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THE INTERFERON RESPONSE TO EXONGEOUS DNA AND DNA DAMAGE IS DEPENDENT ON DNA-PK ALONG WITH THE cGAS/STING PATHWAY

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by

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Abstract

Two pattern recognition receptors (PRRs) known as interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) bind to viral DNA and to an adaptor protein found on the endoplasmic reticulum called STING. This results in downstream signaling to produce interferon. However, it is not clear whether other classes of DNA-binding proteins, such as DNA damage kinases, also participate in the interferon response to viral infections. Additionally, it is unknown whether DNA-sensing PRRs like IFI16 and cGAS play a role in responding to damaged host DNA. In this study we show, by comparing interferon responses to exogenous nucleic acid in cells that were treated with a DNA-PK inhibitor and in untreated cells, that a DNA damage kinase known as DNA-dependent protein kinase (DNA-PK) is involved in PRR signaling. DNA-PK was shown to physically associate with IFI16 and STING. Additionally, we investigated the possibility of phosphorylation of cGAS, STING, and/or IFI16 by DNA-PK through bioinformatics using a protein database known as ScanSite 4.0. In order to see PRRs' role in DNA damage, treating *IFI16* knockout cell lines with DNA damage agents resulted in a decreased type I IFN response to DNA damage. These findings show DNA repair proteins such as DNA-PK playing a role in mediating an interferon response to viral infections as well as DNA-sensing PRR such as IFI16's role in responding to DNA damage. These findings have implications in understanding viral pathogenesis and developing new therapies for viral infections and cancer.

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Abbreviations

ATM: Ataxia Telangiectasia Mutated

ATR: ATM and RAD3-Related

cGAMP: Cyclic Guanosine Monophosphate-Adenosine Monophosphate

cGAS: Cyclic Guanosine Monophosphate-Adenosine Monophosphate Synthase

DAMP: Damage-Associated Molecular Pattern

DDR: DNA Damage Response

DNA-PK: DNA-Dependent Protein Kinase

dsDNA: double-stranded DNA

IFI16: Interferon gamma-Inducible Protein 16

IFN: Interferon

ISD: Interferon Stimulatory DNA

ISG: Interferon-Stimulated Gene

MAMP: Microbe-Associated Molecular Pattern

NHEJ: Non-Homologous End Joining

PMA: Phorbol Myristate Acetate

Poly dAdT: Poly(deoxyadenylic-deoxythymidylic) acid

Poly IC: Polyinosinic-polycytidylic acid

PRR: Pattern Recognition Receptor

STING: Stimulator of Interferon Genes

RPL37a: Ribosomal Protein L37a

VAC70: Vaccinia Virus 70

Introduction

Innate Immunity and PRR Signaling

The immune system prevents and fights infections by utilizing chemical and physical barriers, as well as specialized cells found in the blood and tissues throughout the body. In order to stop an infection from occurring, the immune system utilizes several layers of defense. A successful immune response can stop or slow the replication of a pathogen and can prevent or lessen the onset of disease in a host.

The first layer of immunity consists of barrier defenses, which provide an impermeable layer between the host and pathogen. Examples of such structures include skin and mucus that line the outside of body cavities (Niyonsaba et al. 2017). Chemical defenses also make up of first layer of immunity and consist of antimicrobial enzymes that are found in body secretions such as tears, saliva, and mucus (Niyonsaba et al., 2017). While these structures are mostly effective at keeping microorganisms from entering the host, there are ways that microbes can circumvent these defenses and proceed to infect the host. Thus, there are additional layers of immunity to stop pathogen replication if the pathogen has overcome these barrier defenses.

The next layer of immunity consists of the innate immune response. If a pathogen can penetrate the mechanical barriers and chemical defenses, then specialized innate immune cells can detect pathogens throughout the body and initiate an inflammatory response (Riera Romo et al., 2016). This is a fast-acting response to broad classes of pathogens that results in increased temperature and blood flow to the infected area and the recruitment of innate immune cells such as neutrophils and macrophages. These cells have the ability to engulf pathogens through a process known as phagocytosis. The main

purpose of the innate immune system is to quickly eliminate pathogens before numbers of the pathogen become too high for the body to control and also to activate the adaptive immune system.

Adaptive immune responses are pathogen specific. For example, an adaptive immune response against a human immunodeficiency virus (HIV) infection will not work against the flu (Farber et al., 2016). These responses require recognition of an antigen in order to activate adaptive immune cells such a B and T cells. Unlike other immune responses, adaptive immune responses have memory, which is critical for the immune system to recognize previously encountered pathogens and clear them before the onset of disease occurs (Farber et al., 2016). Proper coordination of these immune responses is needed in order for a pathogen to be recognized in a timely manner and cleared before further damage occurs to the host.

A critical characteristic of the immune system is its ability to distinguish between host and pathogen. The immune system has evolved mechanisms that allows for this distinction. For example, antibodies bind to antigens found on a specific pathogen, which allows for an immune response to be made selectively against a pathogen (Iwasaki and Medzhitov, 2015). This system is not perfect, however. Misregulation of these immune responses can lead to a spectrum of immunological disorders. If an immune response to a pathogen is too weak, there will be little to no protection against an infection as seen in patients with medical conditions that lead to an immunocompromised state such as acquired immunodeficiency syndrome (AIDS). Conversely, if an immune response is too strong or acts broadly against non-harmful antigens, then conditions related to autoimmunity or allergies arise (Taft and Bogunovic, 2018). Thus, there is a need to

understand how immune responses are regulated and the mechanisms by which the immune system recognizes a pathogen.

The innate immune system utilizes a mechanism known as pattern recognition in order to differentiate between host and pathogen. A class of proteins known as pattern recognition receptors (PRRs) bind to components that are well-conserved across broad classes of pathogens called microbe-associated molecular patterns (MAMPs) (Kumar et al., 2011). This characteristic of the innate immune system allows for fast responses to occur against a microbial infection. Recent studies have shown that PRRs can bind to host biomolecules, known as damage-associated molecular patterns (DAMPs), which are affiliated with cellular stress in order to initiate an innate immune response (Matzinger, 1994; Seong and Matzinger, 2004). Upon binding to MAMPs or DAMPs, PRRs signal through cellular pathways that lead to the production of an inflammatory response (Riera Romo et al., 2016). As mentioned previously, the innate immune response can vary in the type of inflammatory response that is elicited depending on the kind of pathogen that is present, such as distinguishing between bacteria and viruses (Mogenson, 2009). Structures found on bacteria are different in composition than structures found on viruses, and consequently will be recognized by different PRRs (Kumar et al., 2011). In the case of a viral infection, replication takes place inside of the host cell so PRRs that bind to viral MAMPs are intracellular receptors that bind to nucleic acid found in the virus's genome (Dempsey et al., 2015). However, host nucleic acids such as RNA and DNA are also present. Therefore, the ability for these PRRs to distinguish between viral and host nucleic acids is a critical hallmark of the immune system being able to discriminate between self and non-self.

The general mechanism of PRR activation during a viral infection starts with PRR binding to viral nucleic acid (Figure 1). This binding event leads to a signaling cascade that results in the activation of a transcription factor known as an interferon regulatory factor (IRF) that can control the transcription of genes coding for a specialized kind of inflammatory cytokine called interferon (IFN). These are signaling proteins that are made in response to an infection. Type I IFN, such as interferon-α and interferon-β are a subtype of inflammatory cytokine that is unique to viral infections and is secreted from virus-infected cells (Figure 1) (Mogensen, 2009). These secretions result in signaling in a paracrine and autocrine manner, where interferon binds to an extracellular receptor known as the interferon α/β receptor (IFNAR) on neighboring cells and on the infected cell itself (Figure 1). Binding of IFN by IFNAR leads to the activation of several cellular signaling pathways in the affected cell that results in the transcription of interferon-stimulated genes (ISGs). These genes code for many different kinds of proteins that lead to the overall restriction of viral replication within cell known as the antiviral state (Figure 1) (Mogensen, 2009). Whether the antiviral state and the interferon response are exclusively activated during a viral infection or can be induced by another stimulus such as a DAMP has yet to be elucidated. Since there are nucleic acid-binding PRRs, there is a possibility that the interferon response can be induced by other non-viral stimuli.

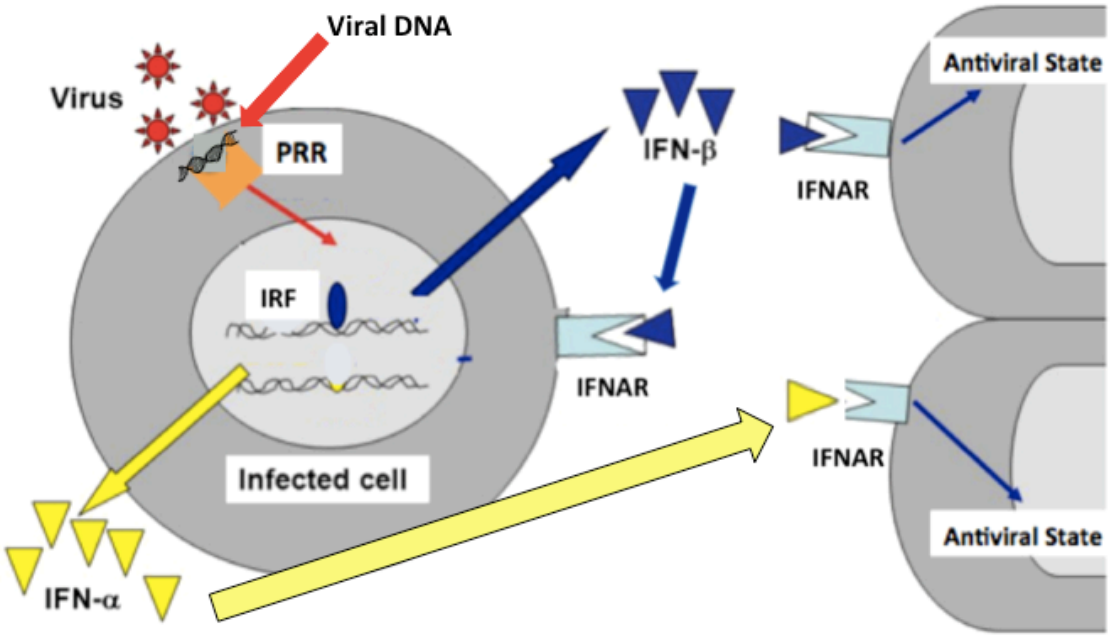

Figure 1: General Mechanism for PRR recognition
During the course of a viral infection, a PRR can bind to a viral component. This binding event results in the activation of a transcription factor known as IRF and the production of type I IFN such as IFN-alpha and IFN-beta, which can bind to IFNAR receptors on both neighboring cells and the infected cell itself. This binding event by type I IFN induces the antiviral state in cells due to transcriptional upregulation of ISGs.

ISGs code for proteins that have the ability to mediate several cellular responses due to their numerous functions. As a result, there are hundreds of ISGs that are transcribed during an IFN response (Der et al., 1998; Stark et al., 1998). ISGs function to stop viral replication by mechanisms such as by increasing host resistance to viral infection, and upregulating antiviral defenses (Stark et al., 1998). In particular, these genes code for intrinsic immune proteins, which are a family of proteins that function to stop viral replication. Specific ISGs can perform different functions to promote the antiviral state such as stopping protein synthesis, cleaving mRNA, or inhibiting cell cycle progression by inducing pro-apoptotic pathways (Mogensen, 2009). Due to the fact that viruses use host translational machinery to synthesize viral proteins, shutting down protein synthesis will inhibit further replication. Additionally, many viruses have RNA genomes; thus, antiviral defenses that degrade cytoplasmic RNA can also inhibit viral replication. While these mechanisms are beneficial for conferring resistance to viral replication in cells, they can also lead to a considerable amount of cellular stress because these responses lead to the halting of normal cellular metabolism and function. Since viral replication uses host machinery, the antiviral state also stops normal cell function like protein synthesis and cellular metabolism. These functions are needed for the overall survival of the cell, which means that the interferon response system can have dangerous effects on cells. If too much interferon is produced over a long period of time, damage to host tissue and immune dysfunction can occur (Lee-Kirsch et al., 2015). Thus, a successful interferon response must produce the right amount of interferon over the right length of time in order to confer protection. Additionally, the ability for PRRs to recognize the correct features that discern between self and non-self is critical. If these

sensors bind to host nucleic acid, this can lead to an unregulated interferon response and result in elevated interferon levels over a sustained period of time. It is evolutionarily advantageous for nucleic acid-sensing PRRs to be separate from host nucleic acid and to recognize structures that are unique to viruses.

Roles of PRRs

Additional downstream effects of PRR signaling include activation of tumor suppressors and apoptotic pathways in order to avoid further propagation of the virus. Even though cell cycle regulators have been classically associated with playing roles in apoptosis and regulating cell cycle entry, they have also been shown to be activated by type I IFN signaling and have additional immune functions, such as inducing antiviral defenses (Muñoz-Fontela et al., 2016). Regulating the cell cycle is a critical aspect of restricting viral replication as shown by the fact that DNA tumor viruses such as human papilloma virus and human adenovirus have evolved mechanisms to degrade both PRRs and tumor suppressors in order to replicate more efficiently (Lau et al., 2015). Viral proteins that can antagonize certain targets of these host pathways involved in cell cycle regulation show the importance of early detection of viral components in order to initiate a type I IFN response in order to prevent cell damage.

Another key effector function of PRR signaling is the activation of the inflammasome. The inflammasome is a complex of proteins that signals in order to induce a pro-inflammatory form of cell death called pyroptosis (Kerur et al., 2011). Inflammasomes contain PRRs that can detect MAMPs and DAMPs (Schroder and Tschopp, 2010). An activated inflammasome induces the production of a pro-inflammatory cytokine called IL-1β, and also initiates pyroptosis (Schroder and Tschopp,

2010). Pyroptosis results in the release of cellular components, which can act as DAMPs, into the extracellular space, and initiate PRR signaling in neighboring cells (Schroder and Tschopp, 2010). The activation of the inflammasome is a function resulting from detection of a virus is another way for cells to induce pro-inflammatory pathways separately from type I IFN signaling.

Elevated inflammatory responses as a result of PRR signaling are seen across a broad range of diseases, including in patients who are chronically infected with viruses such as HIV. Constant immune activation by type I IFN leads to ineffective control of viral replication due to long-term activation of immune cells, which results in cell exhaustion (Wherry et al., 2011). This is one of the reasons that patients with HIV progress to an immunosuppressed state known clinically as AIDS. Additionally, there is a specific class of genetic immunological disorders associated with the misregulation of antiviral responses known as the type I interferonopathies. In healthy patients, the type I IFN system is controlled by proteins that act as negative regulators and repress the type I IFN response in cells (Shannon et al., 2018). Since type I IFN can lead to major changes in cellular metabolism, transcription, translation, and immune activation, elevated levels of type I IFN over extended periods of time lead to immune dysfunction (Rodero and Crow, 2016). This loss of control of the type I interferon responses lead to disease states related to autoimmunity and autoinflammation (Rodero and Crow, 2016). One such group of patients have a substitution of an asparagine residue at position 154 for a serine (N154S) mutation in a protein called stimulator of interferon genes (STING) and a constitutive activation of IRF3 even in the absence of MAMPs; this results in immune cell dysfunction and inflammatory lung disease (Warner et al., 2017). Further

understanding of the signaling mechanisms of these type I IFN pathways could provide new insights into developing therapies for patients with chronic viral infections as well as autoimmune disorders.

The most common viral MAMP that a PRR senses is nucleic acid (Mogensen, 2009). There are many characterized RNA and DNA sensors found intracellularly that can recognize viral nucleic acid and lead to the activation of IRFs. However, many viruses, such as herpes simplex virus and HIV, have portions of their replication cycle that take place the nucleus; thus, hosts have evolved nuclear PRRs in order to detect these viruses (Kerur et al., 2011). Mechanisms that show how a DNA-sensing PRR distinguishes between host DNA and exogenous DNA from viruses are not well characterized.

IFI16 and the cGAS/STING Pathway

One PRR of interest is interferon-gamma inducible protein 16 (IFI16) because of its role in sensing DNA viruses and RNA-containing retroviruses such as HIV (Kerur et al., 2011; Jakobsen et al., 2013; Altfeld and Gale, 2015). IFI16 is a unique DNA-sensing PRR due to its subcellular localization in the nucleus and its ability to translocate to the cytoplasm in order to detect both nuclear and cytoplasmic DNA (Unterholzner et al., 2010; Veeranki et al., 2011). The subcellular localization of IFI16 is regulated by a nuclear localization sequence (NLS) (Briggs et al., 2001). IFI16 is a PRR classified in the family of AIM-like receptors (ALR) and contains a PYRIN signaling domain important for protein-protein interactions as well as two DNA-binding HIN-200 domains (Altfeld et al., 2015; Unterholzner et al., 2010). The HINb domain of IFI16 recognizes and binds MAMPs, such as double-stranded DNA (dsDNA) from herpes simplex virus (HSV) and

HIV provirus, in a non-sequence specific manner (Unterholzner et al., 2010; Jakobsen et al., 2013). This ability to bind to DNA in a non-sequence specific way is due to the fact that the HINb domain can bind to the backbone of DNA (Jakobsen et al., 2013). Previous studies have also shown that IFI16 binds preferentially to dsDNA rather than to single-stranded DNA, although secondary structures such as dsDNA hairpins that can form during reverse transcription of HIV can also be bound by IFI16 (Jakobsen et al., 2013). The PYRIN domain of IFI16 is crucial for the interaction of other proteins in the PRR signaling pathways, such as with STING, and with assembling proteins found in the inflammasome in order to induce pyroptosis (Monroe et al., 2014).

The precise mechanism of IFI16 signaling in the DNA sensing cGAS/STING-pathway is not well characterized; however, studies have shown that IFI16 is essential for activation of this pathway (Jønsson et al., 2017; Almine et al., 2017). In macrophages, upon binding to dsDNA, IFI16 interacts with another PRR known as cyclic GMP-AMP synthase (cGAS) and acts as a cofactor in order to promote the catalysis of a secondary messenger molecule known as cyclic GMP-AMP (cGAMP) (Shannon et al., 2018) (Figure 2). IFI16 and cGAMP bind to STING, an adaptor protein found on the endoplasmic reticulum, in order to promote dimerization of STING through protein-protein interactions found in the PYRIN domain of IFI16 (Jønsson et al., 2017). The STING homodimer, along with IFI16, can recruit TANK-binding kinase (TBK1), which initiates another signaling cascade that results in the phosphorylation of STING by TBK1 at a conserved amino acid found on the pLxIS motif on the STING protein (Liu et al., 2018). This complex then recruits a transcription factor known as interferon regulatory factor-3 (IRF3), which gets phosphorylated by TBK1. Phosphorylated IRF3 dimerizes

and enters the nucleus in order to upregulate the transcription of type I IFN (Figure 2).

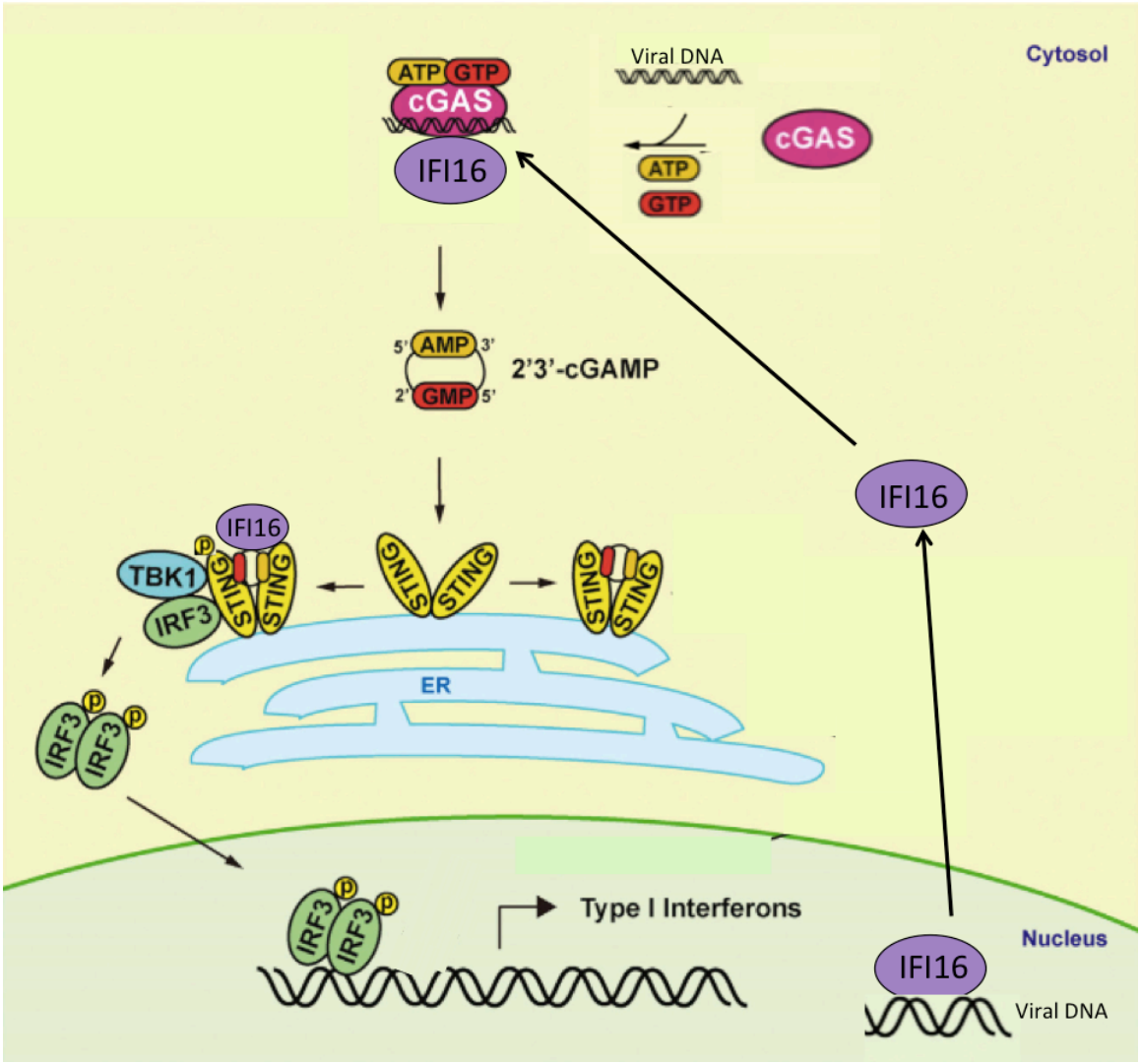

Figure 2: IFI16 and cGAS signaling in the cGAS/STING pathway
IFI16 translocates to the cytoplasm and aids in cGAS-DNA binding. cGAS catalyzes the synthesis of 2'3'-cGAMP, which binds and activates STING homodimerization. TBK1 is activated and phosphorylates IRF3, which shuttles to the nucleus and activates transcription of type I IFN genes. Adapted from Cai et al., 2014.

IFI16 can also signal in a pathway separate from the cGAS/STING pathway in order to induce an inflammasome-mediated pyroptosis (Kerur et al., 2011; Monroe et al., 2014). The inflammasome can activate two signaling molecules: caspase-1 and IL-1 β. The first is procaspase-1, which the inflammasome cleaves to form caspase-1. The formation of caspase-1 results in additional signaling, which eventually produces a second molecule that is an inflammatory cytokine called IL-1β (Altfeld and Gale, 2015). IFI16 can act as the initiator protein after binding dsDNA in order to activate the inflammasome and induce pyroptosis through an adaptor protein known as apoptosis-associated speck-like protein containing card (ASC) (Monroe et al., 2014; Altfeld and Gale, 2015).

How signaling via IFI16 in immune cells leads to the transcriptional changes that results in the production of type I IFN as opposed to cellular changes that causes pyroptosis is not well known. For example, an adaptive immune cell known as a $CD4^+$ T cell can be infected with HIV. IFI16 signaling in response to HIV infection can either lead to an antiviral response or pyroptosis (Monroe et al., 2014). In a resting T cell, IFI16 activates the inflammasome and the cell undergoes pyroptosis. An HIV infection that occurs in an activated T cell results in type I IFN production (Doitsh et al., 2014; Monroe et al., 2014). Thus, this leaves an open question if other cellular proteins interact with IFI16 and help direct these cellular outcomes during IFI16 signaling.

Another PRR of interest is cyclic GMP-AMP synthase (cGAS). cGAS senses cytosolic DNA and signals through STING (Wu et al., 2013). In the presence of dsDNA, the nucleotidyltransferase domain of cGAS catalyzes the synthesis of the second messenger, a cyclic dinucleotide, cGAMP, and leads to transcription of type I IFN genes

in a STING-dependent manner as part of the same pathway as IFI16 (Figure 2) (Wu et al., 2013; Altfeld and Gale, 2015). Similar to IFI16, cGAS also detects dsDNA in a length-dependent, non-sequence specific manner (Shu et al., 2014). cGAS has been shown to bind optimally to dsDNA that is more than 20 base-pairs (bp) but less than 1 kilobase in length (Shu et al., 2014). cGAS binds DNA non-specifically due to the fact that it binds with positively charged amino acid residues to the negatively charged phosphodiester backbone of DNA through electrostatic interactions. This binding event induces a conformational change to cGAS that results in its active, catalytic form that synthesizes cGAMP (Shu et al., 2014).

Though initially thought to function independently of IFI16, cGAS has a low binding affinity for DNA (K_d of 20μM) (Jønsson et al., 2017). This suggested the possibility of other signaling proteins interacting with cGAS as cofactors in order to achieve complete activation (Yoh et al., 2015; Jønsson et al., 2017; Shannon et al., 2018). This may explain why IFI16, another DNA-sensing PRR, can bind to cGAS and participate in the type I IFN response in STING-dependent manner (Figure 2). IFI16 has been shown to increase the half-life of cGAMP by promoting stabilization of this molecule in previous studies (Orzalli et al., 2015). In addition to other PRRs acting as cofactors for cGAS, another possible way of full activation during signaling is post-translational modifications of cGAS, IFI16, or other members of this pathway, but these possible modifications are just beginning to be explored (Du and Chen, 2018).

There are several limitations to describing cGAS exclusively as a cytosolic DNA sensor. One key question regarding cGAS is its ability to distinguish between host and viral DNA, since DNA binding is not sequence specific (Shu et al., 2014). Previous

studies suggest that in order to prevent reactivity to host DNA in the nucleus, cGAS's subcellular localization is exclusively cytosolic (Stetson and Medzitov, 2006; Volkman et al., 2018). Yet cGAS has been described to be a critical sensor for detecting retroviruses, whose DNA intermediates are synthesized in the nucleus as well as DNA viruses whose genomes are also replicated in the nucleus (Gao et al., 2013; Ma et al., 2015; Rasaiyaah et al., 2013). Current hypotheses describe how these viral replication events cause the leaking of host DNA from the nucleus to act as a DAMP for cGAS to detect DNA in the cytosol, and that cGAS can detect these viruses during mitosis due the disassembly of the nuclear envelope (Volkman et al., 2018). However, cGAS has the ability to associate with mitotic chromosomes. In fact, the majority of expressed cGAS is tightly tethered in the nucleus in order to prevent binding to intact chromosomal DNA (Yang et al., 2017; Volkman et al., 2018). These findings further suggest the possibility of additional interactions between cGAS and other nuclear DNA binding proteins that can post-translationally modify cGAS or otherwise regulate its activation state and its ability to distinguish between chromatin and exogenous DNA.

cGAS is a critical host target for many kinds of viruses in order to circumvent the innate immune response. In a normally functioning cell, host DNA is localized to the nucleus and mitochondria. DNA sensed in the cytoplasm acts a MAMP or DAMP since several DNA viruses replicate in the cytoplasm; thus, there are PRRs that detect viral DNA as a MAMP in the cytoplasm (Gao et al., 2013). This pathway is so important for responding to viral infections that DNA viruses such as human papillomavirus and human adenovirus 5 have evolved viral proteins that act as antagonists to target cGAS and STING for proteolytic destruction (Lau et al., 2015). This DNA sensing pathway has

been shown to be essential for detecting retroviruses and other RNA viruses as well. HIV, a retrovirus, reverse transcribes its RNA genome by synthesizing dsDNA, which can be detected by cGAS (Gao et al., 2013). cGAS can detect mitochondrial DNA as a DAMP during a dengue virus infection (DENV) (Aguirre et al., 2017). DENV has an RNA genome, but its replication can activate the cGAS/STING pathway by inducing mitochondrial stress, which results in the release of host mitochondrial DNA in the cytoplasm to be detected by cGAS. This mitochondrial stress is caused by changes in metabolism caused by viral replication. Therefore, understanding cGAS signaling in the scope of a viral infection is critical given that is a key sensor for DNA, RNA, and retroviral infections.

cGAS and IFI16 have other functions outside of antiviral immunity. These PRRs are also involved in pathways that promote cell cycle regulation and cellular senescence (Johnstone et al., 2000; Yang et al., 2017; Liu et al., 2018). This shows that cGAS and IFI16 could have other interactions not associated with innate immune signaling. In a previous study, *cGAS* knockout cells had decreased cellular senescence, which is a state where the cell no longer divides (Yang et al., 2017). This deletion of *cGAS* also increased the susceptibility to cellular transformation, which predisposes cells to oncogenesis (Yang et al., 2017). Interestingly, cancer patients who had high expression levels of *cGAS* had longer survival times compared to patients who had lower expression levels (Yang et al., 2017; Shannon et al., 2018). Therefore, cGAS's role in non-infectious inflammatory signaling has implications in cancer, aging, and autoimmune diseases and could provide an additional therapeutic target in developing treatments for patients with inflammatory diseases (Yang et al., 2017; Li et al., 2018). Cellular processes such as

DNA damage, telomere shortening and oxidative stress are all causes associated with cancer and aging (Yang et al., 2017). All of these cellular processes result in the production of DNA that acts as a DAMP that cytosolic DNA sensors such as cGAS can detect to an initiate an antitumor response.

Before IFI16 was characterized as a PRR involved in antiviral responses, it was described as a transcriptional repressor needed for the activation of tumor suppressor proteins p53 and BRCA1 (Johnstone et al., 2000). IFI16 is involved in the response to genomic stress as a result of DNA damage, and signals in pathways that regulate cell cycle in order to induce the apoptotic pathway mediated by p53 (Aglipay et al., 2003). Apoptosis is a non-inflammatory form of cell death that does not result in the release of DAMPs, unlike pyroptosis (Barber, 2001). IFI16 also has a role in promoting permanent cell cycle arrest called cellular senescence by inducing the production of type I IFN (Xin et al., 2004). Given that type I IFNs have been shown to have effector functions such as regulating proliferation and promote permanent cell cycle arrest (Johnstone et al., 2000), there may be a positive feedback mechanism where IFI16 can detect nuclear DNA and initiate signaling that leads to the production of type I IFN. Since type I IFN production also increases IFI16 expression, type I IFN that binds to the receptor of that same cell can stimulate production of more IFI16 and amplify IFI16 signaling to further drive the cell into senescence (Xin et al., 2004).

IFI16's function in promoting cellular senescence and regulating expression of other cell cycle proteins has implications in understanding its role in tumorigenesis. Studies have shown that cells missing IFI16 are predisposed to uncontrolled cell proliferation that is telomerase mediated (Xin et al., 2004). Similar to cGAS, patients

with various types of cancers showed reduced expression of IFI16 (Fujiuchi et al., 2004; Shannon et al., 2018).

Other dysfunctions associated with the IFI16, cGAS, and STING have implications in misregulated host DNA damage responses, which can lead to suppressed antitumor immunity and the development of cancer. This suggests that there is an inflammatory component to cancer that is mediated by DNA sensing PRRs (Li and Chen, 2018). Studies have shown that patients with chronic inflammation are susceptible to cancer, and that several kinds of innate immune cells, such as macrophages and neutrophils, are present within the tumor microenvironment in order to modulate these inflammatory responses (Grivennikov et al., 2010). Thus, there is a possibility that patients with cancer have genetic mutations that result in deregulation of type I interferon responses, which leads to immunosuppression and a decreased response to DNA damage. Additionally, DNA tumor viruses such as HPV and human adenovirus have viral oncoproteins that inhibit STING signaling (Lau et al., 2015). There is an evolutionary advantage for these viruses to inhibit IFN production in order to avoid immune surveillance. This inhibitory effects of these viral oncoproteins also predispose infected cells to cellular transformation since cGAS and IFI16 also play a role in promoting cellular senescence and also directly regulating cell cycle (Choubey and Panchanathan, 2016; Yang et al., 2017). Understanding links between the DNA damage response (DDR) and PRR signaling can provide new targets for developing anticancer therapies.

Therefore, in addition to understanding how IFI16 and cGAS work to restrict viral replication, there is potential in further understanding their role in responding to DNA damage and promoting antitumor immunity.

The DNA Damage Response and Innate Immunity

While it has been shown that there are PRRs that can bind to microbial DNA (Riera Romo et al., 2016), it is not clear whether these DNA sensors also have the ability to bind to host DNA as well. Conversely, it is not well known whether other classes of DNA binding proteins, not classified as PRRs, have functions in immune signaling. One such class of proteins is DNA repair proteins that can bind to damaged host DNA.

In order to maintain genomic integrity, cells have a DDR system that recognizes DNA lesions, promotes repair, and stops DNA replication and cell cycle progression (Blackford and Jackson, 2017). Damage to DNA may be caused by a number of factors including mis-replication by the DNA replication machinery, chemical assault from reactive oxygen species, and ionizing radiation (Nakad et al., 2016). Cells utilize the DDR in order to detect different kinds of lesions to DNA including single-stranded breaks, double stranded breaks, and incorrect base pairs (Nakad et al., 2016). The main function of the DDR is to stop cell cycle progression and repair the lesions in order to prevent propagation of the damage into daughter cells. Depending on the severity of the damage, pathways may induce DNA repair or apoptosis activation if the damage is beyond repair.

The most severe form of DNA damage is a double-stranded break. This kind of lesion is especially harmful because if it is left unrepaired, double-stranded breaks can result in loss of nucleotide sequence, translocation of chromosomes, or genetic rearrangement caused by the uncontrolled recombination of DNA (Nakad et al., 2016). However, double-stranded breaks are sometimes caused intentionally during some cellular processes (Alt et al., 2013). These cellular processes include homologous

recombination during meiosis and V(D)J recombination during lymphocyte development, which induce double-stranded breaks as intermediates. V(D)J recombination produces small exogenous DNAs; whether nuclear, DNA-sensing PRRs interact with these excised host DNA is not well known. Further characterizing links between the DDR and DNA sensing could provide insight into the mechanisms in how PRR signaling is regulated during these cellular processes.

One mechanism of DDR used to repair double-stranded breaks is non-homologous end joining (NHEJ). This pathway results in a loss of nucleotides as it does not utilize a DNA template such as a homologous chromosome for repair (Kim et al., 2013). When a double-stranded break occurs, two DNA-binding proteins called the Ku70/Ku80 complex can bind to the DSB ends. These proteins recruit the catalytic subunit of this complex known as DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase (Kim et al., 2013). DNA-PK can autophosphorylate as well as phosphorylating additional proteins, such Artemis and protein complex XRCC4-DNA ligase IV, that are needed to re-ligate and repair the damaged DNA. Since DDR proteins such as DNA-PK work to repair double-stranded breaks across the genome, these kinds of DNA-binding proteins also have the property of binding to DNA in a non-sequence specific manner, as seen with cGAS and IFI16.

Another function of the DDR to a double-stranded break is to induce cell cycle arrest. Additional DNA damage kinases structurally analogous to DNA-PK are Ataxia Telangiectasia Mutated (ATM), and ATM and RAD3-Related (ATR) (Blackford and Jackson, 2017); these kinases detect double-stranded breaks and activate the ATR-Chk1 checkpoint pathway, which prevents the cell from entering S phase. ATM and ATR play

a role in activating tumor suppressors, such as p53, which leads to the expression of genes that can further repair DNA damage and inhibit cell cycle progression (Kastan et al., 1992). Additional studies need to be done to determine how DNA damage kinases such as DNA-PK, ATM, and ATR can discriminate between host DNA and exogenous DNA.

While DNA damage kinases like DNA-PK may play a role in antiviral immunity, there is evidence that demonstrates that PRRs play a role in responding to host DNA damage. Given that PRRs have the ability to bind to DAMPs, another possible function for PRRs is sensing damage found in genomic DNA is another possible function for PRRs. Links between type I IFN responses and DNA damage have been shown, and in particular, inducing DNA damage with various chemicals resulted in activation of IRF3, a transcription factor that controls expression of type I IFN and ISGs (Kim et al., 1999). While it is not clear whether PRRs have the ability to bind to damaged genomic DNA in the nucleus, cGAS has been shown to bind to mitochondrial and nuclear DNA that has leaked out into the cytoplasm (Aguirre et al., 2017). DNA damage agents like cisplatin, etoposide, and mitomycin C damage host DNA and cause the DNA to leak into the cytoplasm; thus, there is the possibility for cytosolic PRRs such as cGAS to detect this DNA (Yang et al., 2017). This ability may be evolutionarily advantageous in restricting viral replication since several retroviruses induce double stranded breaks in genomic DNA during their replication cycle (Cooper et al., 2013). Additionally, DNA viruses like HSV and adenovirus seem to inhibit a DNA damage response by utilizing proteins that act to inhibit DNA repair proteins (Evans and Hearing, 2005). Yet, the DNA sensors that

can mediate this type I IFN response to DNA damage and the mechanism by which they induce this response are not well characterized.

DNA damage sensors that detect double stranded breaks in DNA also have functions in antiviral responses (Nakad et al., 2016). Since DNA-PK is also a DNA-binding protein found in the nucleus, this protein is particularly of interest when examining links between DNA damage and innate immunity. DNA-PK has the ability to induce cell death by interacting with AIM2, a cytosolic PRR structurally analogous to IFI16 (Wilson et al., 2015). Interestingly, retroviruses such as HIV, have an integration step in their replication cycle, which involves inducing a double-stranded break in host DNA in order for strand transfer to occur in the host genome (Cooper et al. 2013). This double-stranded lesion during HIV integration seems to induce DNA-PK activation and result in apoptosis (Cooper et al., 2013). However, studies have shown that depending on the secondary structure of the DNA intermediate of retroviruses, the NHEJ pathway can also promote cell survival instead of apoptosis (Li et al., 2001; Monroe et al., 2014). Thus, there may be a possible role for DNA-PK in restricting viral replication by sensing exogenous viral DNA; however, it is currently unknown how DNA-PK has the ability to distinguish between host and viral DNA.

Experimental Goals

The main objective of this study were to see if DNA-PK has a function in PRR signaling and producing type I IFN responses. The first question addressed was whether DNA-PK alters the type I IFN response to exogenous DNA (Figure 3). Since previous studies have shown that DNA-PK interacts with a DNA-sensing PRR structurally similar to IFI16, known as AIM2 (Wilson et al., 2015), we hypothesized that DNA-PK and IFI16

could interact upon detection of viral or damaged host DNA, which would result in increased transcription of type I IFN and ISGs. Thus, the possibility of DNA-PK binding in complex with IFI16, cGAS, and STING was tested. DNA-PK also has the ability to translocate to the cytoplasm, so we also examined the possibility of DNA-PK to phosphorylate IFI16, cGAS, and/or STING. This study examined possible interactions between DNA-PK and IFI16, cGAS, and STING, and proposes a mechanism by which detection of exogenous DNA can also induce a DDR mediated by DNA-PK. We hypothesized that DNA-PK is needed for an interferon response and has the ability to phosphorylate IFI16, cGAS, or STING in order to promote activation of the pathway during a viral infection. This function of DNA-PK provides an additional link beyond the DNA repair pathway that DNA-PK has classically been associated with and PRR signaling. This role of DNA-PK in regulating immune is a novel function. Further understanding of this new avenue of signaling could provide a better understanding of mechanisms related to viral infections and DNA damage responses.

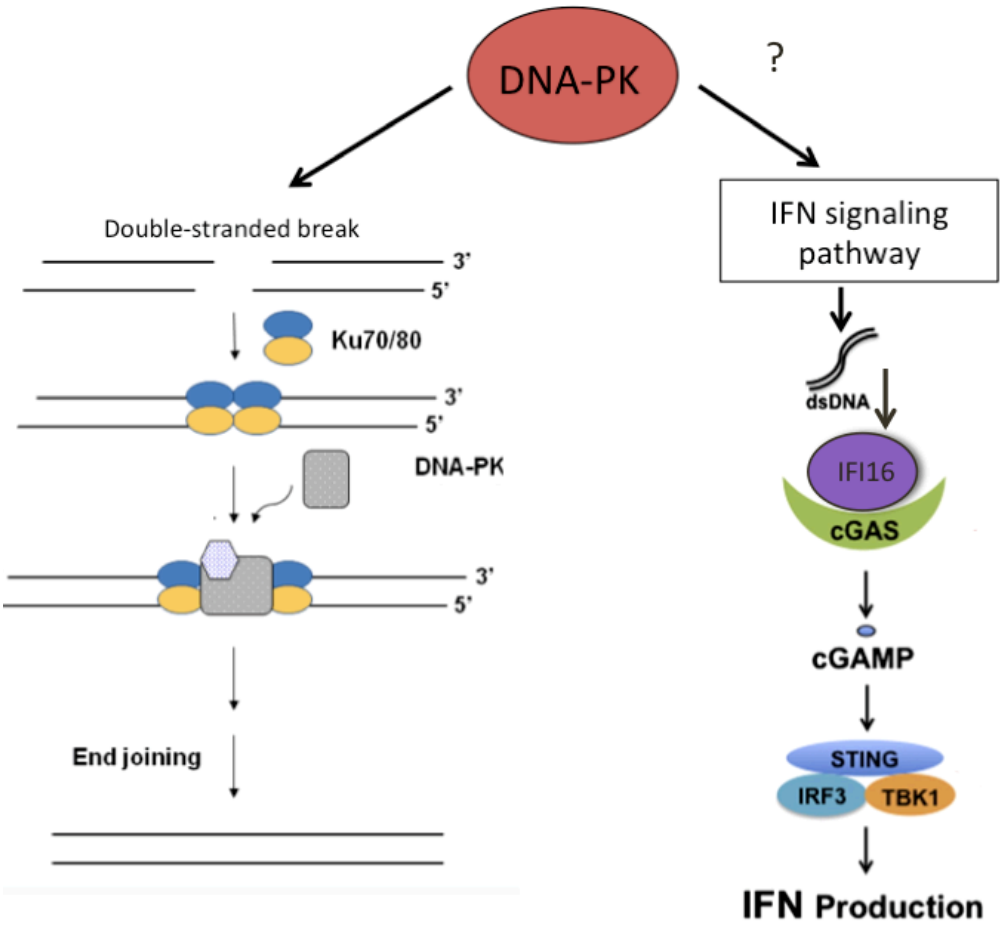

Figure 3: Proposed Model of Innate Immune Activation by DNA-PK
DNA-PK participates in the NHEJ pathway. When a double stranded break occurs, Ku 70/80 binds the DNA and recruits DNA-PK, which phosphorylates itself and other proteins such as Artemis and DNA Lig4/XRCC4 in order to repair and religate the lesion (not pictured). We hypothesize that DNA-PK can interact with components of the cGAS/STING pathway in order to regulate a type I IFN response. Adapted from Kim et al. 2013 and Liang et al. 2014.

In order to test these hypotheses, the role of DNA-PK in the type I IFN response was evaluated. First, DNA-PK activity was inhibited in cells after transfection of exogenous nucleic acid and the type I IFN response was measured using quantitative polymerase chain reaction (qPCR). Next, physical interactions between DNA-PK and IFI16, cGAS, and STING were examined through co-immunoprecipitation (co-IP) and immunoblotting. The mechanism by which DNA-PK interacts with IFI16, cGAS and STING was predicted to be by phosphorylating these proteins since DNA-PK is a kinase. This idea was tested by looking at possible phosphorylation sites on cGAS, IFI16, and STING by using a phosphorylation site predictor ScanSite 4.0.

Another objective of this study was to examine IFI16, cGAS, and STING's role in the DDR and cell cycle regulation. Damage to host DNA could result in activation of IFI16 and cGAS due to their role in tumorigenesis and cell cycle progression (Johnstone et al., 2000; Yang et al., 2017). This stems from the hypothesis that DNA sensing, and more broadly PRR signaling, does not exclusively distinguish between host and microbe, but rather between a cell's healthy and stressed state by detecting danger signals such as damaged DNA (Matzinger, 1994). Characterizing cGAS, IFI16, and STING's role in DNA damage provides another function for this signaling pathway outside of antiviral immunity. A clearer understanding of the interferon response to DNA damage will elucidate new mechanisms by which cells promote antitumor immunity in the case of DNA damage. In order to see if cGAS, IFI16, and STING are involved in the interferon response to DNA damage, knockout cell lines missing either cGAS, IFI16 or STING, were treated with different DNA damage agents, and the type I interferon was measured using qPCR. These responses in the knockout cells were compared to wildtype cells.

Finally, in order to see if IFI16, cGAS, and STING mediate changes in cell senescence in response to DNA damage and if appropriate apoptosis responses are dependent on these PRRs, knockout cell lines missing these proteins were treated with DNA damage agents. The resulting changes in cell senescence were measured by utilizing a colorimetric assay.

Methods

Cell Culture

The THP-1 cell line is an immortal monocytic cell line that can be differentiated into macrophage-like cells through the usage of a chemical called phorbol 12-myristate 13-acetate (PMA) (Yoh et al., 2015). This cell type has been used as a model for studying PRR signaling due to its role as an innate immune cell and its ability to produce inflammatory cytokines such as type I IFN (Eming et al., 2007). THP-1 cells, bought from ATCC, were maintained in R10 medium containing RPMI media, 10% fetal bovine serum (FBS), β-mercaptoethanol (0.1% concentration), penicillin/streptomycin/ glutamine (PSG) (1% concentration), non-essential amino acids (NEAA) (1% concentration), sodium pyruvate (5mL/500mL) and normocin (0.1% concentration). RPMI medium, FBS, PSG, β-mercaptoethanol, NEAA, and sodium pyruvate were all obtained from Invitrogen, while normocin came from Invivogen.

Cells were counted and 2 x 10^6 cells were added to individual wells of a 6-well plate in 3 mL of R10 medium; 5 ng/mL of PMA (Sigma) was added to each well. Cells were incubated for 72 hours before further stimulation at 37°C in a 5% CO_2 humidified cell culture incubator. The Paludan lab at the University of Aarhuis provided *cGAS*, *IFI16*, and *STING* knockout THP-1 cell lines, which were also maintained in R10 media (Luecke et al., 2017).

Cell Transfection and Stimulation

In order to add the DNA ligand for cGAS and IFI16 to cells, we utilized transfection to introduce naked DNA into the cytoplasm (Figure 4). Cells were transfected with Lipofectamine® 2000 (Invitrogen) according to the manufacturer's

instructions. Specifically, 4 micrograms of nucleic acid were diluted in a total of 250 µL of Opti-MEM® medium (Invitrogen). This was mixed with 10 µL of Lipofectamine® 2000 diluted in 240 µL of Opti-MEM® medium (Yoh et al., 2015). A "mock" transfection was done with Lipofectamine® 2000 using 240 µL of Opti-MEM medium mixed with 10 µL of Lipofectamine® 2000; an additional 250 µL of Opti-MEM was added to the Lipofectamine® 2000 -OptiMEM mixture for a volume total of 500 µL. These liposome-containing mixtures were incubated between 20 minutes and 6 hours before the transfection complexes were added to cells for stimulation (Figure 4).

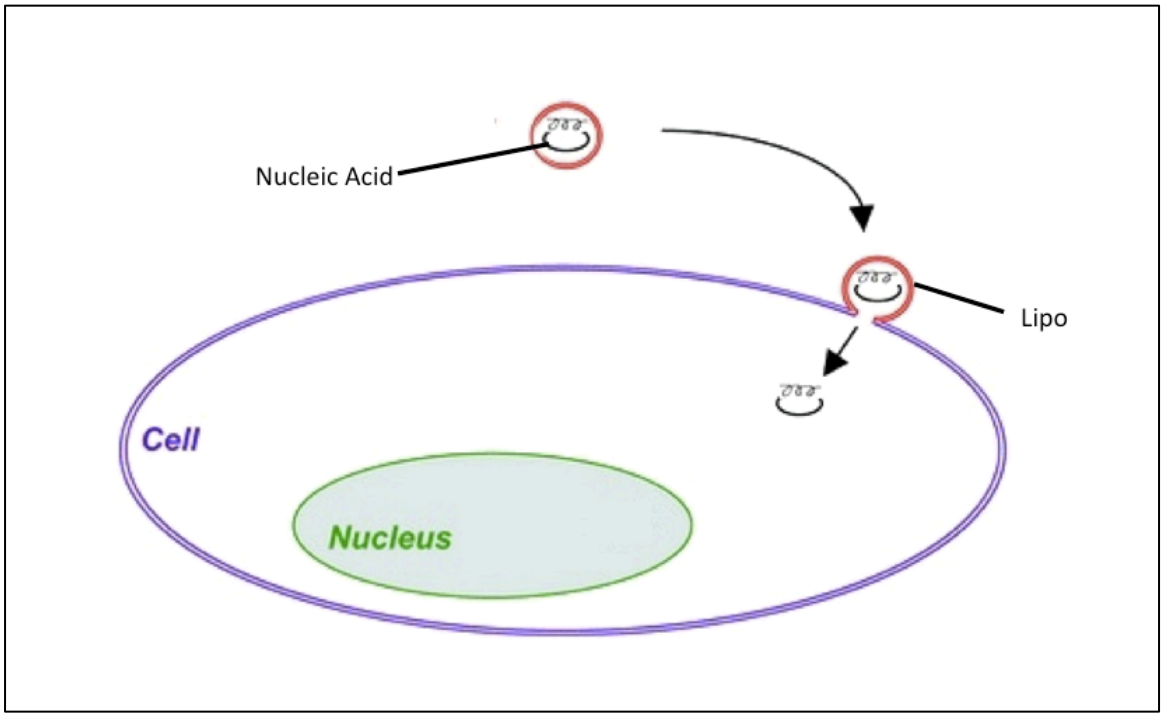

Figure 4: Transfection of Nucleic Acid into the Cytoplasm
Liposomes ("Lipo"), containing either the nucleic acid RNA or DNA, fuse with the plasma membrane of THP-1 cells after being added to the cells' media. This allows for delivery of RNA or DNA to the cytoplasm of these cells.

In order to see if DNA-PK is needed for an interferon response to exogenous nucleic acid, DNA-PK inhibitor NU7026 (Calbiochem) was added to the cells during at the same time as transfection of viral exogenous nucleic acid. NU7026 is a water insoluble solid that can be dissolved in dimethylsulfoxide (DMSO), and acts as a competitive inhibitor for the kinase domain of DNA-PK. It is also highly selective for DNA-PK over other related kinases with an IC_{50} = 0.23 μM (Willmore et al., 2004). Prior to transfection, NU7026 was dissolved in DMSO at a stock concentration of 10 mM and diluted 1:10 in 1X phosphate buffered solution (PBS) (Invitrogen). The diluted NU7026 was added, according to the manufacturer's instructions, so that the final concentration in the well was 20 μM. For cells that did not receive the inhibitor, 60 μL of DMSO diluted 1:10 in 1X PBS was added to the wells at the same volume of NU7026. All of NU7026 or DMSO-treated cells were co-stimulated with nucleic acid and then incubated overnight.

The stimuli used for PRR activation were poly dAdT, poly IC, VAC70, and ISD. Poly dAdT (from Invivogen) is a synthetic form of linear, dsDNA with repeats of adenine and thymine nucleotides. This ligand can induce type I IFN independently of cGAS and IFI16 since it can be transcribed into RNA and is detected by RNA-sensing PRR retinoic acid-inducible gene I (RIG-I) (Ablasser et al., 2009). Poly IC is a synthetic MAMP that consists of double stranded RNA that binds to RIG-I and leads to the production of type I interferon (Matsumoto et al. 2002). VAC70 (Invivogen) is a 70-base pair (bp) linear double-stranded DNA segment derived from the Vaccinia virus genome which is known to induce type I IFN in a STING-dependent manner (Unterholzner et al., 2010; Marcus et al., 2018). ISD is another MAMP that consists of a 45-bp sequence derived from *Listeria*

monocytogenes genome that induces a type I interferon response in a STING-dependent manner (Stetson and Medzhitov, 2006).

The following DNA damage agents were used: mitomycin C, cisplatin, and doxorubicin. The final concentrations of the DNA damage agents were as follows: cisplatin (Sigma) 100 μM, doxorubicin (Selleckchem) and 20 μg/mL, mitomycin C (Selleckchem). Ahn and colleagues (2014) used the concentration given for cisplatin. Brzostek-Racine and colleagues (2011) used the concentrations given for doxorubicin and mitomycin C. These compounds were directly added to the cells' media after cells were matured in PMA. A mock transfection was done as a negative control. All treated cells were incubated at 37^{o} C overnight before RNA extraction.

RNA Extraction and cDNA Synthesis

RNA from treated THP-1 cells was isolated using Zymo Research Quick-RNA MiniPrep Kit (Zymo Research) and instructions from manufacturer. After RNA extraction, the concentration of RNA in each sample was determined by using a ThermoFisher NanoDrop^{TM} 2000. cDNA was synthesized using the ProtoScript II First Strand cDNA synthesis kit and protocol within (New England Biolabs). Samples were stored at -20^{o}C and used for qPCR.

qPCR

In order to quantify the innate immune response in THP-1, mRNA levels of *ISG56* were measured through quantitative polymerase chain reaction (qPCR). *ISG56* is a gene that codes for an ISG that is transcribed in response to type I IFN (McNab et al., 2015). Levels of a housekeeping gene, *RPL37A* ,were also measured; *RPL37A* is transcribed at consistent levels across each of the THP-1 cell conditions (Maess et al.,

2010). Each qPCR sample consisted of 10 µL of iTaq Universal SYBR Green SuperMix which contained DNA polymerase, deoxynucleotidetriphosphates (dNTPs), SYBR Green dye and reference dye ROX (BioRad) as well as 1 µL of forward primer, 1 µL of reverse primer, 1 µL of cDNA sample, and 7 µL of water. Primers were purchased from IDT. The primer sequences for *ISG56* and *RPL37A* are listed below:

Table 1: Primers used for qPCR

Gene	**Primer Sequence**
ISG56 Forward	5'-CCTCCTTGGGTTCGTCTACA-3'
ISG56 Reverse	5'-GGCTGATATCTGGGTGCCTA-3'
RPL37A Forward	5'-ATTGAAATCAGCCAGCACGC-3'
RPL37A Reverse	5'-AGGAACCACAGTGCCAGATCC-3'

SYBR green dye in the PCR mixture binds to double-stranded DNA and fluoresces, which is read by the thermal cycler used to run qPCR, a Bio-Rad CFX96 Real-Time PCR Machine. There was an initial denaturation step that occurred at 95^{o}C for 3 minutes. Following this, a total of 40 cycles were completed that consisted of denaturation that lasted 10 seconds at 65^{o}C, an annealing and elongation step that lasted that lasted for 30 seconds. After each cycle of completed steps, fluorescent dye known as SYBR green was read (Figure 5). These steps were repeated for a total of 40 separate cycles. In order to produce melt curves after the completion of 40 cycles, temperature increased in 0.5^{o}C increments ranging from 65^{o}C to 95^{o}C.

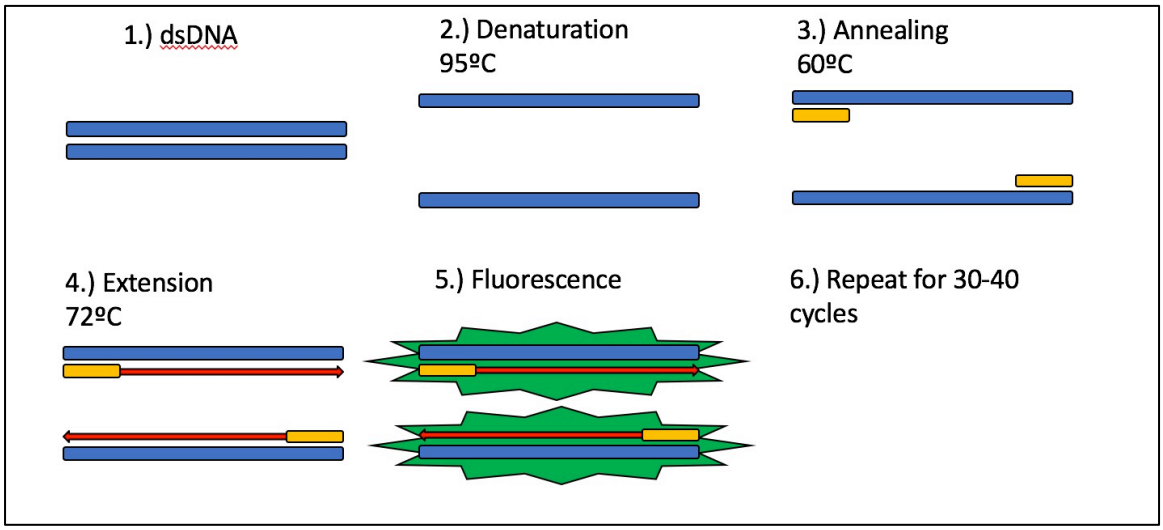

Figure 5: Schematic of qPCR
This shows the general steps involved in qPCR in order to measure mRNA levels. Double-stranded cDNA is denatured by high temperature, which allows for primers specific for genes of interest to anneal to the complementary DNA sequence. Levels of cDNA can be quantified when the SYBR Green dye binds to dsDNA and fluoresces. Figure adapted from Dorrity, 2018.

The resulting threshold values (Cq) were determined the Bio-Rad CFX96 Real-Time PCR machine were converted to fold change for both *ISG56* and *RPL37A.* We calculated the change (Δ) in Cq value for each sample using the following equation:

$$\Delta Cq = Cq \text{ of } ISG56 - Cq \text{ of } RPL37A$$

The resulting ΔCq values for the negative control, which was the mock condition, were averaged and used to calculate the ΔΔCq value by utilizing the following equation:

$$\Delta\Delta Cq = \Delta Cq \text{ of experimental sample} - AVERAGE\ \Delta Cq \text{ negative control}$$

From this ΔΔCq for each sample, the fold change was calculated using the following equation:

$$Fold\ Change = 2^{-(\Delta\Delta Cq)}$$

In order to graph the resulting values for each sample, the fold changes were averaged and plotted on a bar graph, with error bars representing the standard deviation for each sample.

Cell Lysate Preparation and Co-IP

THP-1 cells stimulated as described above for 24 hours were lysed in order to release protein complexes from the cell (Figure 6). After being washed once in 1x PBS, the cells were pelleted at 13.3 xg for 10 minutes in 150 µL of NP-40 buffer (Boston BioProducts) in the presence of a Complete Protease Inhibitor tablet (Pierce). Cells were lysed for 1 hour at 4 $^{\circ}$C rocking. Half of the volume of lysate was saved to be used to do a Co-Immunoprecipitation (co-IP), and the rest was used as a whole cell lysate control sample for the immunoblot.

In order to remove proteins that bind to the agarose bead promiscuously, the co-IP samples were “pre-cleared” of these proteins by incubating with 20 µL of Protein A/G

PLUS Agarose Beads (Santa Cruz Biotechnology) for 30 minutes at 4^{o}C. Samples were centrifuged at 13.3 xg for 1 minute and the supernatant was saved.

To pull down proteins in complex with STING, cGAS or IFI16, 10 μL of STING antibody (Santa Cruz Biotechnology sc-241044), 10 μL of IFI16 antibody (Santa Cruz Biotechnology sc-8023), or 10 μL of cGAS antibody (Cell Signaling Technology 15102) were used. This was done in order for cGAS, IFI16 or STING to be selectively bound by antibodies (Figure 6). These samples were incubated overnight at 4 ^{o}C. Protein A/G PLUS Agarose Beads were added in order to pull down any antibody-protein complexes and incubated for 1 hour at 4 ^{o}C in order for the agarose beads to bind the F_c portion of the antibody-protein complex. Samples were centrifuged at 13.3xg for 1 minute in order to pellet cells. The samples were washed in 500 μL of NP-40 buffer containing a Complete Protease Inhibitor tablet and incubated for 5 minutes at 4 ^{o}C, which was repeated five times. Samples were centrifuged at 13.3xg for 1 minute after each wash and the supernatant was discarded. Final samples were resuspended in 20 μL of NP-40 buffer containing a Complete Protease Tablet (Sigma) and stored at -20 $^{\circ}$C before being used in an immunoblot.

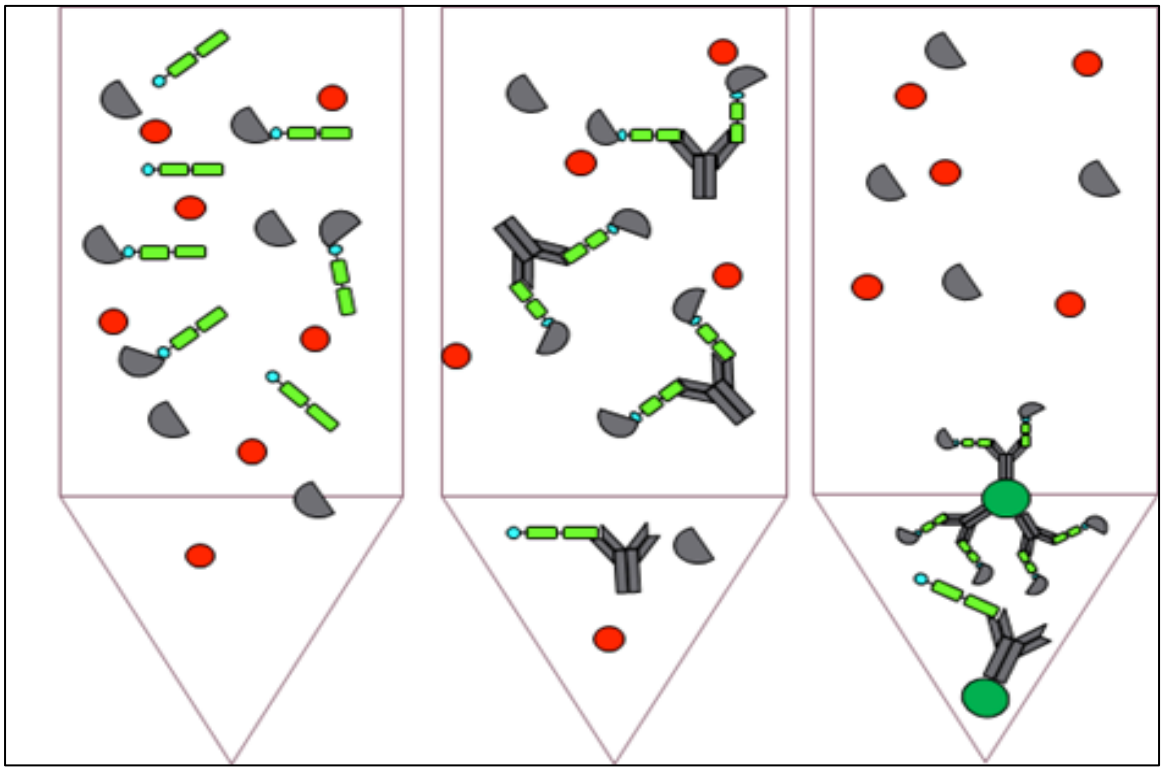

Figure 6: Schematic for Co-Immunoprecipitation
The general steps involved in pulling down a protein complex from a lysate in order to find binding in complex are shown. In the leftmost panel, a whole cell lysate contains protein complexes of interest depicted as circles, half circles, and rectangles. In the middle panel, antibodies, shown as grey "Y"s, bind to protein of interest. In the rightmost panel, these protein-antibody complexes are incubated with A/G PLUS Agarose Beads (depicted as green circles), which bind to the F_c portion of the antibody, and will pull down the protein of interest along with any additional proteins bound in complex. Graphic obtained from Bacas, 2015.

Immunoblot

First, 4X Laemelli dye (Bio-Rad) containing 1,4-Dithiothreitol (DTT) (Sigma) was added to whole cell lysate and Co-IP samples at a final concentration of 1X Laemelli buffer and a final volume of 50 µL. The samples were denatured at 95^{o} C for 5 minutes, loaded into a Mini-PROTEAN TGX Precast Gel (Bio-Rad), and run at 100 volts for 1 hour. Precision Plus Protein Kaleidoscope Prestained Protein Standard (Bio-Rad) was used as the protein ladder. The running buffer consisted of 10x Tris Glycine sodium dodecyl sulfate (SDS) (VWR Life Science) and Millipore water and diluted so that the concentration of the Tris Glycine SDS was 1x. A wet transfer was done using a0.45 µm nitrocellulose membrane (Bio-Rad) and transfer buffer containing 10x Tris Glycine Buffer (from Bio-Rad), methanol, and Millipore water. The transfer ran at 100 volts for 45 minutes at 4^{o}C .

The nitrocellulose membranes containing transferred proteins were placed into blocking solution containing 5% milk powder and 1X TBST; TBST contains 20X TBS (VWR Life Science), 0.5 mL of Tween 20 (Bio-Rad) and was diluted to a total volume of 1 liter in Millipore water. Membranes incubated in blocking solution for 1 hour at room temperature. Membranes were washed three times with 1X TBST for 5 minutes at room temperature. A 1:1,000 dilution of anti-DNA-PKcs primary antibody (Santa Cruz Biotechnology sc-5282) was made in blocking solution and added to the membrane for 1 hour at 4^{o}C. Membranes were washed three times with 1x TBST for 5 minutes at room temperature. A 1:10,000 dilution of goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology sc-2031) was made in blocking solution, and added for 1 hour at 4^{o}C. Membranes were washed with 1x TBST for 5 minutes at

room temperature three times. SuperSignal West Dura Extended Duration Substrate (ThermoFisher) was added to the membranes in order to see the presence of the protein of interest since the Dura solution contains HRP substrate and results in luminol-based chemiluminescence. The membranes were imaged using an Amersham Imager 600.

Bioinformatics

Since DNA-PK is a kinase, one possible way it could interact with components of the cGAS, STING and IFI16 is through phosphorylation. In order to look for the possibility of phosphorylation of cGAS, STING, or IFI16 by DNA-PK or other DNA damage kinases, the protein sequences for cGAS, IFI16, and STING were analyzed using ScanSite 4.0 (Obenauer et al., 2003). This is a phosphorylation site predictor that looks for specific motifs within proteins that are likely to phosphorylated by specific kinases. Other factors that are considered when phosphorylation sites are predicted are subcellular localization, evolutionary conservation and surface accessibility (Obenauer et al., 2003). Polypeptide sequences for cGAS, STING, and IFI16 were accessed from National Center for Biotechnology Information (NCBI) and the following NCBI reference sequence numbers were entered into Scansite: cGAS was NP_612450.2, STING was Q2KI99.1 and IFI16 was NP_001351796.1.

Proliferation Assay

In order to measure cell metabolism across different cells, we measured the conversion of 3-(4,5-dimethyltiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to formazan. This chemical reaction is caused by the reduction of MTS with either NADH or NADPH as a cofactor produced by dehydrogenases and can be extrapolated out to measure cell proliferation or senescence

under the assumption that the more cells that are present, then higher amounts of MTS dye will be converted to formazan by the mitchondria. Quantification of cell senescence in THP-1 cells, and cGAS, IFI16, and STING knockout cell lines was done using a CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). Cells were counted and plated at $1.6x10^4$ cells/mL in a volume of 50 µL in a 96-well plate. PMA was added to cells at 5 ng per milliliter and cells were incubated for two days prior to treatment with cisplatin. Each cell line was either left untreated or treated with cisplatin. The final concentration of cisplatin in each well was added according to concentrations used by Ahn and colleagues (2014) and cells were incubated for 24 hours.

Cells had 20µL of CellTiter 96® AQ_{ueous} One Solution Reagent added to them, which includes MTS dye, and were incubated for an hour to allow cells to metabolize the compound. The wavelength that the absorbance was read at was 490 nm and was read by SpectraMax M Series Microplate Reader in order to measure the amount of formazan generated. Each condition was done in triplicate.

Results

DNA-PK is Required for an IFN Response to Exogenous Nucleic Acid

In order to see if DNA-PK is required for a type I IFN response, we compared transcription of *ISG56* in cells that were either treated with a DNA-PK inhibitor (NU7026) or DMSO as a control. The transcription of *ISG56* indicates an antiviral response since it is transcriptionally upregulated by the production of type I IFN, therefore can be measured in order to observe the downstream effects of PRR signaling (Fensterl and Sen, 2011). As shown in Figure 7, the transcription of *ISG56* varies depending on the kind of stimulus that was transfected into cells. These effects were examined across these two conditions when cells were stimulated with nucleic acid. We expected little to no difference between the NU7026 (DNA-PK inhibitor) and the DMSO control when cells in the "mock" condition were treated with just Lipofectamine® 2000 (Figure 7). Both poly dAdT and VAC70 were able to induce a type I IFN response in the DMSO control cells; yet, when cells were treated with NU7026, the IFN response was attenuated (Figure 7). Since VAC70 is known to signal through both IFI16 and cGAS (Unterholzner et al., 2010), this suggests that DNA-PK works with both these PRRs in order to produce a type I IFN response. However, transfection of poly dAdT in cells that were treated with NU7026 also resulted in a reduced type I IFN response when compared to controlled cells (Figure 7). Poly dAdT activates RNA-sensing PRRs since it can be transcribed into RNA (Ablasser et al., 2009). This pattern indicates that DNA-PK has downstream effects on the transcription of type I IFN and may interact with transcription factors that control gene expression of type I IFNs.

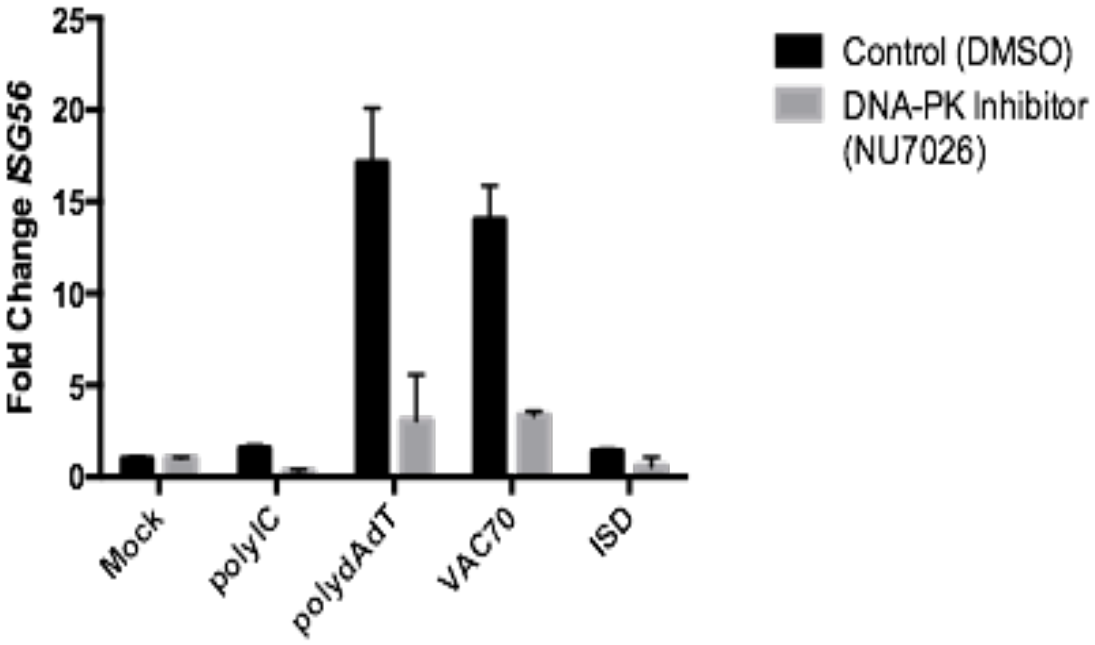

Figure 7: DNA-PK is needed for an Interferon Response to Exogenous Nucleic Acid qPCR results for THP-1 cells stimulated with different kinds of nucleic acid. Mock is Lipofectamine® 2000 alone, poly IC is a synthetic RNA, poly dAdT is synthetic dsDNA, VAC70 is a 70-base pair segment from Vaccinia virus, and ISD is dsDNA that is bacterial in origin. Data for each condition were completed in triplicate, and represent the average fold change for qPCR. Errors bars are the standard deviation.

Unexpectedly, neither poly IC nor ISD resulted in the upregulated transcription of *ISG56* to the extent seen with poly dAdT and VAC70 (Figure 7). Poly IC is a synthetic RNA that can lead to the transcription of type I IFN through the RIG-1 signaling pathway (Palchetti et al. 2015), which is independent of the cGAS/STING DNA-sensing pathway; therefore, we expected that cells that were treated with either NU7026 or DMSO would have similar, elevated levels of type I IFN, since this response is due to the presence of exogenous RNA, not DNA. Given that ISD is an immunostimulatory DNA that has been shown to upregulate transcription of type I IFN, cells that were treated with DMSO were expected to have higher levels of transcription of *ISG56* than cells treated with NU7026, which would have lower levels of *ISG56*. However, both conditions result in low transcription in both NU7026 and DMSO conditions (Figure 7). The pattern that was predicted for ISD was expected to be similar to those observed with cells that were stimulated with poly dA:dT and VAC70, since the IFN response to ISD has been shown to be mediated by the STING pathway (Stetson and Medzhitov, 2006). Instead, cells that were transfected with poly IC or ISD, and were treated with NU7026 inhibitor had similar levels of transcription of *ISG56* than control cells.

Overall, these data show that the induction of type I IFN in response to exogenous nucleic acid is dependent on DNA-PK kinase activity and may indicate DNA-PK's ability to interact with DNA-sensing PRRs as well as downstream transcription factors. These findings support previous findings that DNA-PK can signal in an IRF3-dependent manner in response to cytosolic DNA (Ferguson et al., 2012). Thus, this suggests that DNA-PK participates in sensing of exogenous nucleic acid.

DNA-PK Interacts with IFI16 and STING

In order to see if our hypothesis that DNA-PK could interact with DNA-sensing PRRs was correct, physical interactions between DNA-PK with IFI16, cGAS and STING were examined. Co-immunoprecipitation (Co-IP) is a method that allows for proteins bound in complex to be pulled down due to the specificity of an antibody binding to one of the proteins; the presence of other bound proteins can be probed using other antibodies in an immunoblot. Pull-downs were performed using IFI16, cGAS or STING antibodies, and the presence of DNA-PK in the pull-down complexes was determined by immunoblot using a DNA-PK antibody. THP-1 cells were stimulated under different conditions to see if the presence of exogenous nucleic acid, such as Poly IC, VAC70, Poly dAdT or salmon sperm, increased association of DNA-PK with IFI16, STING or cGAS (Figure 8). VAC70 was transfected to see if there was association between the proteins in the presence of viral DNA while salmon sperm was done in order to see if association still happened in the presence of eukaryotic DNA. Both of these conditions resulted in the associated of DNA-PK with IFI16 and STING, but not cGAS (Figure 8). DNA-PK has a molecular weight of 469 kDa (kildaltons) (Jette and Lees-Miller, 2015); the presence of a band that size was detected in both IFI16 and STING, but not cGAS co-IP pull-downs across all stimulatory conditions (Figure 8).

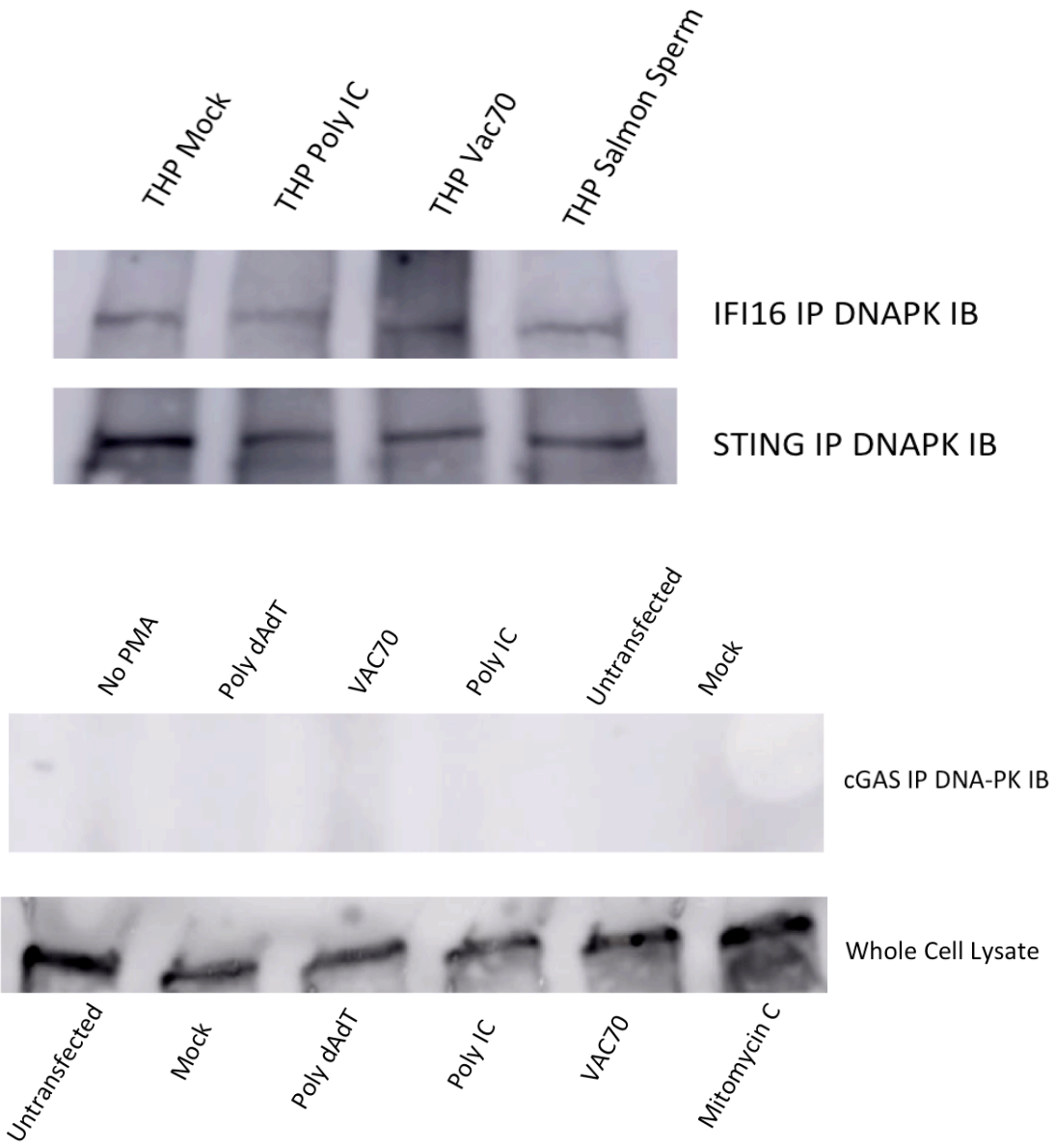

Figure 8: DNA-PK Binds in Complex with IFI16, and with STING in THP-1 cells
THP-1 cells were either matured with PMA or not (no PMA). PMA-treated cells were not treated (untransfected), stimulated with Lipofectamine® 2000 alone (Mock), Poly IC (a synthetic RNA), VAC70 (a 70-base pair dsDNA sequence from Vaccinia virus), salmon sperm (eukaryotic DNA), poly dAdT (a synthetic DNA) or mitomycin C (DNA damage agent). Cells were lysed after 24 hours of stimulation and used in IP followed by IB. IP means immunoprecipitation and IB means immunoblot.

In order to see if these associations were dependent on the presence of exogenous RNA, Poly IC was transfected to THP-1 cells. The no PMA conditions, untransfected and mock what were done as a negative controls. The immunoblot done for the whole cell lysates was completed to confirm the presence of expressed endogenous DNA-PK protein in both PMA and non-PMA conditions in THP-1 cells (Figure 8). Even in the mock and Poly IC conditions, there was binding in complex between DNA-PK and IFI16, and between DNA-PK and STING (Figure 8). This indicates that a background level of association may occur between these proteins even in the absence of exogenous DNA.

DNA Damage Kinases are Predicted to Phosphorylate the cGAS/STING Pathway

Given that DNA-PK was shown to bind in complex with STING and IFI16, we wanted to understand in the mechanism by which it interacts. Phosphorylation is one of the possible post-translational modifications that can be made to a protein. Given that DNA-PK is a kinase and NU7026 specifically alters DNA-PK's kinase activity, phosphorylation by DNA-PK would provide a way in which DNA-PK could signal in this pathway and could also be a method by which it could alter the type I IFN response. Using Scansite 4.0, a DNA damage kinase is predicted to phosphorylate cGAS at threonine-68; the kinase predicted is DNA-PK (Table 2). A score of 0.422 indicates that DNA-PK has a very high likelihood of phosphorylating cGAS as its substrate, with a score of 0 meaning 100% certain and ranging to infinity (Obenauer et al., 2003). Additionally, the percentile associated with DNA-PK phosphorylating cGAS was 0.192%, which means that the prior mentioned score was compared to the entire

proteome. The lower the percentile, the better the score is compared to other entries in the database. These data support the idea that additional post-translational modifications to cGAS could result in activation, due to its low binding affinity for dsDNA (Jønsson et al., 2017). It also demonstrates another role DNA-PK may have in type I IFN signaling and in DNA-sensing (Ferguson et al., 2012). Although we were not able to show that cGAS and DNA-PK could bind in complex, phosphorylation of a protein is a transient reaction so it could still be possible that DNA-PK phosphorylates cGAS. This was a new finding because it has not been previously shown that cGAS has the ability to interact with DNA damage kinases, and vice versa. This finding also supports the idea that DNA-PK has ability to translocate into the cytoplasm and perhaps respond to damaged host DNA that has leaked out from the nucleus (Cooper et al., 2013). Overall, this finding demonstrates a possible link between DNA-PK and the type I IFN response.

Table 2: Predicted Phosphorylation Sites of cGAS and STING

Substrate	Predicted Kinase	Phosphorylation Site	Score	Percentile
cGAS	DNA-dependent protein kinase	Threonine-68	0.422	0.192%
STING	Ataxia Telagiectasia Mutated kinase (ATM)	Serine-326	0.341	0.025%

DNA-PK is predicted to phosphorylate cGAS; ATM is predicted to phosphorylate STING. DNA-PK is predicted to phosphorylate cGAS at threonine-68 (T68) and ATM is predicted to phosphorylate STING at serine-326. A score is the likelihood of a peptide to become a substrate for a kinase and the percentile is generated by comparing all of the scores in the proteome (Obenauer et al., 2003). The lower the score and percentile, the better the quality of the match. Data adapted from: https://scansite4.mit.edu/4.0/#scanProtein

Predicted sites that could be phosphorylated by DNA-PK or other DNA damage kinases were also examined for STING and IFI16. Unlike cGAS, STING has a different DNA damage kinase, ATM, predicted to bind and phosphorylate STING based on motifs within STING that are likely to be phosphorylated (Table 2). The score and percentile associated with ATM predicted to phosphorylate STING also support the idea that DNA damage kinases can interact with components of the PRR signaling pathways in order to alter the type I IFN response (Table 2). While these findings for STING do not support the idea that DNA-PK can phosphorylate STING, this further validates the possibility that DNA damage kinases can also signal in other DNA-sensing pathways, such as the cGAS/STING pathway. ATM has been shown to modulate STING-dependent type I IFN responses caused by DNA damage, which supports the idea that ATM can phosphorylate STING as a substrate (Hartlova et al., 2015). Additionally, these data suggest that type I IFN responses may not be exclusively dependent on one DNA damage kinase. A phosphorylation analysis was also run on IFI16; however, there were no sites that were predicted to be phosphorylated by DNA-PK or any other DNA damage kinases. This was unexpected because both DNA-PK and IFI16 are nuclear proteins that both bind DNA. While these findings do not reinforce the possibility of phosphorylation by DNA-PK, it does not necessarily rule out binding between DNA-PK and IFI16 due to the fact we show the DNA-PK associates with IFI16 in complex, and that IFI16 can interact with cGAS and STING, which are predicted to be phosphorylated by DNA-PK and ATM, respectively.

The Type I IFN Response to DNA Damage is Dependent on IFI16

While these data show the involvement of DNA-PK in the type I IFN response to exogenous DNA, we also wanted to see if IFI16, cGAS, and STING are involved in the DDR. The type I IFN response to DNA damage has been shown to be dependent on DNA-sensing PRRs such as cGAS and IFI16 (Yang et al. 2017; Dunphy et al., 2018). However, these observations have yet to be replicated in THP-1 cells in response to drugs other than etoposide (Dunphy et al., 2018). In order to see if this IFN response was dependent on IFI16, cGAS, and STING, PMA-matured PRR knockout cells (-/-) were treated with DNA damage agents mitomycin C and doxorubicin, and the type I IFN responses were measured. Mitomycin C is a DNA crosslinker that can prevent the DNA replication from synthesizing new DNA and induce double-stranded breaks (Tomasz, 1995). Doxorubicin can intercalate DNA base pairs and prevent the synthesis of DNA by stopping the replication machinery (Tacor et al., 2013).

As a negative control, all four cell lines were treated with Lipofectamine® 2000 for a mock condition. If these proteins are positive regulators needed for transcription of IFN, then we would see less transcription of *ISG56* across these knockout cell lines following damage due to the fact that these PRRs would help upregulate transcription of IFN in wildtype cells. Conversely, if these proteins function as negative regulators of transcription of IFN following damage, then we would expect to see higher levels of expression of *ISG56* compared to the wildtype cells. In the mock condition, *IFI16* knockout cells and the control cells showed similar levels of transcription at a fold change of about 1 (Figure 9). However, *STING* and *cGAS* -/- cells had lower levels of transcription in both unstimulated and stimulated conditions. Compared to the wildtype

PMA-treated THP1 cell line, *IFI16* -/- cells showed attenuated transcription levels of *ISG56* when they were stimulated with both mitomycin C and doxorubicin (Figure 9). These data demonstrate that DNA damage can lead to a type I IFN response dependent on IFI16. However, given that transcription of *ISG56* in *STING* -/- and *cGAS* -/- cells were not consistent with the control cells, this conclusion cannot be expanded towards these proteins. These findings are novel because it has not been shown previously that the IFN response to DNA damage is dependent on IFI16 and that without this PRR there is a diminished type I IFN response (Figure 9).

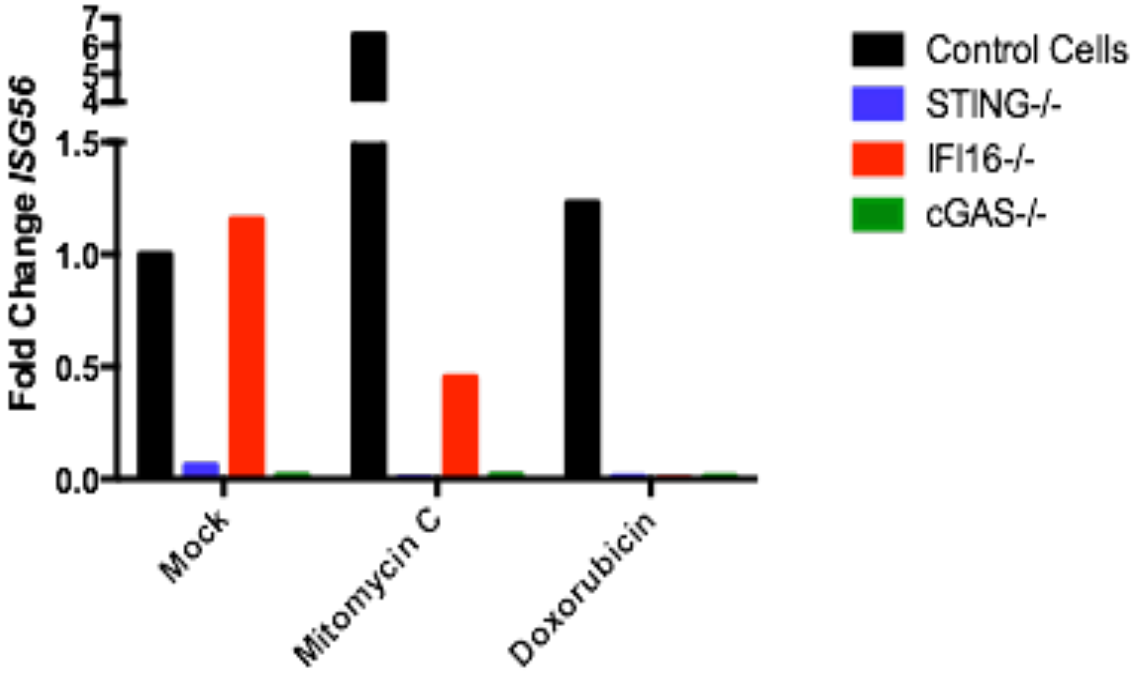

Figure 9: Induction of Type I IFN in response to DNA Damage is IFI16 dependent PMA-treated THP cells and knockout (-/-) THP-1 cells were treated with Lipofectamine® 2000 (mock), mitomycin C, or doxorubicin. After 24 hours of stimulation, cells were lysed and transcription of *ISG56* was measured using qPCR. Fold change standardized to transcription of *RPL37A*.These data are representative of a single experiment.

DNA-Sensing PRRs are Essential for Cell Senescence

Since the IFN response to DNA damage was shown to be dependent on IFI16, we next asked if our PRRs of interest also had roles in cell senescence in response to DNA damage (Figure 10). In normally functioning cells, DNA damage causes cells to stop proliferating in order to avoid propagation of mutations in DNA caused by the damage (Blackford and Jackson, 2017). We tested whether this phenomenon is dependent on our PRRs of interest by utilizing a cell proliferation assay that measured the number of respiring cells in culture due to the production of a formazan product generated by the reduction of MTS by the cell. Across both the PMA and non-PMA conditions, THP-1 cells proliferated more slowly than any of the three knockout cell lines, even without any treatment (Figure 10). However, when PMA-matured cells were treated with cisplatin, there was minor decrease in respiring cells seen in the THP-1 cells indicating cell senescence (Figure 10a) There was a significant increase in proliferation in *STING* -/- cells, and a slight increase in proliferation in the *cGAS* -/- cells and *IFI16* -/- knockout cells when treated with cisplatin. A similar pattern was seen in the non-PMA matured cells. In the presence of cisplatin, *STING* -/- and *IFI16* -/- cells proliferated significantly more than the untreated cells while *cGAS* -/- proliferated slightly more and there were similar amounts of respiring THP-1 cells (Figure 10).

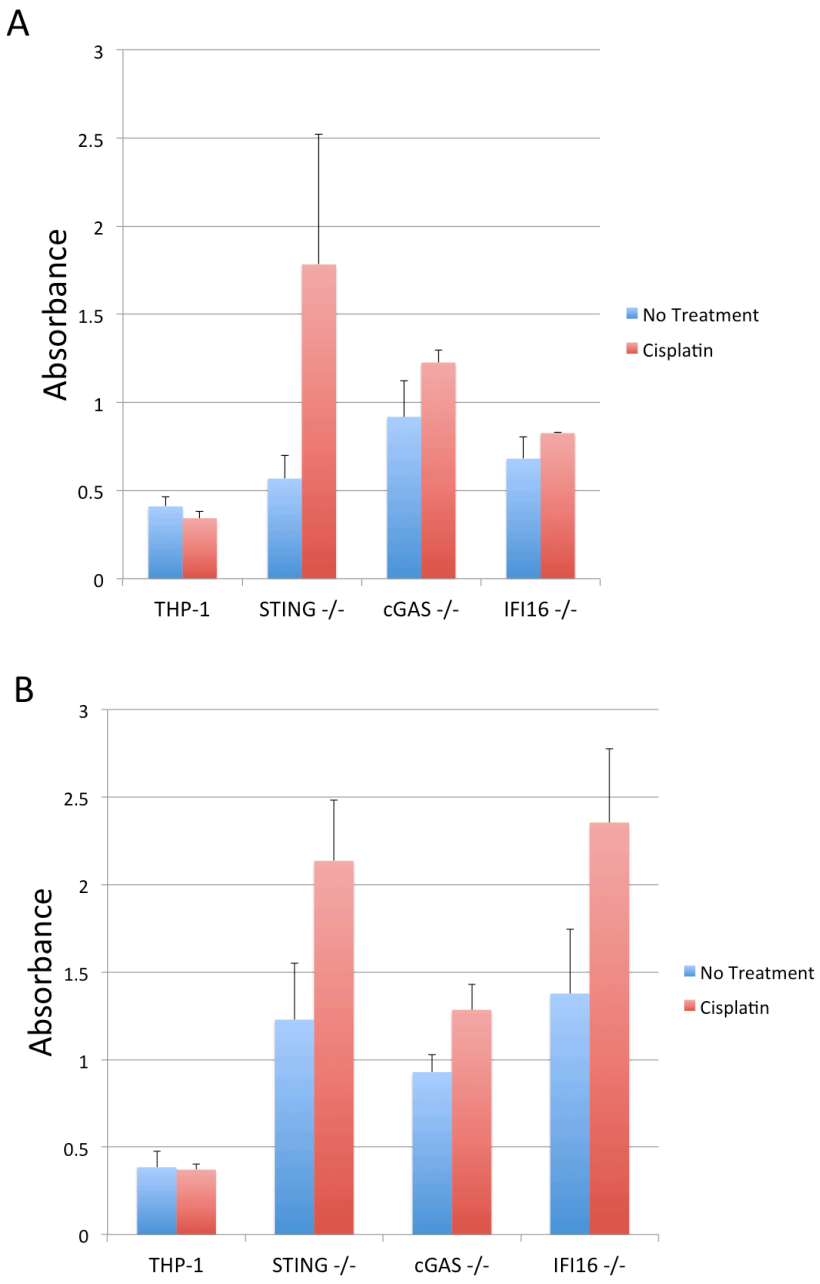

Figure 10: Proliferative Activity of THP-1 Cells is STING, cGAS, and IFI16-dependent
Measurement of cell proliferation changes in response to DNA damage after treatment for 24 hours using an MTS assay **A.** cells that were matured with PMA and **B.** cells not matured with PMA. Data represent the mean of biological triplicates from one experiment, and the error bars represent standard deviation.

These results suggest that these components of the DNA-sensing pathway are involved in cell senescnce. In the presence of PMA, the proliferation of the *IFI16* -/- cells is less than seen in cells that were not treated with PMA (Figure 10). This may be because IFI16 are not expressed at high levels in fully matured macrophages in comparison to monocytes (Shannon et al., 2018). In addition, proliferation of these knockout cells is increased in the presence of DNA damage when compared to the untreated cells. Since STING, cGAS and IFI16 are involved in the type I IFN response to DNA damage, their ability to play a role in cell senescence and apoptosis in response to DNA damage further shows their role in responding to host DNA.

Discussion

The aim of this study was to see if there is crosstalk between DNA sensing by cGAS, IFI16, and STING and DNA repair proteins. We hypothesized that due to the subcellular localization of DNA-PK and IFI16 in the nucleus and their ability to bind DNA, that there could be interactions between these proteins. Additionally, we also hypothesized that DNA-PK can phosphorylate these PRRs due its kinase activity and ability to translocate to the cytoplasm (Cooper et al., 2013). Overall, since IFI16, cGAS and DNA-PK have roles in DNA binding, cell cycle regulation, and inducing cell death, we predicted that these proteins would have functions in signaling together. We specifically wanted to understand the mechanism of crosstalk that occurs between DNA-sensing PRRs, such as IFI16 and cGAS, with DNA-PK, a DNA damage kinase. This was done by first examining DNA-PK's role in the type I IFN response to viral infections. Additionally, physical interactions between DNA-PK with IFI16, cGAS and STING were determined through co-IPs and immunoblots. Further aspects of crosstalk were examined by looking at cGAS, IFI16, and STING's role in mediating cellular responses to DNA damage. Type I IFN responses to both viral nucleic acid and host DNA damage were measured by quantifying the transcription of *ISG56*, an antiviral gene upregulated during a type I IFN response. These findings demonstrate that there is crosstalk between these two classes of DNA binding proteins, and bioinformatics studies were done to predict the way in which these additional functions happen.

DNA-PK is Essential for the Type I IFN Response

The addition of a DNA-PK inhibitor to cells transfected with viral nucleic acid resulted in a reduced type I IFN response; this pattern was seen in cells that were

transfected with poly IC, ISD and poly dAdT and VAC70 (Figure 7). However, this pattern was significantly different in cells stimulated with poly dAdT and VAC70 (Figure 7). Since VAC70 is known to be detected by cGAS and IFI16, this finding supports the idea that DNA-PK can signal with these two PRRs (Unterholzner et al. 2010). Since poly dAdt can be transcribed into RNA that can be detected by RNA-sensing PRRs such as RIG-I, this finding indicates that DNA-PK may also play a role in type I IFN production that is also downstream of DNA-sensing PRRs such as regulating IRFs (Ablasser et al., 2009). Since DNA-PK is a protein that is localized to the nucleus, it could interact with these IRFs in order to regulate transcription of type IFN. Cells that were stimulated with poly IC and ISD did not have a statistically significant difference between the condition that received the inhibitor and the condition that received DMSO as a control, but there was still a small decrease in the transcription of *ISG56* in the presence of NU7026 (Figure 7). Poly IC is known to induce type I IFN responses independently of DNA-sensing PRRs and ISD is known to induce type I IFN responses in a cGAS/STING-dependent manner (Matsumoto et al., 2002; Stetson et al., 2006). These results demonstrate the ability for DNA-PK to participate in PRR signaling and in the induction of type I IFN. This also suggests that the ability of DNA-PK to alter expression of *ISG56* is perhaps due to its interactions with components of a DNA-sensing pathway. Additionally, these findings show that DNA-PK may have the ability to regulate transcription of type I IFN given that there was a reduction in *ISG56* transcription across both DNA and RNA stimulatory conditions, which demonstrates its possible role in downstream signaling.

This prediction that DNA-PK could interact with DNA-sensing PRRs was examined by looking at physical interactions between DNA-PK with IFI16, cGAS and STING. These interactions were confirmed by a co-IP and immunoblot showed binding in complex between DNA-PK and IFI16, and DNA-PK and STING across both mock and stimulatory conditions (Figure 8). Even in the mock condition, there was binding, which indicates that there are interactions between the proteins even when there is no exogenous DNA present (Figure 8). Given that DNA-PK and STING were shown to bind in complex, DNA-PK could localize in the cytosol in order to detect damaged host DNA that has leaked out of the nuclear and initiate a type I IFN response by associating with the cGAS/STING pathway. This hypothesis could be tested by doing a confocal microscopy experiment that sees if DNA-PK changes subcellular localization in the presence of exogenous DNA or DNA damage.

Additionally, there was association between DNA-PK and IFI16, and DNA-PK and STING when poly IC, VAC70 and salmon sperm were transfected, however there was no difference in levels of association in any of the stimulatory conditions compared to mock (Figure 8). Salmon sperm DNA may have acted as a DAMP in order to initiate signaling and the association of DNA-PK with IFI16 and STING due to its structural similarity to host DNA. While it is not clear whether IFI16 can distinguish between host and microbial DNA, binding in complex in both of these conditions supports the idea that IFI16 plays role in both responding to DNA as both a MAMP and a DAMP.

A major limitation of these findings was that there was little to no difference between the mock and other stimulatory conditions. Another reason for why there was association seen even in the mock condition was that these experiments were completed

in immortalized cells, which are known for having background levels of DNA damage due to mutations in DNA repair pathways. Thus, association between DNA-PK with IFI16 and STING may have occurred in the mock condition as result of the types of cells that were used. Additional experiments to confirm if levels of association vary across different stimulatory conditions would be to complete this co-IP in primary cells and to see if binding in complex is still consistent across these cell types.

These data demonstrate the ability of DNA-PK to associate with STING and IFI16 in the presence of both viral and eukaryotic DNA, and poly IC RNA. The ability for these interactions may also depend on the subcellular localization of DNA-PK, and could also suggest that it can translocate from the nucleus to the cytoplasm upon transfection of cytosolic DNA.

The mechanism by which DNA-PK interacts with cGAS, STING, and IFI16 was examined by looking at predicted phosphorylation sites on these three proteins. Earlier experiments showed that inhibiting the kinase activity of DNA-PK with a drug reduced the IFN response (Figure 7). We hypothesized that DNA-PK could activate IFI16, STING or cGAS by phosphorylation. cGAS was predicted by Scansite 4.0 to be phosphorylated at theorine-38 by DNA-PK (Table 2). This supports that DNA damage kinases, particularly DNA-PK have a possible role in post-translationally modifying cGAS, and perhaps activating it in order to induce a type I IFN response. Coupled with the data showing that DNA-PK is needed for an IFN response, this suggests a possible way in which DNA-PK is involved in PRR signaling (Figure 7). While STING was not predicted to phosphorylated by DNA-PK, it is important to note that it is predicted to be phosphorylated by another DNA damage kinase known as ATM that can sense double-

stranded lesions to DNA (Table 2). This phosphorylation mechanism has been confirmed in a previous study, where ATM sensed double stranded breaks, phosphorylated p53, and associated with STING (Härtlova et al., 2015; Dunphy et al., 2018). Experiments looking at direct association between ATM and STING have yet to be completed; however, we were able to show that STING binds in complex with DNA-PK (Figure 8). There was no indication that DNA-PK or any other DNA damage kinase has the ability to phosphorylate IFI16. This could be due to the fact that IFI16 also interacts with both cGAS and STING (Almine et al., 2017); therefore, phosphorylation by DNA-PK may be redundant. Further experiments that look at the role of phosphorylation sites on these proteins could provide additional links between these two classes of DNA-binding proteins.

Type I IFN Response to DNA Damage is Dependent on the cGAS/STING pathway

The pathway that mediates the type I IFN response to DNA damage is not well characterized. One potential pathway is the cGAS/STING pathway, which involves IFI16; components of this signaling pathway have been shown to participate in cell cycle regulation and promote antitumor immunity, further relating them to DNA damage (Ahn et al., 2014; Li et al., 2018; Johnstone et al., 2000). Our hypothesis that these PRRs have dual roles in responding to viral infections and DNA damage is relevant because different kinds of viruses induce DNA damage (Evans and Hearing, 2005). Additionally, both damaged DNA and viral DNA are cytoplasmic DNA species that could be bound by sequence-independent DNA binding proteins, such as cGAS and IFI16 (Aguirre et al., 2017). After treating different cell lines that were missing either *IFI16*, *cGAS,* or *STING* with DNA damage agents and comparing levels of transcription of *ISG56*, we observed

ISG56 induction was reduced across in the *IFI16* -/- cells when compared to control cells (Figure 9). Doxorubicin is a drug that can induce double stranded breaks, thus its ability to induce type I IFN through IFI16 (Figure 9) provides an additional link between DNA-PK and IFI16 (Swift et al., 2006; Blackford and Jackson, 2017). DNA sensors such as IFI16 and cGAS, can to bind to damaged host DNA and initiate an interferon response (Aguirre et al., 2017; Dunphy et al., 2018). Another reason for why the type I IFN response to DNA damage is dependent on IFI16 could be due to DNA-PK's possible involvement in type I IFN signaling in response to DNA damage. We have shown that both DNA-PK and IFI16 can bind in complex (Figure 8), and DNA-PK has been shown to be activated when double-stranded breaks are detected (Cooper et al., 2013); in addition to signaling through the NHEJ pathway, DNA-PK could also be activating PRRs like IFI16 as well.

There were also changes in cell senescence in *STING*, *cGAS*, and *IFI16* knockout cells both without stimuli and due to DNA damage (Figure 10). These patterns were seen with no treatment, which demonstrates these PRRs' role in regulating cell senescence both with and without DNA damage; however, cell proliferation increased in the presence of DNA damage as well (Figure 10). These findings show that when these PRRs are missing in cells in the presence of DNA damage agents, there are inappropriate DNA damage responses that cause these cells to not go to apoptosis (Figure 10). A key effect of the DNA damage response is cellular senescence, which has previously been shown to be dependent on cGAS and STING (Ahn et al., 2014; Yang et al. 2017). Additionally, IFI16, cGAS and STING have been shown to play a role in cellular senescence, and tumorigenesis (Xin et al., 2004; Ahn et al., 2014; Gluck et al., 2017; Li et al., 2018; Liu et

al., 2018; Yang et al., 2017). IFI16 has also been shown to be involved in apoptosis by interacting with p53 (Aglipay et al., 2003). Thus, cells that are missing these proteins lack regulators of cell senescence and proliferate more than cells that have an intact pathway.

These observed phenotypes also show the importance of IFI16, cGAS and STING in the DNA damage response. In a normally functioning cell, double-stranded breaks activate the NHEJ pathway and stops cell cycle progression. In the presence of cisplatin, all three knockout cell lines proliferated more than the THP-1 wildtype cells (Figure 10). These findings show that IFI16, STING and cGAS may interact with components of the NHEJ pathway such as DNA-PK and work to stop cell cycle progression. Wilson and colleagues (2015) show that another DNA-sensing PRR known as AIM2 is associated with the development of colon cancer in mice, and AIM2 could interact with DNA-PK in order to promote apoptosis. A possible way that DNA damage could cause more proliferation in these IFI16, cGAS or STING deficient cells is due to their inability to induce an type I IFN response to this damage, which can stop cell proliferation.

This has been supported in previous studies where cGAS and IFI16 have been shown to respond to DNA damage through inducing a type I IFN response, and that IFI16 is involved in the activation of ATM in order to associate with STING (Yang et al., 2017; Dunphy et al., 2018). Overall, these findings demonstrate that type I IFN response to DNA damage is needed for cell cycle arrest and that the absence of cGAS, IFI16 and STING in cells leads to inappropriate responses to DNA damage and sustained survival.

Future Directions and Relevance

The results from this study demonstrate that there is crosstalk between DNA-PK and IFI16, STING and cGAS. These data show that DNA-PK is required for a type I IFN

response, and that IFI16 is needed for the IFN response to to DNA damage. However, there are many experiments that must be done in order to further clarify these findings. One follow-up to the bioinformatics data would be to see if the predicted phosphorylation sites at threonine-68 for cGAS and at serine-326 for STING are needed for an IFN response. Site-directed mutagenesis that substitutes these residues could be done and IFN responses would be compared to cells that contain the wildtype versions of these proteins. Another experiment would be to see if DNA-PK's role in the response to viral infections is location dependent by infecting cells with a retrovirus in order to deliver nucleic acid to the nucleus instead of the cytoplasm (Shannon et al., 2018). Another regulator of the pathway, PQPB1, regulates the type I IFN response to exogenous DNA depending on the subcellular localization (Yoh et al., 2015; Shannon et al., 2018). Therefore, this follow up experiment would elucidate if DNA-PK has one function related to DNA repair in the nucleus and whether or not it has a role sensing exogenous DNA exclusively in the cytoplasm. Differences in the type I interferon response due to subcellular localization of exogenous DNA could be done by measuring transcription of *ISG56.*

Determining which other DNA damage kinases are involved in the type I IFN response would also be important. Seeing if additional proteins involved in NHEJ, such as ATM and ATR, play a potential role in responding to exogenous DNA would clarify if these type I IFN responses to viral infections are specific to DNA-PK or if multiple DNA damage proteins are involved as well. While it has been shown the IFI16 mediates a type I IFN response to DNA damage by etoposide via ATM, ATM's role in responding to viral DNA has not been shown (Dunphy et al., 2018). These studies could show possible crosstalk between DNA sensing PRRs and DNA damage proteins. This could be done by

utilizing drug inhibitors against ATM and ATR such as KU-55933 and VE-821 in cells that were transfected with exogenous nucleic acid, and analyzing expression of type I IFN by qPCR.

The mechanism by which cGAS and IFI16 detect DNA damage and induce a type I IFN response has yet to be fully characterized. While it was shown in this study that the type I IFN response to DNA damage is mediated through IFI16 (Figure 9), a mechanistic study needs to be done to confirm these findings. Other studies have shown that cGAS is involved in DNA repair of double stranded DNA breaks and promotes tumor growth (Yang et al., 2017; Liu et al., 2018), and that IFI16 is involved in responding to DNA damage by activating p53 and STING (Dunphy et al., 2018). Thus, a possible experiment that could be performed is site directed mutagenesis of IFI16 in order to delete the nuclear localization signal. Cells could then be treated with DNA damage agents, and type I IFN responses could be compared to cells with the wildtype form of IFI16 that were also treated with DNA damage agents.

These studies demonstrate crosstalk between DNA repair proteins and DNA sensing PRRs, which could lead to new understandings of their role in inducing type I IFN responses. In order for these studies to be more clinically applicable, these effects should also be corroborated in human primary cells rather than in immortalized cells. Understanding the mechanisms for how these NHEJ and PRR signaling pathways interact with each could lead to additional targets for new therapies against viral infections such as HIV and cancer. There is an inflammatory component to both of these diseases, and overproduction of these cytokines can lead to an immunosuppressed state (Snell et al.,

2017). Therefore, better insights on how to modulate these responses in patients could lead to better treatments and clinical outcomes.

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