-5' backbone linkage as well, R231 is dispensable for cGAMP binding and is hypothesized to be important in distinguishing host-produced cGAMP from a bacterial-produced CDN (Ablasser et al., 2013). Eukaryotic cGAMP has been shown to be the CDN with the highest affinity for STING, and this is partially due to the fact that the bound form of cGAMP is the most entropically favored as it is the most similar to the solvent form (Shi et al., 2015). Also, it seems that the mixed 2'-5'/ 3'-5' backbone of cGAMP is vital for STING binding, as cGAMP isomers with different sets of linkages have much lower affinities (Zhang et al., 2013). These findings indicate that while STING can bind several CDNs, it preferentially binds cGAMP, which presumably produces a larger IFN response. This is interesting because the presence of cGAMP is indicative of a viral infection while other CDNs indicate bacterial infection. Robust IFN

production following CDN-STING binding also requires gamma-interferon-inducible protein 16 (IFI-16), which has been shown to modulate the structure of STING to increase signaling and CDN binding affinity (Almine et al., 2017). IFI-16 is a PRR that responds to viral infections (Unterholzner et al., 2010), and its importance for STING-cGAMP signaling further highlights the importance of cGAMP as an indicator of viral infection.

While the STING-cGAMP response tends to produce IFN in response to a virus, there are other modes of STING signaling that result in different outcomes that are more suited to combatting other types of infection. Different signals to STING can cause the protein to traffic to lysosomes and lead to efflux of potassium ions from the cell (Gaidt et al., 2017). This potassium efflux can activate NOD-like receptor protein 3 (NLRP3), which is one of a few proteins capable of forming an inflammasome, a multi-protein complex that is responsible for activating caspase-1 in cells (Parham, 2015). Active caspase-1 is able to cleave the pro-form of the cytokine IL-1β into its active form. IL-1β is a proinflammatory cytokine that tends to be associated with an immune response to bacteria and can lead to recruitment and development of white blood cells at the site of infection (Parham, 2015). However, one additional effect of an active inflammasome is to induce a form of programmed cell death called pyroptosis. The main purpose of pyroptosis is to burst an infected cell to release many different cytokines and other molecules all at once, and this helps to spread the immune response to neighboring healthy cells (Parham, 2015). Inflammasomes normally require two signals to start signaling: a priming signal that induces transcription of inflammasome components, and an activation signal that causes the inflammasome to form (Guo et al., 2015). cGAMP

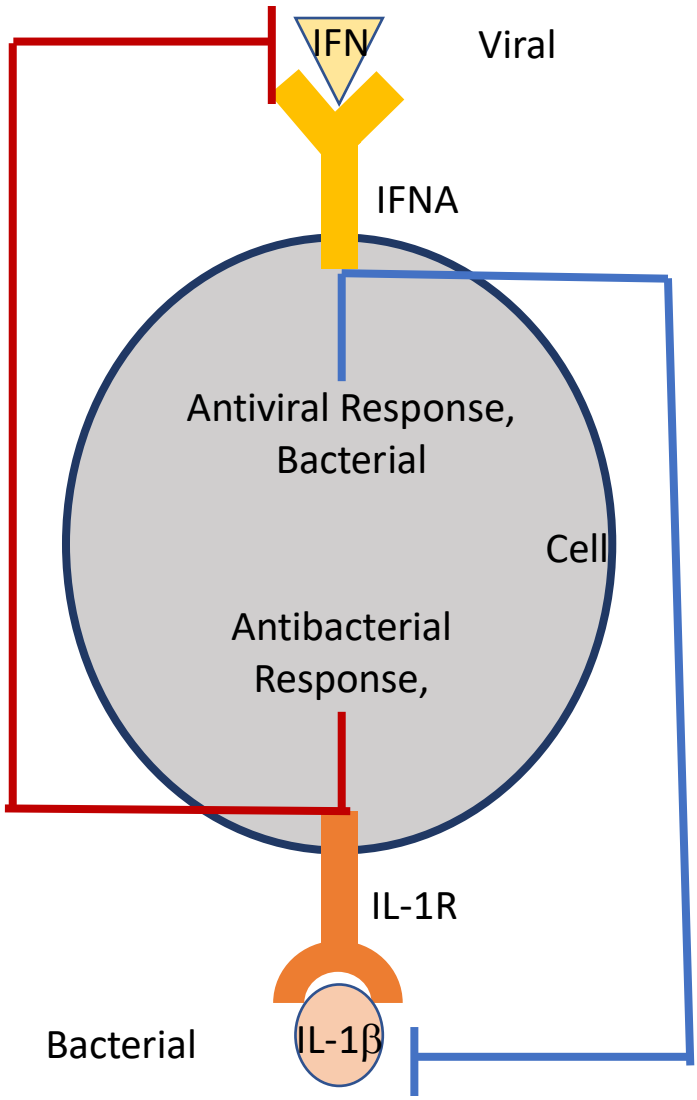
has been found to induce both signals, with priming coming from the production of IFN, which transcriptionally regulates inflammasome components (Swanson et al., 2017), and activation occurring through the aforementioned NLRP3-dependent pathway (Swanson et al., 2017; Gaidt et al., 2017). So not only can STING induce IFN, but through this inflammasome signaling, STING and cGAMP can also induce IL-1β and pyroptosis. This indicates that many current assumptions about cGAMP are not comprehensive; cGAMP does not only induce IFN. There is subtlety in the STING-cGAMP signaling axis, and this process can lead to a variety of immune responses that function to fight different types of infections.

Production of IFN, proinflammatory cytokines, and inflammasome activation are several different pathways that can activate innate immunity in the presence of some kind of PAMP; however, activating these different pathways will lead to functions that are specialized for fighting certain types of infections. For example, IFN activation tends to be associated with viral infection and IFN signaling tends to have strong antiviral effect (McNab et al., 2015). Small amounts of IFN can help in initiating an antibacterial immune response, such as in inflammasome priming, but sustained, high-level IFN signaling will suppress this response (McNab et al., 2015). In comparison, NF-κB activation is linked to PRRs that sense bacterial infection and signaling of NF-κB products leads to bacterial clearance (Kawai et al., 2008). These two distinct pathways can inhibit one another; during infection with influenza virus, patients are much more susceptible to infections of bacterial pneumonia (Morens et al., 2008). Part of this is explained by the fact that Type I IFNs can inhibit certain molecules upregulated by NF-κB product signaling, such as the macrophage chemoattractant CCL2 (Nakamura et al.,

2011). Sustained IFN signaling can also reduce B-cell activation (McNab et al., 2015), which is an adaptive immune subset vital for producing antibodies to mainly fight bacterial infections (Parham, 2015). This confers an advantage during viral infections, as B-cells have very active translation machinery, which viruses could hijack to replicate faster. IFNβ can also directly decrease the expression of caspase 1 and pro-IL1β (Guarda et al., 2011). IFN can also lead to feedback loops that increase the expression of PRRs like *cGAS* (Ma et al., 2015), so inhibiting IFN can actually lower the expression of PRRs. While the IFN response can repress antibacterial immune responses, the converse has also been shown to be true. For example, NLRX1 (NLR family member X1) is a protein vital for sensing bacterial infection, especially infection with *Heliobacter pylori* (Philipson et al., 2015). NLRX1 is upregulated by the host during bacterial infection, and the protein has been shown to associate with the CTT of STING to block the phosphorylation sites necessary for signal transduction, in turn blocking IFN transcription (Guo et al., 2016). This all supports the idea that the immune response to one type of pathogen can repress the immune response to another type of pathogen. Since cGAMP can generate either an IFN response or an inflammasome response, maybe these differing cGAMP responses can interact with each other.

These models of co-infection with both a virus and a bacterium provide strong evidence that an immune response to one class of pathogen can repress the immune response to the other classes of pathogens and predispose the host to infection with the secondary pathogen (Figure 3). One effect of the antiviral state induced by IFNs is a shutdown of protein translation in healthy cells (Parham, 2015). While this slows viral replication, it also hinders the ability of immune cells to produce antibacterial proteins.

This is one reason why an antibacterial response could inhibit long-term IFN activation. Conversely the antibacterial response can recruit cell types that produce large amounts of proteins, such as antibody-producing plasma cells (Parham, 2015). Prolonged exposure to any type of immune response can lead to immunopathology, with damage occurring to tissues both localized at the site of infection and at other locations throughout the body (Parham, 2015). Regulation of the immune response is necessary in order to turn off prolonged immune signals and prevent immunopathology. Activating the bacterial immune response could attenuate IFN signaling, and vice versa, functioning to regulate both types of immunity. Following this, by shutting down the bacterial immune response, IFN increases the efficacy of the antiviral response and reduces the ability of viruses to replicate while also limiting pathology from the bacterial response. Still, there needs to be an effective PAMP to activate either one of these systems.



<u>Figure 3:</u> Example of Repression of Immune Responses During Co-Infection: Both antiviral and antibacterial cytokine signals are received in both a paracrine and autocrine fashion. These signals can then inhibit one another to enhance the desired immune response. This figure does not take into account intrinsic signaling that occurs inside of a single cell. This intrinsic pathway could be activated within one cell and would not require the secretion and reception of other messenger molecules, thus increasing the speed of an intrinsic response compared to paracrine.

CDNs make for an effective PAMP because they are used across many species of bacteria and they are small molecule products of cellular processes that are hard to structurally alter through evolution. Therefore, it makes sense that a receptor such as STING would evolve to sense the presence of CDNs. Yet STING remains intracellular

(Chen et al., 2016) and tends to generate IFN responses that are most effective against viruses Levy et al., 2001). Most bacteria replicate outside of cells and secrete their signaling CDNs into the extracellular environment (Schaap et al., 2013). CDNs are unable to cross the plasma membrane due to their charge, and no known eukaryotic CDN channels exist that would allow for transport of CDNs. This means there may be evolutionary pressure for some sort of extracellular CDN receptor. This receptor would likely induce a bacterial immune response, as any extracellular CDN would indicate the presence of bacteria. cGAMP has not been shown to be secreted, and all transport of cGAMP between cells occurs either using gap junctions (Almine et al., 2017) or enclosed virions (Bridgeman et al., 2015). cGAMP may be outside of cells under physiological conditions due to either abortive viral infection or pyroptosis, but any extracellular cGAMP would be quickly degraded by the 2'3'-cGAMP-specific phosphodiesterase ENPP1 (Li et al., 2014). This means that physiologically, extracellular CDNs are only in the extracellular environment due to the presence of bacteria, never because of viral-derived cGAMP. This puts a selective advantage on an extracellular CDN receptor to activate an anti-bacterial immune pathway.

While an extracellular CDN receptor that activates bacterial immunity has not been identified, an intracellular receptor with these qualities has been found. The reductase controlling NF-κB, otherwise known as RECON, is a cytoplasmic protein that functions to inhibit the activation of NF-κB (McFarland et al., 2017). RECON can specifically bind c-di-AMP but not other CDNs, which can serve two purposes: first to prevent RECON from inhibiting NF-κB, thus activating bacterial immunity, and second to sequester c-di-AMP from STING, thus preventing the activation of viral immunity.

Since intracellular c-di-AMP is indicative of a bacterial infection with *L. monocytogenes* (Woodward et al., 2010), which is an intracellular bacteria, NF-κB activation would lead to the most effective immune response to combat the bacterial infection. Another receptor known as ER membrane adaptor (ERAdP) has also been found to bind c-di-AMP and initiate NF-κB pathways (Xia et al., 2018). The functionality of RECON and ERAdP shows that CDN receptors that activate antibacterial immunity exist and that they can activate pathways that inhibit IFN. The ability of extracellular cGAMP to repress a viral immune response has been found in preliminary data (Figure 1 Results), further supporting the existence of an extracellular CDN receptor.

Experimental Goals:

The original goal of this study was to deliver cGAMP to cells to mimic cGAS activation and measure the flowing IFN response. However, due to lack of consensus in the literature on how to deliver cGAMP, multiple delivery methods were attempted, delivering cGAMP either to the cytosol or extracellular environment. Since preliminary findings showed that extracellular cGAMP can repress *ISG56* and the viral immune response (Figure 7), we concluded that there may be a novel cGAMP signaling pathway. The new main goal of this study was to further explore the nuances of cGAMP signaling in response to differing delivery methods. The signaling of cGAMP and other CDNs has been shown to be much more diverse than previously thought, activating conflicting immune pathways depending on the location of the CDN stimuli. This study proposes the existence of an extracellular receptor for CDNs that activates an antibacterial immune response through NF-κB activation. It is hypothesized that structural similarities between

eukaryotic cGAMP and the bacterial CDNs allows for cross-activation of this receptor by cGAMP. While not the primary target for this receptor, cGAMP would activate an antibacterial immune response in this manner, and this response could in turn inhibit the IFN induced by intracellular CDNs. This receptor would represent a novel signaling pathway for CDNs and could potentially have a role in both generation of an immune response and regulation of the immune response. Studying this new mode of signaling would lead to better understanding of innate sensing of bacteria and regulation of conflicting immune responses.

In order to test these hypotheses regarding cGAMP-induced repression and an extracellular CDN receptor, monocytic THP-1 cells were matured and stimulated with DNA, cGAMP, or other CDNs. The subcellular localization of the cGAMP and CDNs was varied by using two methods of delivery: lipid transfection for intracellular delivery, and addition to the media for extracellular delivery. The immune response of the stimulated cells was measured by analyzing transcriptional changes of various immune products from either viral or bacterial immune pathways by qPCR. Responses at the protein level were measured by HEK-Blue assay. A rationale for experimental choice and progression is seen in Figure 4.

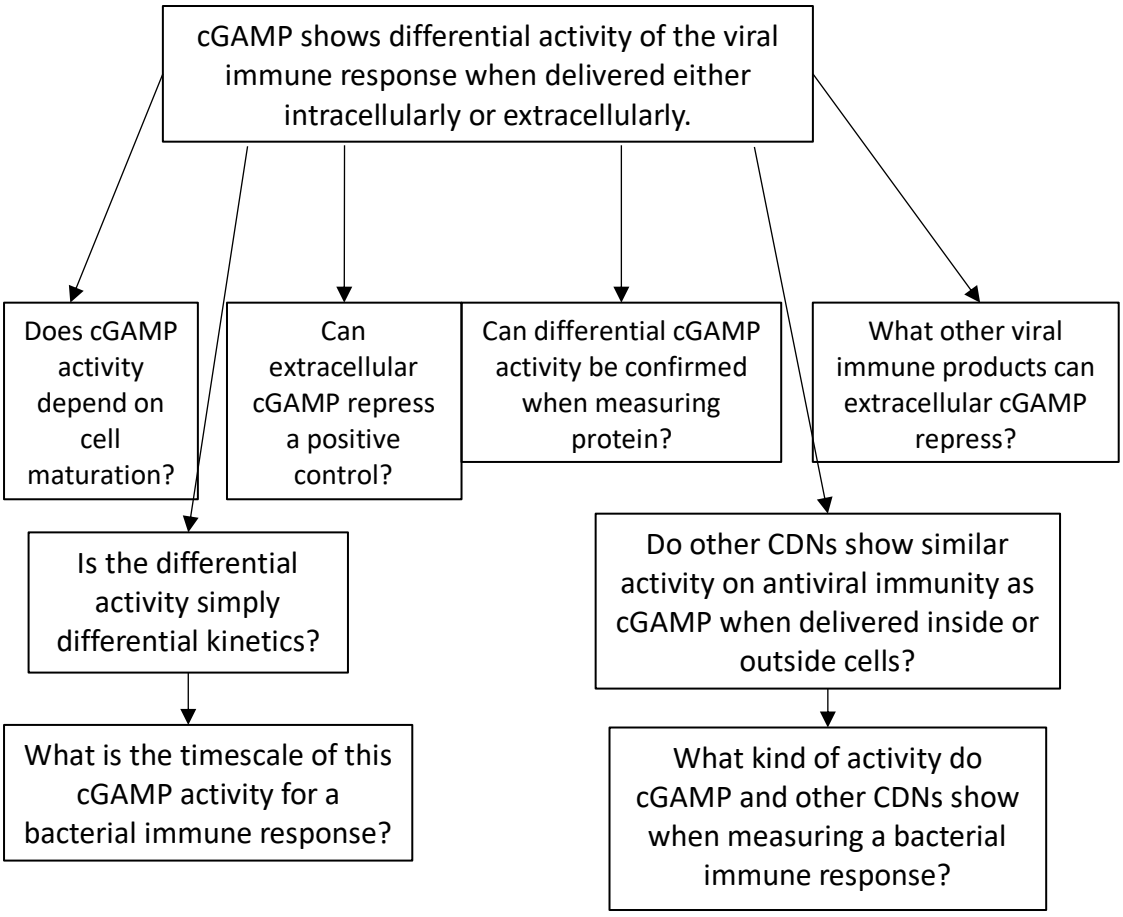


Figure 4: Experimental Flowchart: This flowchart details the order of experiments conducted in this study and what data was used to rationalize next steps.

Methods:

Cell Culture:

RPMI medium (obtained from Invitrogen) was supplemented with fetal bovine serum (FBS; obtained from Invitrogen) (10%), penicillin/streptomycin/glutamine (obtained from Invitrogen) (5 mL/500 mL media), Normocin (obtained from Invivogen) (0.5mg/mL), non-essential amino acids (obtained from Invitrogen) (1 mL/100 mL media), sodium pyruvate (obtained from Invitrogen) (1 mM) and β-mercaptoethanol (obtained from Invitrogen) (50 nM), hereafter referred to as R10 media. THP-1

monocytic cells (obtained from ATCC) were cultured in R10 media at 37°C and 5% CO_2 and were plated at the following concentrations: $2 * 10^6$ cells in 3 mL of media in a 6-well plate, or $1 * 10^5$ cells in 1 mL of media in a 24-well plate. CRISPR knockouts of IFI16 and STING (both obtained from the Paluden lab) in THP-1 cells were cultured in the same manner described above.

DMEM media (obtained from Invivogen) was supplemented in the same manner as R10 media and is hereafter referred to as D10 media. D10 media was further supplemented with 30 μg/mL of blasticidin (obtained from Invivogen) and 100 μg/mL of zeocin (obtained from Invivogen) to make HEK-Blue media. HEK-Blue IFN α/β cells (obtained from Invivogen) were maintained in HEK-Blue media at 37°C and 5% CO_2.

Cell Stimulation:

THP-1 cells resemble undifferentiated human monocytic cells (Tsuchiya et al., 1980); however, maturation into macrophages results in changes of expression of many proteins, especially PRRs like cGAS and STING. In order to mature THP-1 cells, phorbol myristate acetate (PMA) maturation was performed by adding 5 ng of PMA per milliliter of cells (Yoh et al., 2015). Cells were then incubated for 72 hours to allow for recovery before further stimulation.

To stimulate cells in a 6-well plate, 10 μL of Lipofectamine 2000 (obtained from Invitrogen) was mixed with 240 μL of Opti-MEM (obtained from Invitrogen), a buffered, simplified form of media. A 250 μL mixture of stimulus (either CDN or nucleic acid) and Opti-MEM was also made, and both mixtures were allowed to incubate at room temperature for 5 minutes. After incubation, both mixtures were combined and incubated

at room temperature for 20 minutes to 6 hours before addition to the cells, as per the manufacturer's instructions. For a 24-well plate, a mixture of 2 μL of Lipofectamine 2000 and 48 μL of Opti-MEM was made before being combined with 50 μL of stimulus and Opti-MEM using the same timeframe described above. Use of this procedure with a CDN stimulus constitutes the "IN" condition described in the results because lipofectamine delivery results in cargo delivery to the cytosol (Figure 5). "Mock" samples were generated by combining the Lipofectamine 2000 mixture with pure Opti-MEM. "No stim" samples were prepared with just Opti-MEM and no lipofectamine or nucleic acid.

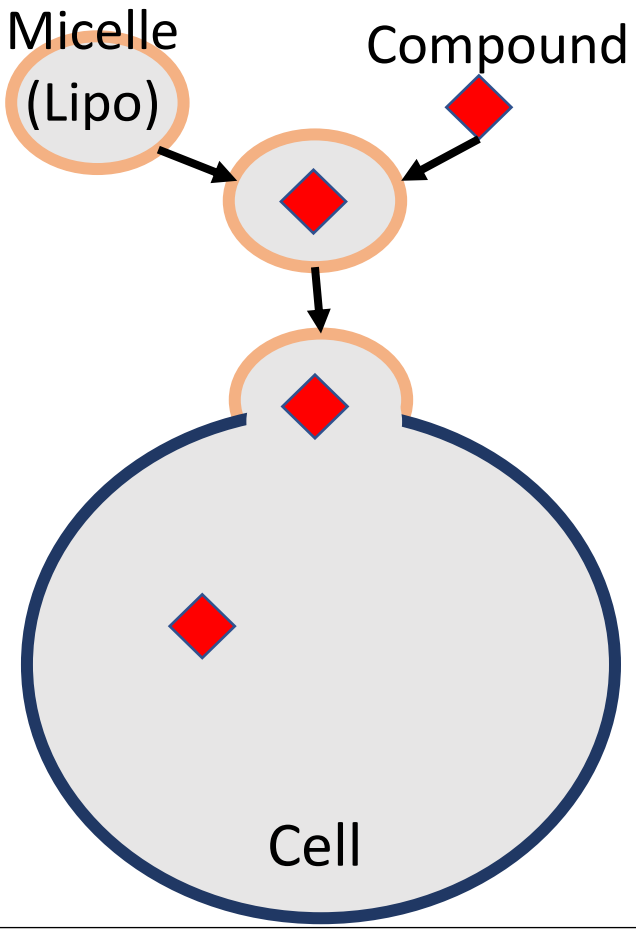


Figure 5: Cytosolic Delivery of Stimuli: By using Lipofectamine 2000, compounds such as CDNs or DNA can be delivered to the interior of a cell. This occurs as the Lipofectamine forms a lipid micelle around the compound, and then that micelle merges with the cell membrane to deliver the cargo to the cytosol. Large or polar compounds delivered without Lipofectamine will remain outside of cells due to their inability to diffuse across the plasma membrane.

When delivering CDNs for the "OUT" condition in a 6-well plate, a 500 µL mixture was made of the CDN and Opti-MEM which was allowed to sit for 25 minutes before being added to the cells. A 100 µL mixture was used for 24 well plates. This method delivers the CDN to the extracellular environment since the molecules are too polar to diffuse through the membrane and channels for entry are not known. For the

"OUT+L" condition, both mixtures were made as were described in the Lipofectamine 2000 protocol above; however, the two mixtures were not mixed together before addition to the cells. This was used as a control because the CDN would still be delivered outside of the cells because Lipofectamine requires 20 minutes to absorb the cargo, but fusion involving empty lipofectamine micelles would still occur.

DNA stimuli, such as polydA:dT, Vac70, and G3Y-form were used at a concentration of 2 μg DNA/mL of cells. PolydA:dT (obtained from Invivogen) is a synthetic, positive control B-form dsDNA that is known to induce IFN through STING-independent pathways (Kalantari et al., 2014). Vac70 (obtained from Invivogen) is 70 base pairs of DNA from the genome of Vaccinia virus that is known to induce IFN in a STING-dependent manner (Unterholzner et al., 2010). G3Y-form (obtained from IDT) DNA is a synthetic strand of DNA modelled on the genome of HIV. It has a repetitive sequence of guanines that allows for secondary structures to form that cause recognition by cGAS (Herzner et al., 2015). Various CDNs, such as cGAMP, cdi-AMP, cdi-GMP, and cdi-UMP (all obtained from Invivogen) were used at a concentration of 10 ng/mL of cells. Lipopolysaccharide (LPS; obtained from Invivogen) was used at a concentration of 5 μg/mL of cells and was not delivered using Lipofectamine. LPS is a bacterial cell membrane component that, when delivered extracellularly, induces an innate immune response composed mainly of NF-κB products (Alexander et al., 2001).

Following stimulation, cells were incubated overnight before they were pelleted. Cell pellets were stored in RNA lysis buffer (see RNA extraction protocol below) and a sample of the supernatant was taken. Both the pellets in lysis buffer and the supernatant samples were kept at -80°C.

RNA Extraction and cDNA Synthesis:

RNA Extraction was performed using a Quick-RNA miniprep kit (obtained from Zymo Research) following the manufacturer's protocol. The concentration and purity of purified RNA was confirmed via Nanodrop and RNA samples were stored at -80°C. A Nanodrop allows for spectroscopic determination of nucleic acid concentration using a small volume of sample, usually 1-2 μL. cDNA was then synthesized using the ProtoScript II First Strand cDNA Synthesis Kit (obtained from New England BioLabs) following the manufacturer's protocol. RNA amounts added during cDNA synthesis were controlled to ensure that every sample in an experiment was made using the same amount of RNA. For example, in a given experiment, if the smallest amount of RNA used to make cDNA was 100 ng, all cDNA samples were made with 100 ng of RNA. This would ensure that qPCR measurements would not differ due to varying amounts of cDNA across samples. cDNA samples were stored at -20°C.

qPCR:

For each sample, 2 μL of cDNA was mixed with 10 μL iTaq Universal SYBR Green mix (obtained from BioRad), 6 μL nuclease-free water, and 1 μL each of the forward and reverse primers at a concentration of 10 μM. Amplification primers were obtained from IDT and the primer sequences are as follows:

ISG56 Forward: 5'-CCT CCT TGG GTT CGT CTA CA-3'

ISG56 Reverse: 5'-GGC TGA TAT CTG GGT GCC TA-3'

IFNα Forward: 5'-CCA TTC TGG CTG TGA GGA AAT A-3'

IFNα Reverse: 5’-TGA TTT CTG CTC TGA CAA CCT C-3’

IFNβ Forward: 5’-CAC GCT GCG TTC CTG CTG TG-3’

IFNβ Reverse: 5’-AGT CCG CCC TGT AGG TGA GGT T-3’

IL-1b Forward: 5’-GGA TAT GGA GCA ACA AGT GG-3’

IL-1b Reverse: 5’-ATG TAC CAG TTG GGG AAC TG-3’

IL6 Forward: 5’-AGA ATT GCC ATT GCA CA-3’

IL6 Reverse: 5’-CTC CCA ACA GAC CTG TCT ATA-3’

cGAS Forward: 5’-GGG AGC CCT GCT GTA ACA CTT CTT AT-3’

cGAS Reverse: 5’-CCT TTG CAT GCT TGG GTA CAA GGT-3’

RPL37a Forward: 5’-ATT GAA ATC AGC CAG CAC GC-3’

RPL37a Reverse: 5’- AGG AAC CAC AGT GCC AGA TCC-3’

Samples were run on Bio-Rad CFX96 qPCR machine for 40 cycles. Before cycling started, there was a 3 minute 95°C initial denaturation. One cycle consisted of a 10 second 95°C denaturation step followed by a 30 second 60°C annealing and elongation step. Then the machine would read fluorescence. After 40 cycles, the machine went from 65°C to 95°C in 0.5°C increments in order to generate melt curves to confirm products. A simplified diagram of the qPCR process can be seen in Figure 6.

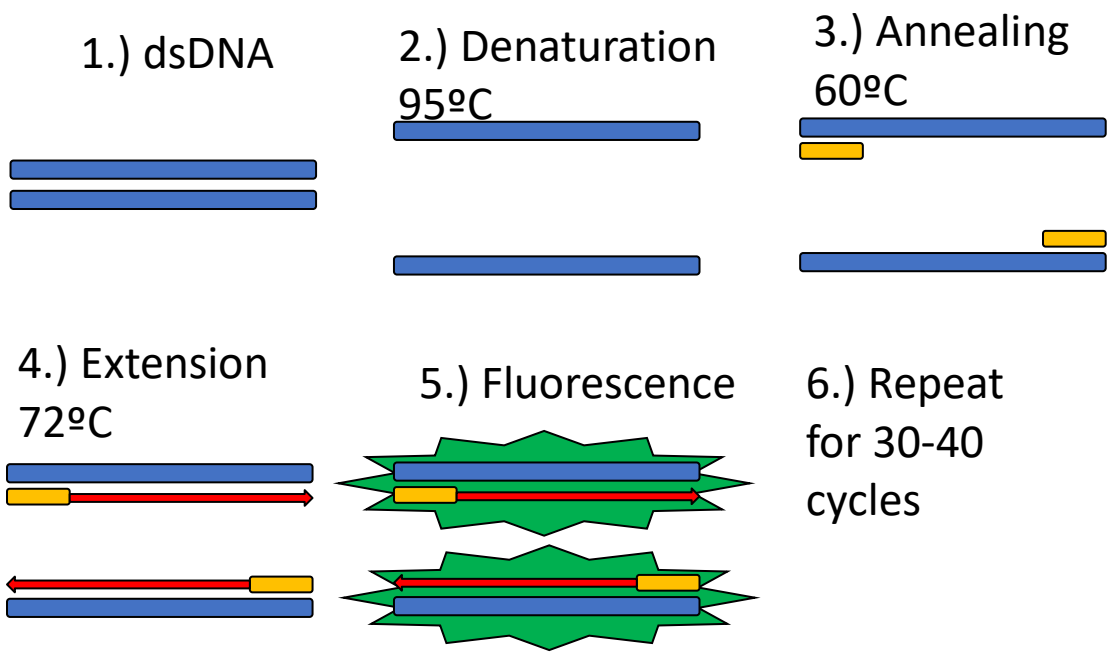


Figure 6: Process of qPCR: qPCR measures the relative number of mRNA transcripts of a gene of interest to both background expression and the expression of a housekeeping gene. The most important step in qPCR is the fluorescence reading; by using a dye, such as SYBR Green, that binds only to dsDNA, the amount of dsDNA in each sample can be quantified every cycle. This is what allows for comparison between samples, as a fluorescence reading during the same exponential phase of elongation will be compared for each sample.

Threshold cycle (Ct) values for SYBR Green fluorescence of both the gene of interest (GOI) and the housekeeping gene *RPL37a* were collected. *RPL37a* encodes a protein of a ribosomal subunit and it was chosen as a housekeeping gene because it is expressed at similar levels across our cell type and its expression is not influenced by our stimuli (Popovici et al., 2009; Maess et al., 2010). Ct values are determined by a horizontal line placed in the exponential phase of each data set. For each sample, a ΔGOI value was calculated by subtracting the Ct *RPL37a* value from the Ct GOI value. This normalized all expression values to a gene with consistent background level expression to control for potential differences due to variation in cell count. Next a ΔΔGOI value was

generated by subtracting the average ΔGOI value for either the "mock" or "no stim" samples from the ΔGOI of the sample in question. This normalized the expression of the GOI in the stimulated samples to background GOI expression in the controls. Lastly, fold change for each sample was calculated using the following equation: $2^{-\Delta\Delta GOI}$. The value of fold change is representative of how many times more expression of the GOI is in the indicated sample over background levels of GOI expressions. A fold change of 1 indicates background expression, while a fold change greater than 1 shows increased expression and a fold change less than 1 indicates inhibition below background levels.

Samples containing Lipofectamine 2000 were further normalized to "mock" values, while samples without Lipofectamine 2000 were normalized to "no stim" values. This allowed for any immunogenic effects of the lipofectamine reagent to be normalized for in final fold-change values.

HEK-Blue Assay:

Initially, 20 μL of supernatant sample from the above stimulation conditions was added to a well of a 96-well plate. A suspension of HEK-Blue cells at 280,000 cells/mL was made and 180 μL was added to each well, followed by incubation overnight. QUANTI-Blue (obtained from Invivogen) was prepared according to the manufacturer's instructions and 180 μL of this solution was added to a new 96-well plate. Then 20 μL of supernatant from the corresponding HEK-Blue well was added. This was allowed to incubate for one hour before being read on a plate reader at 620 nm. Absorbance readings from experimental samples were normalized to control values, with samples containing Lipofectamine 2000 normalized to "mock" values, and samples without Lipofectamine

2000 normalized to “no stim” values. These normalized absorbance values were averaged and compared.

Results:

ISG56 is one of the many genes activated by Type I IFNs, and its presence is generally indicative of an innate immune response to a virus (McNab et al., 2015), so by measuring *ISG56*, the viral immune response can be measured. When analyzing transcriptional changes of *ISG56* in response to various stimuli, it can be seen that the two different delivery methods for cGAMP result in varying outcomes: cGAMP delivered via lipid transfection increases *ISG56* transcription, while cGAMP added to the media represses this activity below background levels (Figure 7). Both polydA:dT and Vac70 DNA were able to increase the expression of *ISG56* as was expected from the literature, and while cGAMP transfected into the cytosol had a lower fold change comparatively, it too increased expression of *ISG56* (Figure 7). This suggests that cytosolic cGAMP is activating a viral immune response, probably through a STING-dependent pathway. However, cGAMP delivered without lipofectamine transfection, and so stuck in the extracellular environment, repressed expression of *ISG56* below background levels (Figure 7). This was a novel finding that had not been shown in any previous literature. This repressive pattern of extracellular cGAMP also matched the repressive activity of extracellular LPS (Figure 7), which is a bacterial stimulus that will activate a bacterial immune response. This suggests that extracellular cGAMP may be acting as a stimulus of a bacterial type response. Overall, these data suggest that there are

two different pathways through which cGAMP can signal, and these pathways depend on the location of cGAMP.

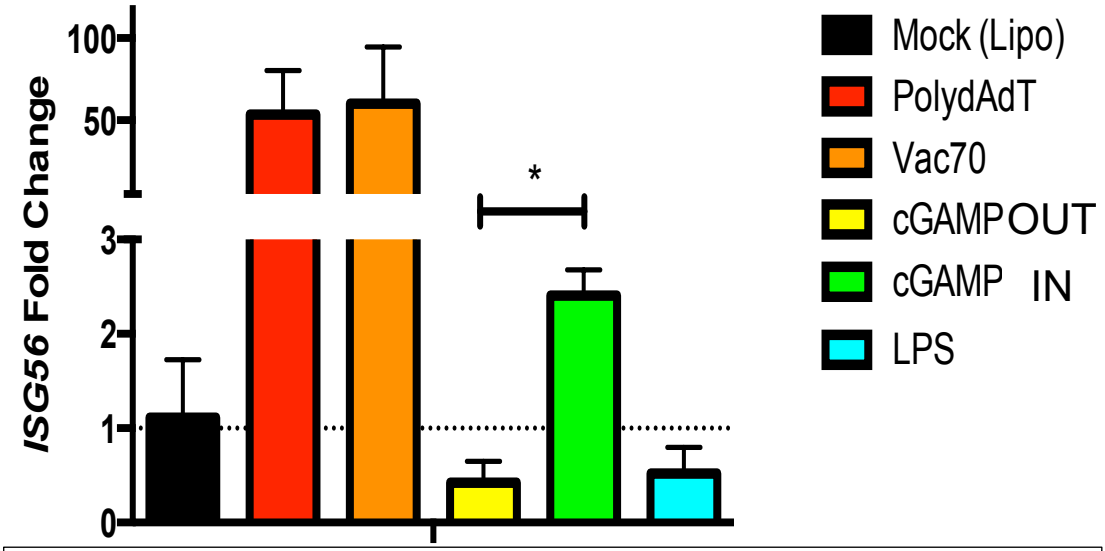


Figure 7: Untransfected cGAMP represses transcription of *ISG56*. PMA-treated THP-1 cells stimulated with polydA:dT and Vac70 DNA, cGAMP IN (cGAMP tft), cGAMP OUT (cGAMP), or LPS (delivered without transfection). The dashed line at one indicates background expression. Measurements were taken 24 hours after stimulation. Data are representative of the mean oftechnical triplicates from three experiments, and error bars represent the standard deviation. A Kruskal-Wallis non-parametric ANOVA was run followed by a Mann Whitney T-test was performed for statistical analysis. *- $p<0.05$

Alternatively, it is possible that cGAMP delivered outside of cells does induce an ISG56 response, but that response has a different timing than the intracellular cGAMP response. Thus, the kinetics of the responses were examined. The activity of cGAMP on *ISG56* in either location peaks at 24 hours after stimulation (Figure 8). Both cytoplasmic and extracellular cGAMP induce little change in transcription of *ISG56* from 0 to 12 hours after stimulation, but the two conditions diverge at the 24-hour mark (Figure 8). This is when cytoplasmic cGAMP increases expression of *ISG56*, and when extracellular cGAMP decreases expression. This lag in transcription for cytoplasmic cGAMP is attributed to the lag in the creation of IFN, as IFN must first be made, secreted, and bind to IFNAR before *ISG56* can be transcribed.

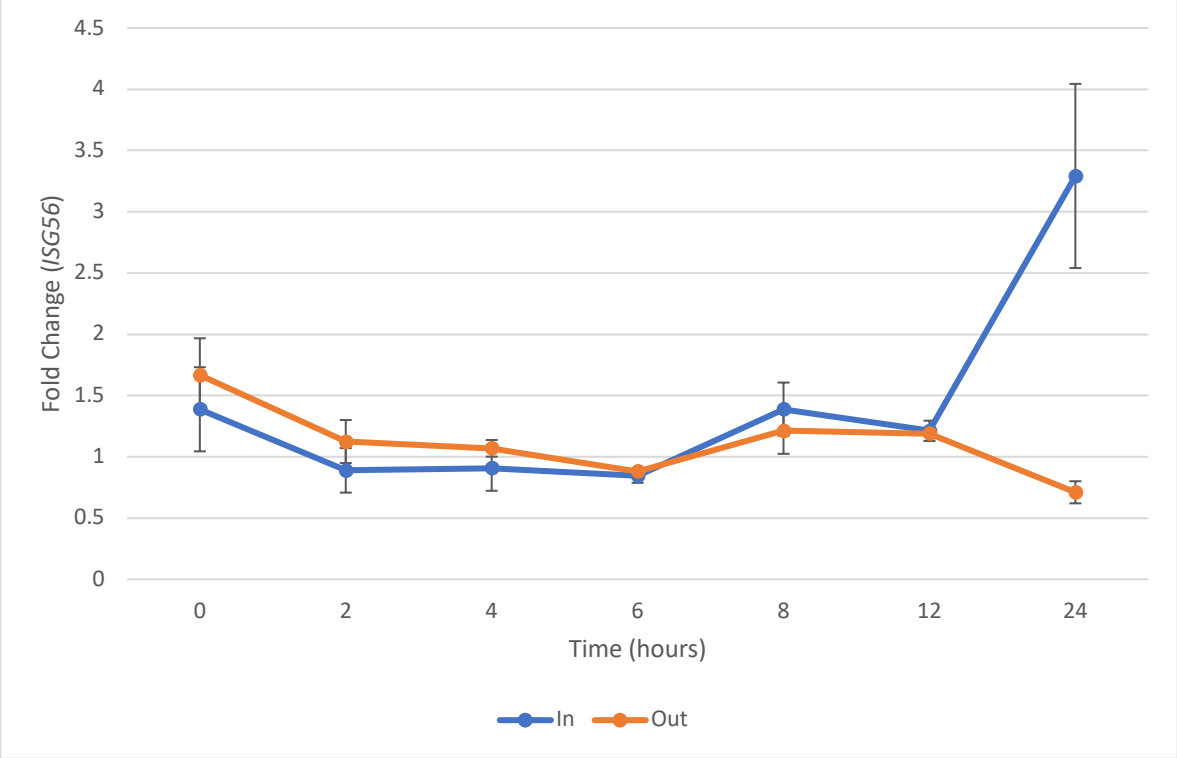


Figure 8: Kinetics of the cellular ISG56 response to cGAMP. PMA-treated THP-1 cells were stimulated with cGAMP delivered either with Lipofectamine 2000 (IN), or addition to the media (OUT). Data are representative of the mean of technical triplicates from two experiments and error bars represent the standard deviation.

As the inhibitory response was shown to be real, we next determined if this response was dependent on THP-1 maturation. The activating activity of lipid-delivered cytoplasmic cGAMP is lost in THP-1 cells not treated with PMA (Figure 9). This suggests that the components of this pathway are only present in THP-1 cells after maturation into macrophages. However, the repressive activity of extracellular cGAMP is present before maturation, since *ISG56* is repressed with or without PMA treatment

(Figure 9). When measuring the activity of cGAMP on either *IFNb* or *IL-1β*, no difference is observed for either when cGAMP is in different locations (Figure 9). With Type I IFNβ, this is attributed to the 24 hour measurement, as IFNs tend to be transcribed earlier (Parham, 2015). *IL-1β* is a common product of inflammasome activation, so the data suggest that neither cGAMP pathway is signaling through the inflammasome.

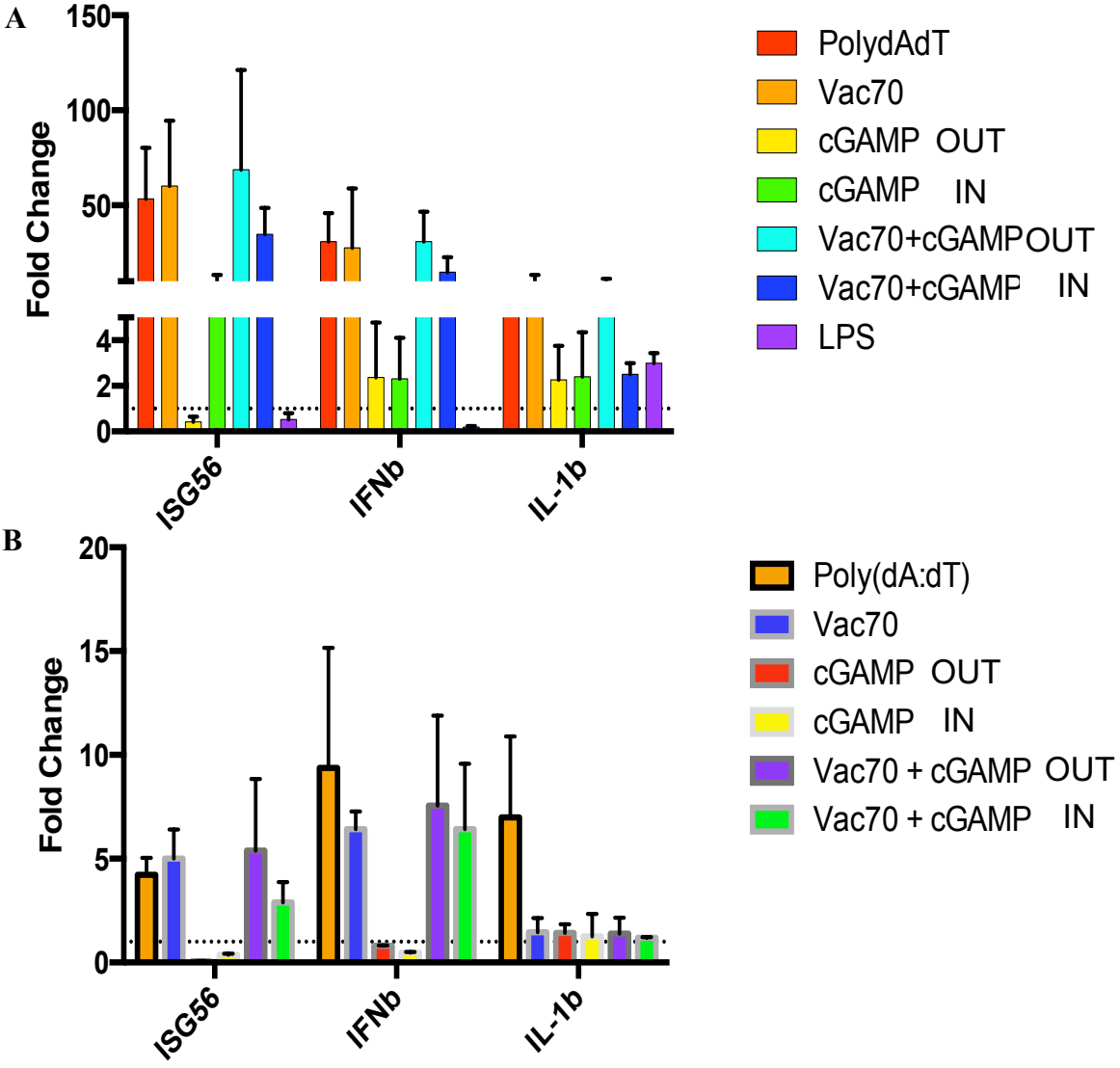


Figure 9: Repressive action of untransfected cGAMP is PMA-dependent.

Measurement of transcriptional changes of *ISG56, IFNb, or IL-1b* in response to the indicated stimuli after 24 hours. A shows cells matured with PMA, B shows cells not treated with PMA. The dashed line at one indicates background expression. Data are representative of the mean of technical triplicates from one experiment and error bars represent the standard deviation.

Knowing that the activity of cGAMP partially depends on maturation of THP-1 cells, it was decided to observe whether extracellular cGAMP could inhibit the response to a known, activating stimuli. When stimulating cells with both a known DNA stimulus and extracellular cGAMP, *IFNα* expression falls below that of the DNA stimulus alone (Figure 10). This suggests that this repressive pathway is active and can repress the activity of normally excitatory stimuli such as polydA:dT and Vac70. Since extracellular cGAMP can repress the activity of Vac70, which signals through STING, this indicates that the extracellular cGAMP pathway can interact with the cytoplasmic cGAMP pathway. In addition, extracellular cGAMP can repress the activity of polydA:dT, which induces IFN independent of STING, indicating that extracellular cGAMP can broadly inhibit IFN production.

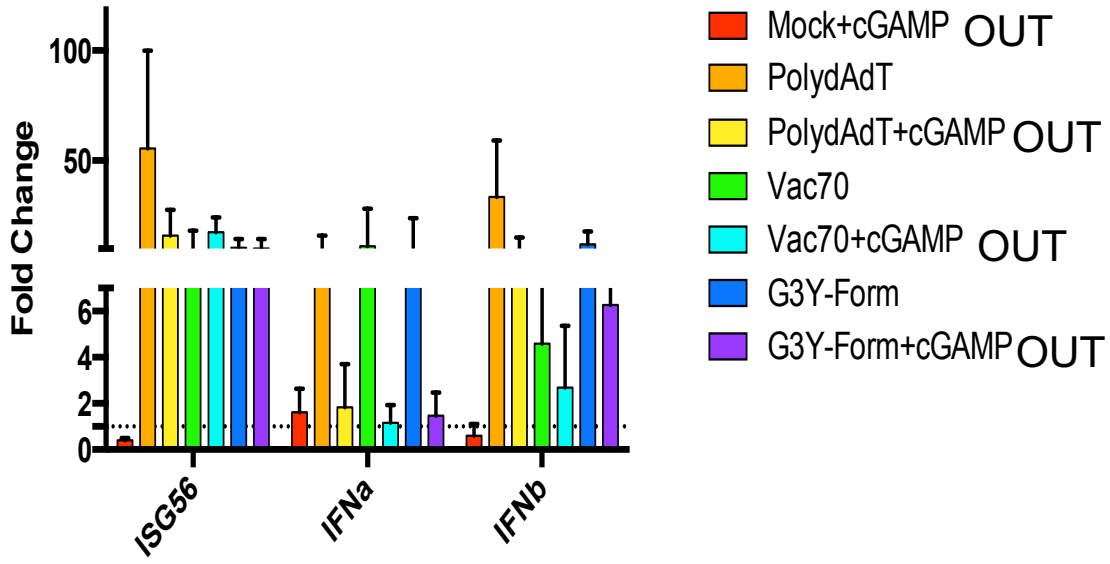
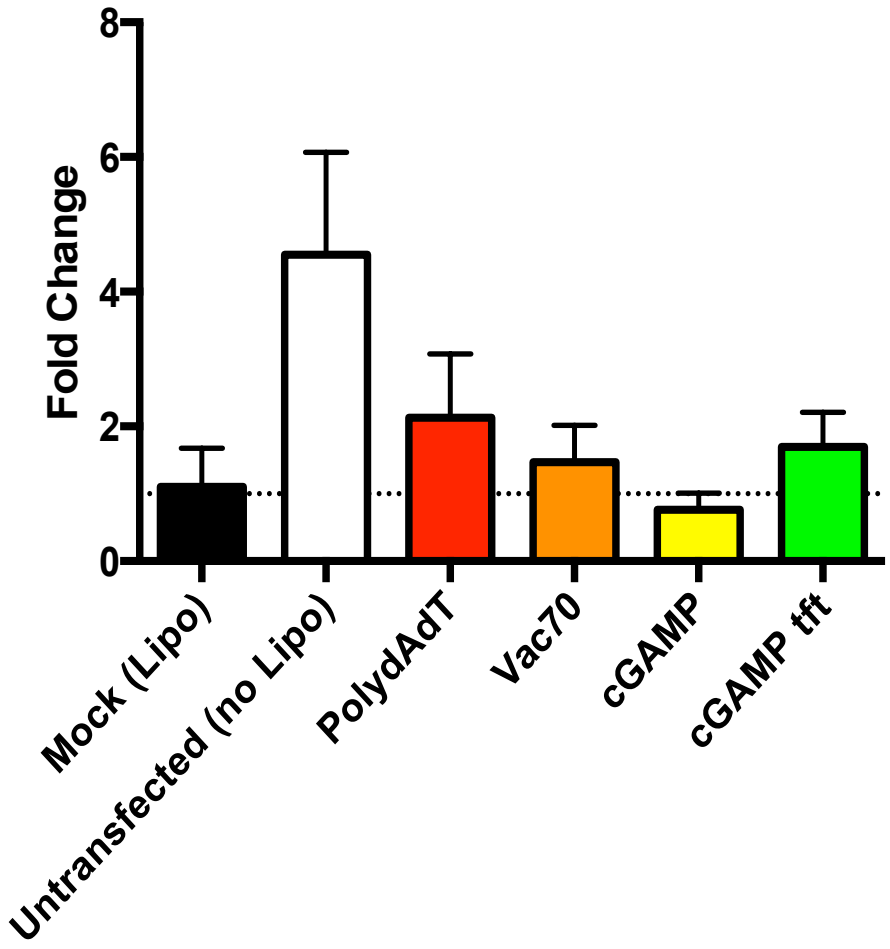


Figure 10: Extracellular cGAMP is able to repress active signaling. PMA-treated THP-1 cells were stimulated with either a DNA stimulus or the DNA in tandem with extracellular cGAMP. The dashed line at one indicates background expression. Data are representative of the mean of technical triplicates from one experiment, and error bars represent the standard deviation.

Another product of interest that may be involved in the inhibitory extracellular cGAMP pathway is cGAS; maybe cGAMP can act in a feedback loop to repress expression of *cGAS*. When measuring transcription of *cGAS*, it was found that all stimuli repress its expression below expression in the untransfected, unstimulated condition (Figure 11). Vac70 and polydA:dT DNA both repress the expression of *cGAS*, likely in a negative feedback mechanism to prevent the production of pathological levels of IFN. Higher expression of cGAS would also lead to a higher concentration of cytosolic cGAMP. Extracellular cGAMP is the strongest repressor of *cGAS*, suggesting that the novel extracellular cGAMP pathway described previously is targeting the known

pathway in order to lower levels of IFN. With less *cGAS* expression, the PRR pathway cannot be activated by DNA and so cannot make *ISG56*.



<u>Figure 11:</u> Repression of *cGAS* transcription by cGAMP. This experiment is measuring the change in transcription of *cGAS* in response to cGAMP, DNA, and Lipofectamine 2000. The dashed line at one indicates background expression. "Tft" means transfection, so the "IN" condition. Data are representative of the mean of technical triplicates from one experiment and error bars represent the standard deviation.

Repression via extracellular cGAMP would require a receptor, and this receptor would likely have evolved to sense other bacterial CDNs, as extracellular CDNs would be indicative of a bacterial infection. While extracellular cGAMP is rarely seen physiologically, this receptor would exist to sense both c-di-GMP and c-di-AMP as both of these CDNs are bacterial in origin and are widely conserved among prokaryotes (Schaap et al., 2013). cGAMP is not secreted and is degraded extracellularly by ENPP1 (Li et al., 2014), so the proposed extracellular receptor would not bind cGAMP as a main target. However, cGAMP may bind this extracellular receptor due to similarity in structure to other CDNs, and then repress the viral response seen above. Binding to multiple CDNs would be possible as STING is already capable of binding many different CDNs (Burdette et al, 2011). To examine this assumption, other CDNs were tested to see if they showed the same patterns of activation and repression as cGAMP. Other CDNs were found to exhibit the same differential activity as cGAMP, repressing *ISG56* expression when extracellular, and increasing expression when cytoplasmic (Figure 12). Activation of *ISG56* by intracellular c-di-GMP is likely due to cross-activation of STING, since c-di-GMP is known to bind STING, albeit with a lower affinity than cGAMP (Burdette et al., 2011; Shu et al., 2012). Synthetic c-di-UMP has been shown to not bind many CDN-binding proteins like STING, due to the smaller size of the pyrimidine preventing efficient ring-stacking in the binding domain (Yin et al., 2012), which explains why intracellular c-di-UMP does not activate *ISG56*. Although other studies have shown that bacterial intracellular c-di-AMP has been shown to induce IFN through STING (Woodward et al., 2010), however, these data show otherwise (Figure 12). This may be due to a secondary pathway that c-di-AMP can activate, which leads to

ER-phagy of STING and removes STING from the cytosol (Moretti et al., 2017), or due to difference in timing of activation of the two pathways. However, cGAMP and c-di-GMP both show some level of repression of *ISG56* when delivered extracellularly, indicating that the similar structures of these two CDNs could be of relevance within the proposed repressive pathway. The finding is supported when measuring IFN secretion via HEK-Blue assay. While not significant, all CDNs show a general pattern of increased IFN secretion when delivered intracellularly and decreased IFN secretion when delivered extracellularly (Figure 13).

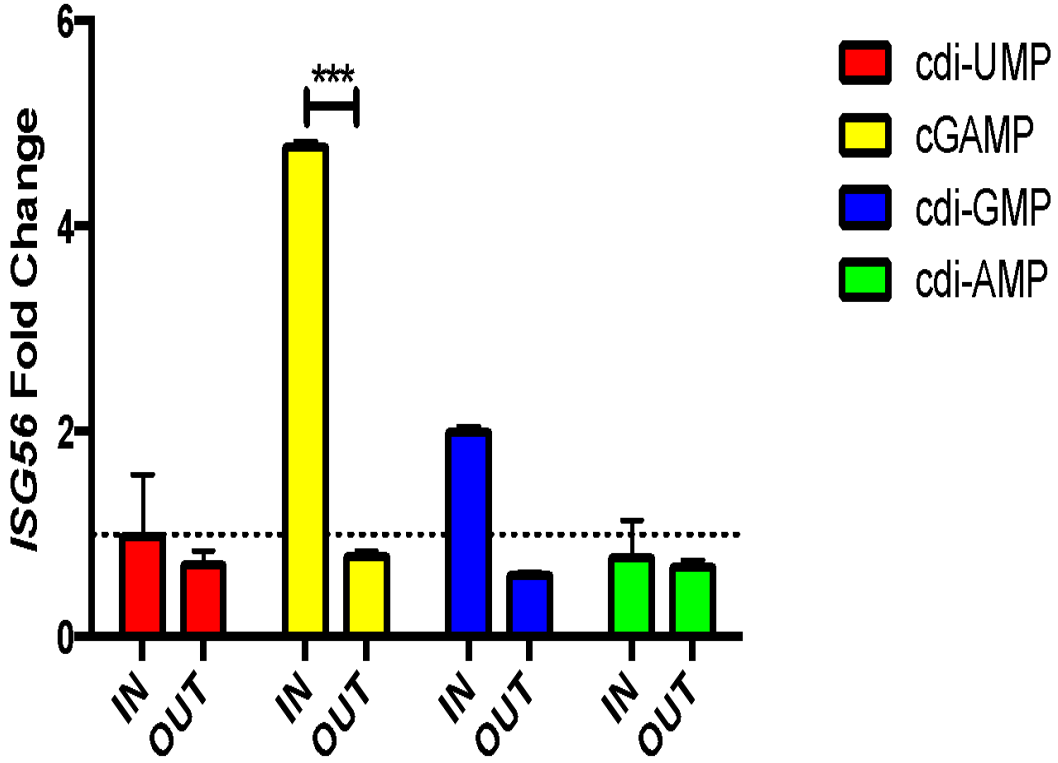


Figure 12: Repression of the ISG56 response with other CDNs. PMA-treated THP-1 cells were stimulated with either c-di-UMP, cGAMP, c-di-GMP, or c-di-AMP delivered to the cytoplasm or the extracellular environment. Changes in the transcription of *ISG56* were measured via qPCR. The dashed line at one indicates background expression. Data are representative of the mean of technical triplicates from three experiments and error bars represent the standard deviation. A Mann Whitney T-test was run for statistical analysis.

*** - $p<0.001$

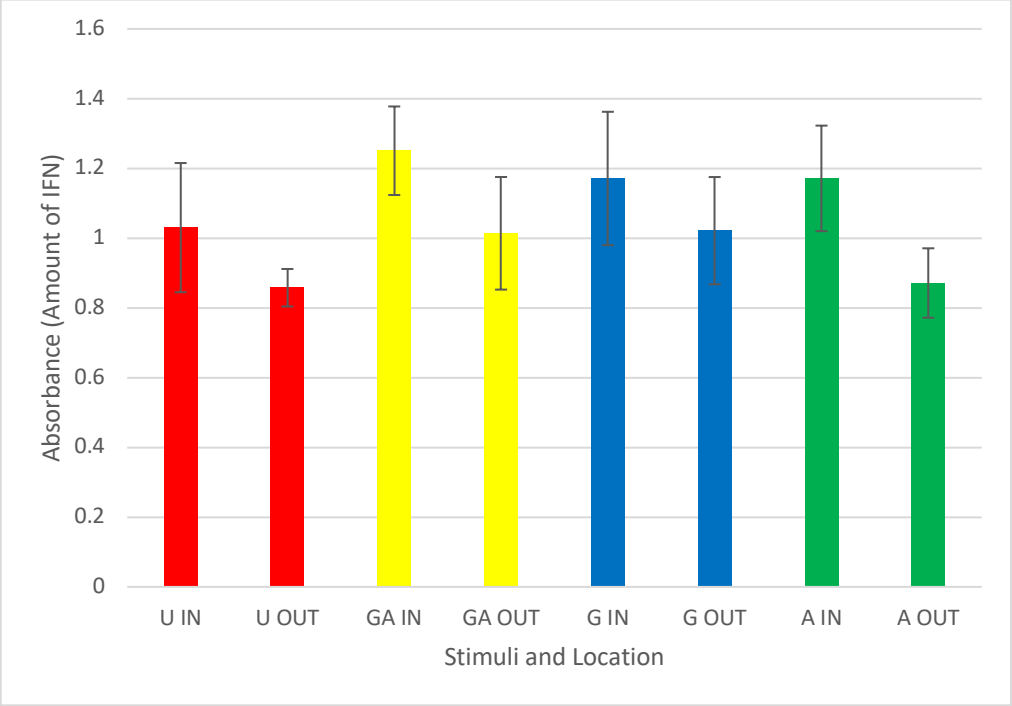


Figure 13: Repression of IFN secretion with cGAMP and other CDNs. Non-PMA-treated THP-1 cells were stimulated with either c-di-UMP, cGAMP, c-di-GMP, or c-di-AMP delivered to the cytoplasm or the extracellular environment. Changes in the secretion of Type I IFNs were measured via HEK-Blue assay. Data are representative of the mean of technical triplicates from one experiment and error bars represent the standard deviation.

In order to further study the extracellular *ISG56* repressive pathway, we recalled the repression of *ISG56* by LPS observed in Figure 1. LPS is bacterial in origin and would activate bacterial immunity such as the interleukin response when delivered extracellularly (Parham, 2015). It was hypothesized that cGAMP could signal through interleukin pathways that have been shown to inhibit IFN responses (D'Andrea et al., 1993). This is also broadly shown in the fact that a viral infection can predispose the host to bacterial infection, and vice versa (Morens et al., 2008; McNab et al., 2015). Thus, we examined the effects of cGAMP and other CDNs on the transcription of *IL6*, a prominent cytokine within the bacterial immune response (Ghosh et al., 1998). The previously observed patterns of activation and repression for *ISG56*, a viral immune product, are reversed when the bacterial immune product *IL6* is measured (Figure 14). All tested CDNs strongly repress *IL6* when delivered to the cytoplasm, which can be due to the activation of IFN and its products (Figure 12), which are broadly inhibitory of members of the interleukin family (Guarda et al., 2011). All tested CDNs show some slight activation of *IL6* when extracellular, which suggests that the proposed extracellular receptor for CDNs could signal through an IL-6-inducing pathway.

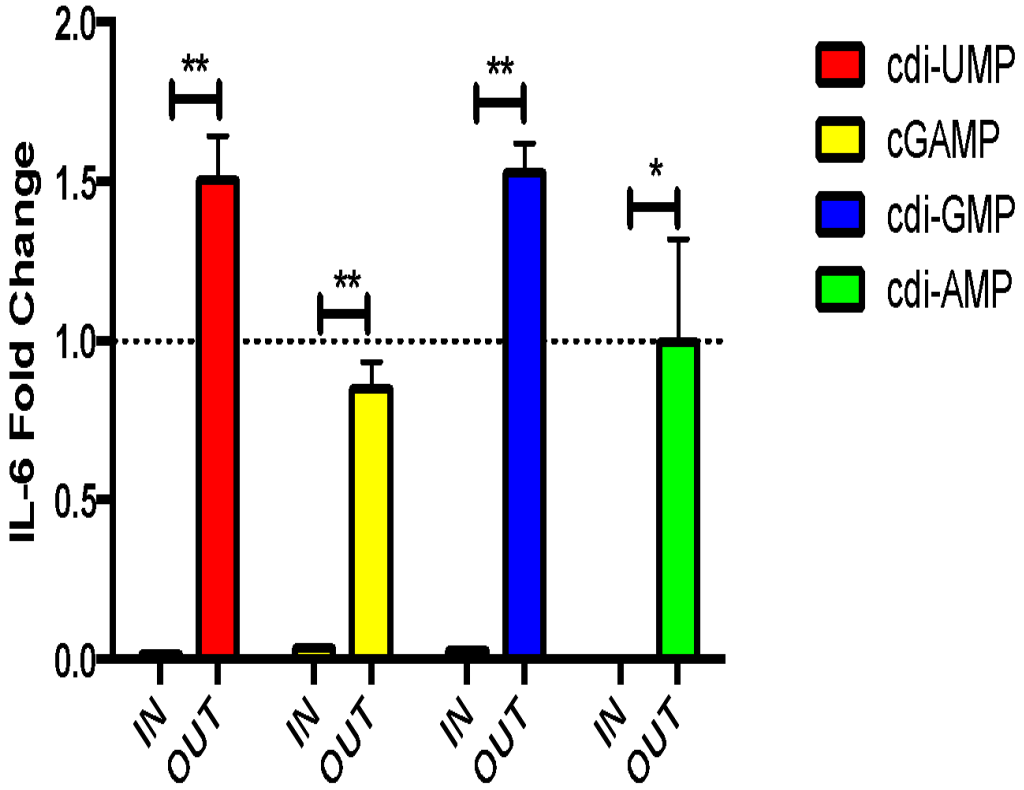
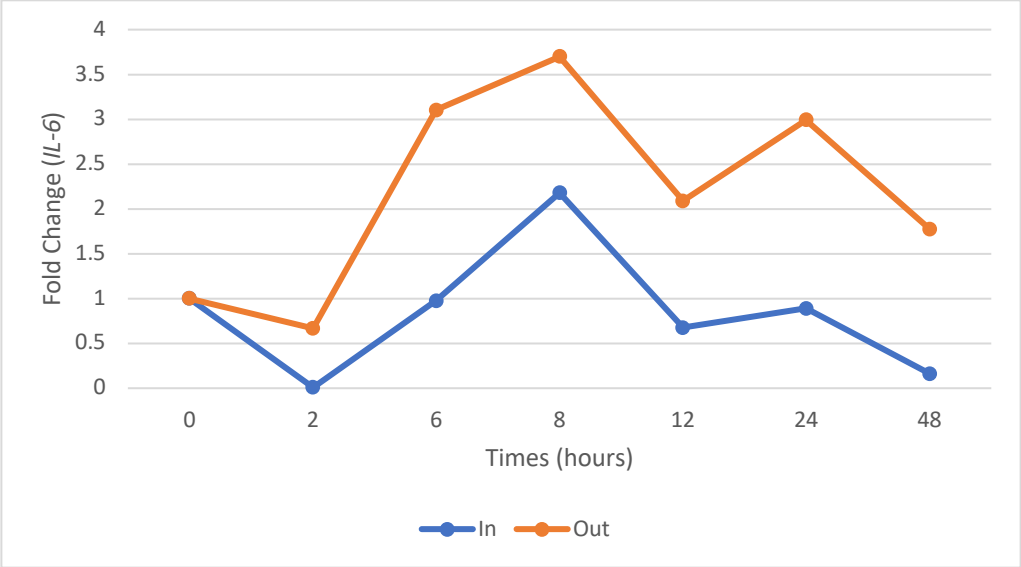


Figure 14: Repression of the IL-6 response with other CDNs. PMA-treated THP-1 cells were stimulated with either c-di-UMP, cGAMP, c-di-GMP, or c-di-AMP delivered to the cytoplasm or the extracellular environment. Changes in the transcription of *IL6* were measured via qPCR after 24 hours of stimulation. The dashed line at one indicates background expression. Data are representative of the mean of technical triplicates from three experiments and error bars represent the standard deviation. A Mann Whitney T-test was run for statistical analysis. *- $p<0.05$ **- $p<0.01$

In order to ensure that differences in *IL6* activity were not due to differences in timing of the response, a kinetics experiment was run. The activity of cGAMP on *IL6*, a cytokine indicative of a bacterial immune response, shows changes in transcriptional activity much faster (Figure 15). The "In" and "Out" conditions diverge in activity after two hours, and repressions stabilizes after 12 hours (Figure 15). Due to this increase in response time when measuring *IL6*, it can be assumed that the method of repression for *IL6* is different than the method of repression for *ISG56*.



<u>Figure 15:</u> Kinetics of the cellular IL-6 response to cGAMP. PMA-treated THP-1 cells were stimulated with cGAMP delivered either with Lipofectamine 2000 (IN), or addition to the media (OUT). Data are representative of the mean of technical triplicates from one experiment.

Discussion:

The goal of this project was to explore cGAMP-induced immune signaling resulting from different delivery methods. This cGAMP was delivered in different methods, which surprisingly altered the resulting immune response, so this effect was further explored. The activity of cGAMP delivered to either the intracellular or extracellular environment was measured by qPCR of the antiviral product *ISG56* and was compared to other characterized stimuli. These findings showed that extracellular cGAMP can repress transcripts associated with an antiviral response, and this activity was further characterized via kinetics experiments, combined stimulation experiments, and experiments where cell differentiation varied. From here, it was hypothesized that extracellular cGAMP could mimic bacterial CDNs and signal through an extracellular CDN receptor to activate antibacterial transcripts. To examine this hypothesis, the activity of cGAMP and other CDNs on *ISG56* was compared. This was accompanied by an experiment exploring the activity of cGAMP and the CDNs on the antibacterial product *IL6*.

Extracellular cGAMP Induces Repression of *ISG56*:

When cGAMP is delivered using either lipid transfection or addition to the media, *ISG56* expression is either increased or repressed, respectively (Figure 7). The increase in expression of *ISG56* by intracellular cGAMP is not as high as either STING-dependent Vac70 DNA or STING-independent polydA:dT. This disparity in the increase of *ISG56* expression can be attributed to three factors. The first is that the concentration of cGAMP used to stimulate cells is below levels made by DNA-stimulated cGAS, and so STING is

activated less. The second is that by delivering cGAMP directly to cells, we bypass physiological production of cGAMP and so the signaling pathway is accelerated. This would mean that cGAMP-induced *ISG56* would peak before the DNA-induced process. Thirdly, there could be a STING-independent pathway activated by the DNA stimuli. Conversely, extracellular cGAMP induced repression of *ISG56* (Figure 7) and can even repress the expression of *IFNα* in the presence of the known IFN inducers polydA:dT, Vac70, and G3Y-form DNA (Figure 10). This demonstrates the ability of extracellular cGAMP to repress a viral immune response even in the presence of known activators, supporting the hypothesis of a robust repressive signaling pathway. The effects of cGAMP on *ISG56* are dependent on PMA maturation of the THP-1 cells (Figure 9). While extracellular cGAMP can repress *ISG56* independent of maturation, intracellular cGAMP can only increase expression of *ISG56* in matured cells. This suggests that the intracellular receptor for cGAMP, STING, is expressed at higher levels in matured THP-1 cells. The ability of cGAMP to alter *ISG56* expression is likely dependent on IFNα, but not IFNβ, as cGAMP can induce variations in *IFNα* (Figure 10) but has no significant effect on *IFNβ* (Figure 9). This indicates that the pathway that the extracellular CDN receptor uses communicates with the IFNα pathway and can repress some components. This also could indicate a difference in regulation between the two classes of Type I IFNs.

While extracellular cGAMP was able to repress *ISG56* expression, the extracellular bacterial cell wall component LPS could do the same (Figure 7). Since *ISG56* is produced during a viral immune response, and extracellular LPS activates an antibacterial response that represses the antiviral response (Alexander et al., 2001), we

hypothesized that extracellular cGAMP could be activating antibacterial pathways in a similar manner to extracellular LPS. During intracellular bacterial infections, there is a separate intracellular LPS-sensing pathway involving the NLRP3 inflammasome (He et al., 2016). Activation of antibacterial immunity by extracellular cGAMP would necessitate some type of extracellular receptor for cGAMP. However, cGAMP showed no effect on *IL-1β* levels (Figure 9), indicating that this predicted antibacterial pathway did not involve the inflammasome. Yet extracellular cGAMP can repress *cGAS* expression (Figure 11). This shows that the repressive action of extracellular cGAMP is targeting a member of an antiviral immune pathway, and this could be one mechanism by which extracellular cGAMP can reduce IFN and ISG levels.

These two differing modes of action for cGAMP on *ISG56* both take at least 24 hours to take effect, with neither significant repression for extracellular delivery or increased expression for intracellular delivery appearing until that timepoint (Figure 8). This suggests that both cGAMP pathways are acting through some form of paracrine signaling because of the slow response time. This was expected for the *ISG56*-activating cGAMP pathway, as cGAMP would activate STING to induce IRF3 and eventually lead to IFN production (Wu et al., 2013), agreeing with the canonical pathway. This IFN signals in a paracrine fashion in order to make ISGs (Parham, 2015), and this paracrine signaling will take time to induce as IFN must be transcribed, translated, and secreted before *ISG56* activation can occur. This lag to change *ISG56* levels is also seen when extracellular cGAMP represses the response, and so this repressive pathway may also signal in a paracrine fashion.

cGAMP Activity is Mirrored by Other CDNs:

While an extracellular receptor for cGAMP was proposed, this receptor would not likely have evolved to sense cGAMP as a primary target. This is because cGAMP is self in origin and is not generally extracellular under physiological conditions due to the activity of the extracellular cGAMP-specific phosphodiesterase ENPP1 (Li et al., 2014). Yet other cGAMP receptors, such as STING, have been shown to have affinity for other CDNs such as c-di-GMP and c-di-AMP (Burdette et al., 2011). Since these other CDNs are secreted and used solely by prokaryotes (Schaap, 2013) and bacteria tend to be extracellular pathogens, an extracellular receptor would likely evolve to recognize these conserved, prokaryotic CDNs. This receptor would likely also recognize eukaryotic cGAMP due to the structural similarities between CDNs (Figure 2). This hypothesis led to tests comparing the activity of various CDNs to cGAMP when delivered either intra- or extracellularly.

While cGAMP was the only CDN to significantly alter *ISG56* transcription depending on location, c-di-GMP followed the same pattern as cGAMP: increasing *ISG56* expression when intracellular, and repressing expression when extracellular (Figure 12). *ISG56* repression via extracellular c-di-GMP was similar to that of extracellular cGAMP, but increased expression of *ISG56* by intracellular c-di-GMP was lower than levels observed by intracellular cGAMP. This indicates that shared structure between cGAMP and c-di-GMP may be enough to have shared signaling via the proposed extracellular CDN receptor, but that reduced affinity of c-di-GMP for STING (Shi et al., 2015; Zhang et al., 2013) reduces activation of IFN and, subsequently, *ISG56*. This indicates that the proposed extracellular CDN receptor may not differentiate

between phosphodiester linkages of CDNs, as both 2'3'-cGAMP and 3'3'-c-di-GMP can inhibit *ISG56* through this extracellular pathway. This could be tested in the future by comparing the effects of eukaryotic 2'3'-cGAMP to the prokaryotic 3'3'-cGAMP; if both can activate the antibacterial response when extracellular, then the CDN receptor would not differentiate between backbone linkages.

In comparison, intracellular and extracellular c-di-AMP do not differ; yet extracellular c-di-AMP results in repression of *ISG56* from background levels while intracellular deliver does not alter *ISG56* expression (Figure 12). In the intracellular environment, c-di-AMP likely activates ER-phagy of STING (Moretti et al., 2017), leaving IFN unaltered or slightly repressed, but extracellular c-di-AMP clearly represses *ISG56*, supporting the concept of the extracellular CDN receptor. c-di-UMP was meant to be a negative control, as it cannot bind and activate STING due to insufficient ring stacking effects due to the size of the single-ringed pyrimidine (Yin et al., 2012), and so it was unsurprising that intracellular c-di-UMP did not alter *ISG56* expression from background levels (Figure 12). Yet c-di-UMP too shows repression of *ISG56* when extracellular, indicating that the extracellular CDN receptor may not rely on ring stacking to bind CDNs.

Intracellular CDNs Repress the *IL6* Response:

While the proposed extracellular CDN receptor can signal to repress *ISG56* and the antiviral immune response, the pathway that leads to this repression is unknown. One potential pathway would involve NF-κB and IL-6; this pathway functions to activate antibacterial immunity (Mogensen, 2009; Kawai et al., 2008), which would make sense

as extracellular CDNs would be indicative of a bacterial infection. This antibacterial immune pathway could inhibit viral immunity to either regulate the antiviral immune response or to increase the effectiveness of the antibacterial immune response (Nakamura et al., 2011; Morens et al., 2008). After testing to see if CDNs could influence *IL6* expression, it was found that all intracellularly delivered CDNs strongly repress *IL6* (Figure 14). Some shared structural homology of the CDNs could be inducing an intracellular antiviral response that greatly represses the antibacterial IL-6 response. When delivered extracellularly, c-di-UMP, c-di-GMP, and to a lesser extent c-di-AMP all induce expression of *IL6*. This indicates that the extracellular CDN receptor is inducing some form of an IL-6 response, and the response is not dependent on nucleotide residues or ring stacking. However, cGAMP does not influence *IL6* beyond background levels (Figure 14), suggesting that the difference in linkage between the prokaryotic and eukaryotic CDNs may be a factor in activation of this antibacterial pathway.

This is not supported by kinetics data for the cGAMP induced *IL6* response; cGAMP can induce *IL6* expression around 8 hours after stimulation (Figure 15). These data conflict with Figure 7 because all samples in Figure 7 were taken at 24 hours after stimulation. The activity of intracellular cGAMP on *IL6* peaks at 8 hours after stimulation while the repressive action of extracellular cGAMP is lowest between 12 and 48 hours (Figure 15). The *IL6* response to both diverges within 2 hours, which differs from the 24 hours it took for the *ISG56* responses to diverge (Figure 5). Because the *IL6* responses diverge so quickly, it suggests that the repression is occurring through intrinsic cell signaling, as the necessary proteins would already be expressed and there would be little lag time in between stimulus and response. This shows that the viral and bacterial

immune responses to CDNs are signaling through separate pathways with different mechanisms of action. The complete mechanisms of signaling for both intracellular and extracellular CDNs is shown in Figure 16.

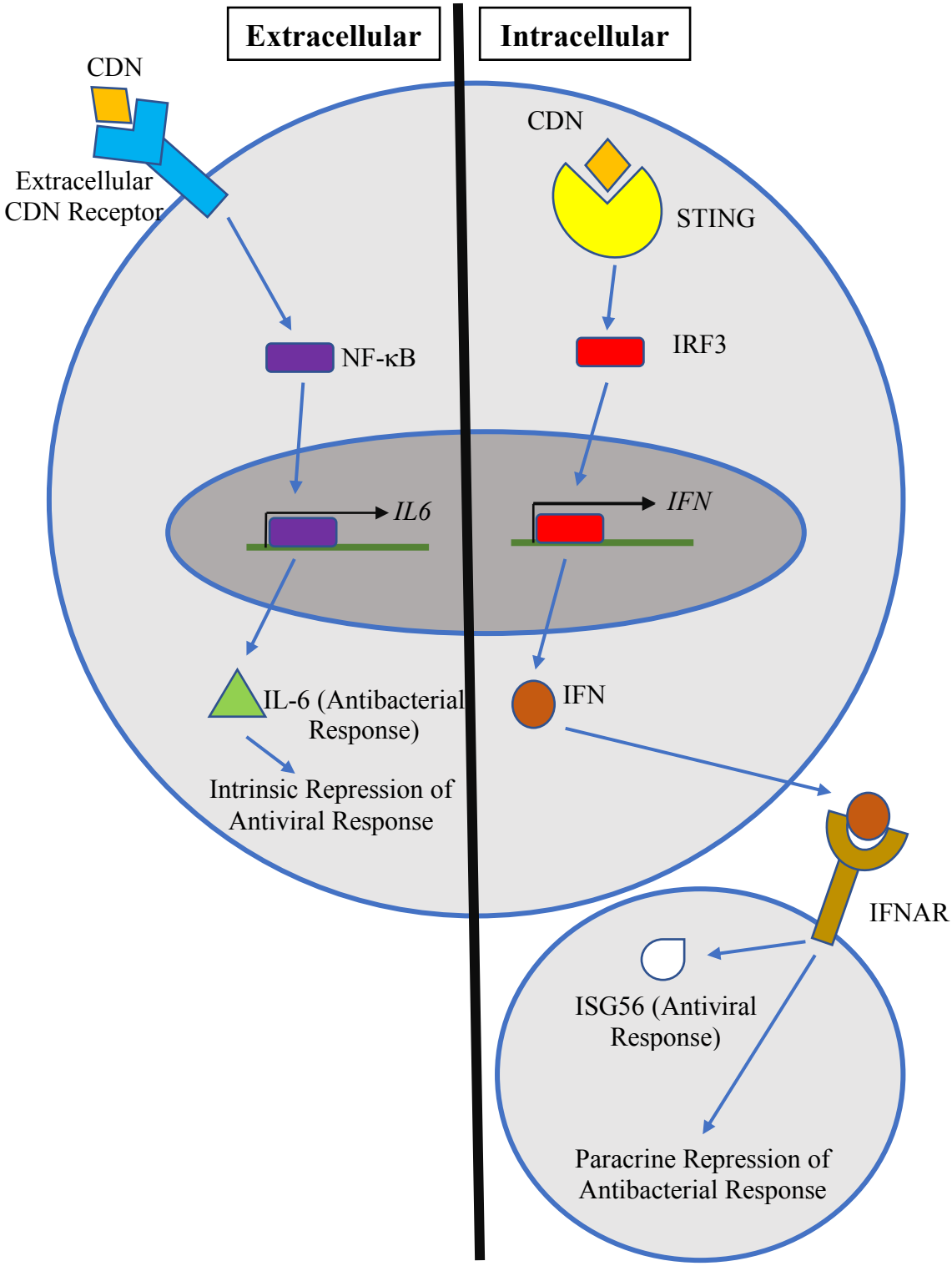


Figure 16: Model Pathways for Differential Activity of CDNs Dependent on Location: The activity of CDNs depends on delivery method and, subsequently, location. Extracellular CDNs activate an antibacterial immune response through a proposed extracellular CDN receptor and repress the antiviral response in a cell-intrinsic manner. Intracellular CDNs activate an antiviral immune response through STING and repress the antibacterial response in a paracrine manner.

Future Directions and Relevance:

The above findings indicate that cGAMP and CDN signaling is not as simple as described in the literature; CDNs signal differently depending on delivery method and, subsequently, localization. The data also support the existence of an extracellular CDN receptor that induces an antibacterial immune response. In light of these conclusions, there are many further experiments that could be conducted to expand on the findings described above. One future direction for this project could be to further scrutinize the delivery methods for cGAMP. While this study exclusively uses lipid transfection with Lipofectamine 2000 reagent to deliver cGAMP to the cytosol, other cytosolic deliver methods exist. For example, perfringolysin-O (PFO) is a bacterial toxin that will perforate the cell membrane (Wu et al., 2013). This method of delivery could be used to show that fusion of a micelle is not necessary for the observed immune response and that the cGAMP response is not Lipofectamine-dependent. This finding would further support the hypothesis of cGAMP-mediated regulation of the bacterial and viral immune responses, distancing the observation from Lipofectamine. However, PFO is bacterial in origin, and interfere with our ability to measure antiviral immune responses. Plus, while many teams use PFO to deliver cGAMP to cells, they only follow up with Western blots for IRF3 phosphorylation, not qPCR analysis (Gao et al., 2013). This could be due to PFO-related cell death, which would preclude analysis by qPCR and prevent easy differentiation of the bacterial and viral immune responses.

Also, it would be prudent to show that the delivery methods are delivering the cGAMP to the assumed locations. Fluorescently labeled cGAMP has been used previously (Hall et al., 2017), and this system could be used to image and show that lipid-

delivered cGAMP is intracellular and that cGAMP added to the media remains extracellular in microscopy experiments. These proposed experiments would support the location-dependent portions of the hypotheses described earlier.

More experiments could be conducted to determine what proteins are necessary for the cGAMP responses. By using a library of CRISPR-knockout cell lines, specific proteins may be found to be vital for cGAMP-induced repression of the viral immune response. For example, by removing NF-κB from cells, *ISG56* may no longer be repressed by extracellular cGAMP, showing that NF-κB is required for this repressive pathway. These first-identified proteins could then be used to highlight other proteins of interest that are likely involved in the repressive pathways. Eventually, this would identify the extracellular CDN receptor that has been proposed. It would also be important to show if this proposed receptor could distinguish between eukaryotic and prokaryotic cGAMP, as the two differ in structure only in their phosphodiester linkages (Davies et al., 2012). This could be tested by repeating experiments with prokaryotic cGAMP and measuring both the viral and bacterial immune responses.

By continuing to study the signaling capabilities of cGAMP, we can further understand the extent of this molecule to both induce and regulate immune responses. Sufficient study of cGAMP signaling could lead to the development of an *in vivo* model of bacterial and viral coinfections, as different delivery methods of cGAMP can induce one immune response or the other as necessary. This would necessitate studying the effects of cGAMP in an *in vivo* system, such as laboratory mice. Yet continued experimentation could reveal that members of either cGAMP pathway are effective drug targets. For example, one could potentially prevent immunopathology induced by IFN by

activating the repressive cGAMP pathway. This treatment could prove effective in cases of coinfection or severe cytokine storm. For example, in patients infected with influenza virus, the patient is made more susceptible to subsequent bacterial infections such as bacterial pneumonia, and bacterial comorbidities account for many flu-related deaths (Morens et al., 2008). By using a cGAMP treatment, we could initiate a patient's bacterial immune response to fight the more prominent comorbidity first. Developing these treatments could only follow further study of cGAMP signaling, so as to ensure that off target effects can be minimalized. Current findings suggest that cGAMP signaling is more nuanced than previously thought, and while much more experimentation is necessary to fully understand the multiple pathways cGAMP can signal through and how cross-talk is regulated, this system could prove experimentally and medically useful. As such, continued study of cGAMP and the other CDNs is suggested to understand the systems of immune activation and regulation.

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