Drew University

College of Liberal Arts

# ROLE OF CHROMATIN STRUCTURE IN IMMUNE SENSING OF DNA

A Thesis in Biochemistry and Molecular Biology

By

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**Abstract**

The innate immune system uses pattern recognition receptors (PRRs) to sense microbial-associated molecular patterns (MAMPs). When a PRR binds a MAMP, this leads to a signal cascade through the cell resulting in transcription and production of inflammatory cytokines, such as interferon. MAMPs are molecules that are typically distinct from any molecules expressed by the human body. However, some PRRs bind to DNA, which is necessary for and not distinct from human cells. These PRRs binds DNA in order to detect viral infections and then create an interferon response to fight off the viral infection. This begs the question of how the PRR can distinguish between host and non-host DNA to prevent the erroneous production of interferon. Since the host cell has a tremendous amount of DNA, the DNA must be extremely organized and structured to fit all of the DNA into the cell. The cell does this by wrapping the DNA around a circular protein called a histone and together, the DNA wrapped around the histone is called a nucleosome. Perhaps this structure also serves a dual purpose of organizing the DNA and preventing host DNA from being sensed by PRRs. We hypothesized that host cell DNA in a nucleosome will not cause an interferon response while nucleosomal DNA without the histone will cause an interferon response. Also, we hypothesized that histones will actively inhibit the DNA sensor instead of just blocking the DNA sensor from binding to the DNA. We investigated the first hypothesis by stimulating cells with either nucleosomes or nucleosomal DNA and measuring the interferon response. We tested the second hypothesis by stimulating cells with either plasmid, histone and plasmid, or histone and measured the interferon response. Our data showed that a smaller interferon response is produced by nucleosomes versus nucleosomal DNA and that histones actively inhibit DNA sensors. This provides a mechanism for how cells can distinguish between host and non-host DNA and avoid erroneous interferon signaling.

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## List of Abbreviations

cGAMP: Cyclic Guanosine Monophosphate-Adenosine Monophosphate

cGAS: Cyclic GMP-AMP Synthase

IFI16: Interferon-Inducible Protein 16

IRF3: Interferon Regulatory Factor 3

MAMPS: Microbial-Associated Molecular Patterns

PMA: Phorbol Myristate Acetate

PRR: Pattern Recognition Receptors

STING: Stimulator of Interferon Genes

TBK1: TANK-binding kinase 1

RPL37a: Ribosomal Protein L37a

qPCR: Quantitative Polymerase Chain Reaction

## Introduction

The immune system is a complex system that helps organisms to fight off foreign invaders, such as bacteria, viruses and fungi. When describing the immune system, the term foreign invader describes the immune system's intended target. Distinguishing between foreign invaders and host cells allows the immune system to specifically attack microbes while leaving the host organism's cells alone and alive. The immune system can distinguish between host cells and non-host cells by sensing molecules that only appear on these non-host cells while tolerating molecules that are on host cells (Takeuchi and Akira, 2010). This ability to distinguish between host and non-host cells is extremely important and without this ability, the host organism can suffer from autoimmune diseases where the immune system attacks the host organism's cells (Gonzalez et al., 2011). The only cure for these diseases is to inhibit part of the immune system to prevent large amounts of damage being caused by immune cells. However, the large trade off of inhibiting the immune system is that foreign invaders can invade much easier. The immune system needs to be able to distinguish between host and non-host in order to keep the organism alive and healthy.

Host defense is usually classified into to three different sections: the barrier defenses, innate immunity, and lastly, adaptive immunity. These layers differ in many ways, such as cells involved, processes involved and specificity, which allows the immune system to fight off foreign invaders while protecting and not harming the host cells.

The first layer is the barrier defenses that provide an impermeable wall to most pathogens (Niyonsaba et al., 2017). This layer consists of the skin and mucosal layers to block pathogens from entering the body. Included in this layer are many peptides and proteins that help to prevent pathogens from entering and replicating in the body. These peptides or proteins have extremely broad defensive capabilities and can defend the organism from many different pathogens. These

peptides can also have another function known as immunomodulation, which allows for the recruitment of cells from the innate immune system to join the fight against pathogens (Niyonsaba et al., 2017). The immunomodulation function of these peptides shows that the immune system is interconnected even though humans try to break it up into three distinct sections for simplicity. Even though these peptides have broad effects against pathogens, they typically do not harm host cells and show specificity against only pathogens. However, over expression of some of these peptides can actually cause some autoimmune diseases such as psoriasis (Niyonsaba *et al.*, 2017).

The innate immune system also typically does not harm the host's cells and is more specific than the previous layer, but still responds to broad classes of pathogens (Riera Romo et al., 2016). Some of the cells that are involved with the broad responses of the innate immune system are macrophages and neutrophils. These cells respond to pathogens that manage to get through the barrier defenses by congregating and phagocytosing the pathogen. This congregation of cells and blood at the site of the pathogen causes the familiar symptoms of pain, redness and swelling. Not only do these cells respond to the pathogens, they also signal the adaptive immune system for help if the pathogen is not cleared. However, similarly to the barrier defenses the innate immune system can also cause problems for the host when it becomes dysregulated and causes autoimmune diseases (Zierhut and Funabiki, 2020). This pattern of dysregulation leading to problems for the host and autoimmune diseases continues when discussing adaptive immunity.

The final layer of the immune system is the adaptive immune system. This layer is the most specific but takes the longest to begin fighting the pathogen (Farber et al., 2016). The slow response is due to the fact that a new adaptive response must be made for each new pathogen and previous adaptive responses will not work against a new pathogen. However, the adaptive

immune response has a unique feature called memory. This allows the cells of the adaptive system, such as T-cell and B-cells, to respond very rapidly and robustly to a pathogen that has been seen before by the adaptive immune system and fight off the pathogen before any disease is seen in the organism (Farber *et al.*, 2016). The pathogen is destroyed by the highly specific adaptive immune response and this leads to the clearance of the pathogen from the organism with very little or no damage to the host organism.

To make such a highly specific response, the adaptive immune system must create a large repertoire of receptors for the T-cells and B-cells so that the cells can be specific for many different pathogens. However, there is great possibility for some of these many receptors to recognize a specific molecule from the organism that makes them (Gonzalez *et al.*, 2011). The cells with these receptors must be prevented from leaving the site of maturation and sensing the host cells which potentially could lead to problems within the host. If the cells were allowed to leave and start self-sensing, this could lead to the host attacking its own cells and causing autoimmune diseases. Organisms have many ways to prevent this from happening called tolerance (Gonzalez *et al.*, 2011). Central tolerance happens where the cells mature; in either the bone marrow for B-cells or the thymus for T-cells, and central tolerance is the process of self-reactive cells getting deleted or inactivated. If these cells escape central tolerance, the cells can still be deleted or inactivated through peripheral tolerance mechanisms that can happen anywhere in the body (Schwartz, 2012). However, central and peripheral tolerance mechanisms to deal with self-reactive adaptive immune cells do not apply to innate immune cells that might be self-reactive.

Since the innate immune system is extremely broad in what it senses and there are very few clearly defined tolerance mechanisms, this leads to the question of how self-versus non-self-

recognition happens. The receptors the innate immune system uses to get broad specificity are called pattern recognition receptors (PRRs) (Takeuchi and Akira, 2010). PRRs bind to microbe-associated molecular patterns (MAMPs), which are molecules that are uniquely foreign from the human body such as lipopolysaccharides, which are only found on Gram negative bacteria. Since PRRs bind molecules that are expressed by many different microbes, this allows the innate immune system to sense broad classes of microbes with the PRRs. PRRs should only bind uniquely foreign molecules therefore avoiding the possibility of self-sensing in humans.

Specifically, in the defense against DNA viruses, the cell uses PRRs that sense DNA in order to detect the virus (Paludan and Bowie, 2013). As this process will be a major focus of this thesis, the specifics and details of this pathway will be presented. The process of sensing DNA starts when a PRR binds to DNA, which in turn leads to a signal cascade. This signal cascade ends with the activation of the transcription factor interferon regulatory factor 3 (IRF3), which regulates the production of interferon (Stetson and Medzhitov, 2006). Interferon is an inflammatory cytokine that is produced and released from the cell after IRF3 is activated from the signal cascade (Mogensen, 2009). Interferon then binds to the interferon receptor (IFNAR) on nearby cells, causing another signal cascade (Schneider et al., 2014). This signal cascade ends with the production of interferon stimulated genes (ISGs) that have a multitude of effects on the cell such as enhanced pathogen sensing and blocking viral replication (Schneider *et al.*, 2014).

ISGs block viral replication at many steps of the viral life cycle. They can block entry into a cell through trapping the viral genome in the capsid, such as what TRIM5α does, or blocking the virus at the lysosome and endosomes like the IFITM proteins (Schneider *et al.*, 2014). The ISGs can also block viral replication and translation by degrading RNA or could tag

proteins for degradation. Lastly, ISGs can also effect the packaging and export of viruses. This could happen through blocking budding of viruses or tethering the virions to the cell membrane.

However, interferon is not always good for the human body and misregulation of interferon could lead to autoimmune diseases called interferonopathies (Boxx and Cheng, 2016; Davidson et al., 2018). Many well know autoimmune diseases are interferonopathies, such as Lupus and rheumatoid arthritis, and are linked to excess interferon in the body (Muskardin and Niewold, 2018). Not only can interferon lead to autoimmune diseases, interferon has also been shown to worsen bacterial infections and lead to humans being much more susceptible to bacterial infections (Boxx and Cheng, 2016). These show the need for interferon to be tightly regulated and not being produced unless necessary to fight off a viral infection. If the innate immune system was to be sensing DNA that was not part of a viral infection, that could lead to excess interferon and lead to the negative effect on the human body.

To further understand DNA sensing in the innate immune system, we must examine the two best described DNA sensors, cyclic GMP-AMP synthase (cGAS) and Interferon Gamma Inducible protein 16 (IFI16) (Almine et al., 2017; Unterholzner et al., 2010). The protein cGAS has been shown to be a key DNA sensor that synthesizes the small molecule 2'3' cyclic GMP-AMP (cGAMP) after sensing DNA in order to signal to the protein Stimulator of Interferon genes (STING) (Sun et al., 2013). The protein STING then continues the signal cascade in order to start production of interferon through the activation of IRF3. The protein cGAS is localized in the nucleus and the cytoplasm (Liu et al., 2018; Volkman et al., 2019). The protein cGAS binds DNA non-specifically by binding the backbone of the DNA, which leads to a conformational change that allows the cGAS catalytic site to be active in order to create cGAMP (Shu et al., 2014). However, cGAS has a very low binding affinity for DNA, which suggests that another

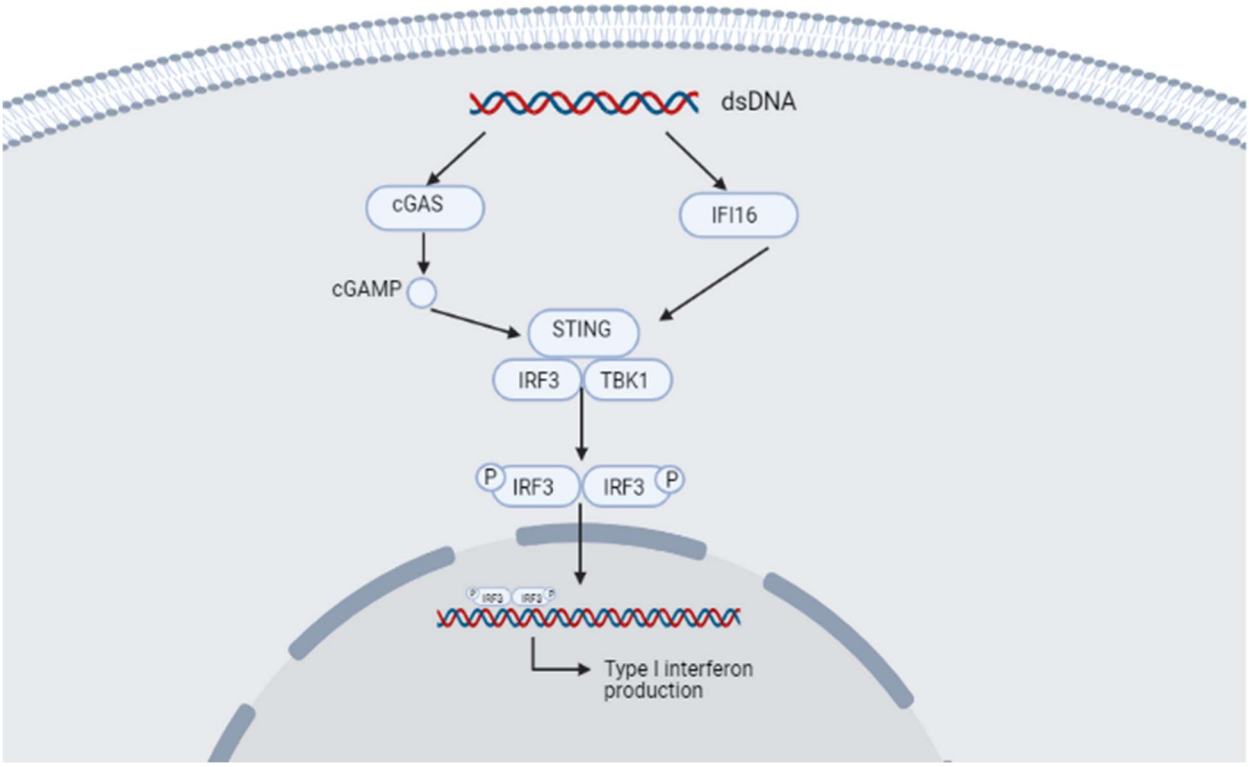


Figure 1: The pathway for IFI16 and cGAS to sense DNA and then produce interferon.

The sensing of DNA started when either IFI16 or cGAS binds DNA. IFI16 then goes and activates STING while cGAS creates a small molecule called cGAMP which then signals STING. STING then dimerizes and binds to IRF3 and TBK1. TBK1 then phosphorylates IRF3 which then dimerizes and moves to the nucleus to act as a transcription factor for type I interferon.

protein is helping cGAS bind to the DNA (Jønsson et al., 2017). Next, we must examine the other well described DNA sensor, IFI16, to further understand the role of DNA in the innate immune system.

IFI16 is the second DNA sensing PRR and is part of the PYHIN family (Veeranki and Choubey, 2012). A PYHIN protein consists of a PYRIN domain for protein-protein interactions and at least one HIN-200 domain for binding DNA (Goubau et al., 2010). In the case of IFI16, this protein has one PYRIN domain but has two HIN-200 domains, which is unique to IFI16 since all other PYHIN proteins have one HIN-200 domain (Unterholzner *et al.*, 2010). These HIN-200 domains bind DNA nonspecifically by binding to the backbone of the double-stranded DNA (Jakobsen et al., 2013; Unterholzner *et al.*, 2010). This allows the HIN-200 domains to bind many different viral genomes and allow broad response to many different viruses. Next, the PYRIN domain is important for the protein-protein interactions to activate STING and then signal through the same downstream pathway as discussed with cGAS. IFI16 has been shown to bind with cGAS and help promote the production of cGAMP and the stimulation of STING (Almine *et al.*, 2017; Jønsson *et al.*, 2017). However, another interesting feature of IFI16 is that IFI16 resides mostly in the nucleus but can translocated between the nucleus and the cytoplasm (Dell'Oste et al., 2014; Veeranki and Choubey, 2012). This is interesting because IFI16 binds DNA nonspecifically, which means it could bind other DNA than viral DNA, such as the host genome that is in the nucleus just as IFI16 is.

PRRs do not only respond MAMPs; they have also been shown to responds to damage-associated molecular patterns (DAMPs) (Roh and Sohn, 2018). DAMPs are self-proteins that are typically released when a cell is stressed and undergoing necrosis. These DAMPs are important to the organism because their sensing can alert the cell to tissue damage and lead to

inflammation. Some examples of DAMPs are the S100 proteins, histones, and ATP, which have been shown to bind to specific receptors such as TLR4, TLR2, and P2X7, respectively (Roh and Sohn, 2018). Sensing of DAMPs can cause problems for humans and this is shown because of the role of DAMPs in some autoimmune disease such as rheumatoid arthritis and osteoarthritis. The S100 proteins, that are commonly found in the cytosol and bind to TLR2, TLR4 or RAGE to be sensed as DAMPs, are found outside the cells and in the fluid of joints and seem to lead to play a huge role in the inflammation in both rheumatoid arthritis and osteoarthritis (Roh and Sohn, 2018). However, the sensing of DAMPs is not always bad. There is some evidence that DAMPs play a role in the immune system's ability to mount an immune response against cancer and fight against cancerous cells (Apetoh et al., 2007; Ghiringhelli et al., 2009). This shows that PRRs can sense self-molecules and not only distinctly foreign molecules. This is both good and bad for the organism: on one hand, the organism can sense tissue damage and respond appropriately; and on the other hand, this could possibly allow for erroneous self-sensing and autoimmune disease. Another critical example of sensing of self-molecules is seen in relation to the viral DNA sensing pathway above. DNA is not a clearly foreign molecule and is in every living cell which creates the interesting topic of how these DNA sensors can distinguish between self and non-self DNA or how DNA may sometimes act as a DAMP.

The DNA PRRs are activated in autoimmune diseases when there are mutations of one of the proteins that clear exogenous DNA from the cell, such as DNase II (Motwani et al., 2019). The lack of the DNase II allows DNA in phagosomes to leak out and not be digested, which in turn stimulates the cGAS and the rest of the pathway and leads to interferon being produced when there is no virus. The DNA sensor cGAS has also been shown to be involved in some

autoimmune diseases, such as Aicardi-Goutieres syndrome, and the reduction of cGAS levels in mice have been shown to abolish these symptoms.

There a few of mechanisms of how non-self DNA can be detected while cellular DNA stays undetected. Possible mechanisms are sequence specificity of the DNA sensors, compartmentalization of these DNA sensors, and the structure of self DNA (Figure 2). Sequence specificity of nucleic acid sensors is something that has been studied and has been identified as a mechanism for sensing of specific viruses and not host nucleic acid (Vabret et al., 2017). It has been shown that certain nucleic acid sensors, such as TLR7 for RNA and Sox2 for DNA, are sequence specific and need a certain sequence in order to elicit a response. While this method of sensing specific sequences might be good to avoid self-sensing, this mechanism also has some severe downsides. One of these downsides is that as the nucleic acid sensor becomes specific, it starts to lose its ability to detect a wide variety of microbes and mutations can allow microbes to avoid detection. As PRRs, the nucleic acids sensors should sense many different types of viruses and not be restricted to one specific virus because then some viruses could be missed by the immune system and cause severe disease in the organism. Luckily, the two DNA sensors of interest, cGAS and IFI16, are not sequence specific and can lead to broad responses to a wide variety of viruses (Jakobsen *et al.*, 2013; Shu *et al.*, 2014). However, this does not lead us any closer to deducing how these DNA sensors are controlled to prevent sensing of self DNA.

Another mechanism that could allow for the discrimination between self and non-self DNA could be compartmentalization of the DNA sensors or host DNA. There have been many papers that show DNA sensors, such as TLR9, sense DNA in endosomes of the cell and cause immune activation against this DNA (Roers et al., 2016). This mechanism seems to present a clear and distinct way that the cell can sense foreign DNA without sensing host DNA; simply

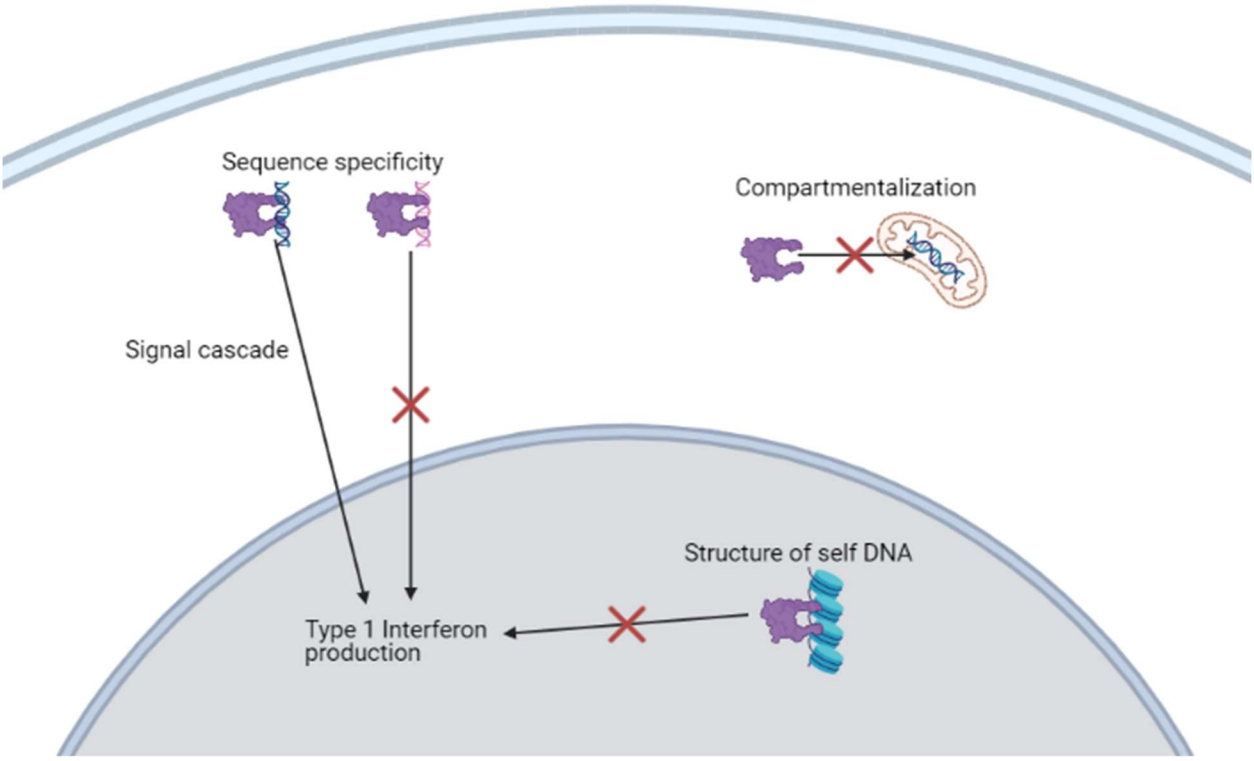


Figure 2: Mechanisms for discrimination between self and non-self DNA.

Each of these three mechanism could be a potential way that self DNA could be discriminated from non-self DNA. The first possible way is that the sequence of non-self DNA could be different than the sequence of self DNA. Therefore, a DNA sensor would not bind to self DNA and would not elicit a response while non-self DNA would elicit a type I interferon response. Another way of discrimination could be that the DNA cannot be accessed by the DNA sensor. This would prevent sensing of the DNA that is compartmentalized and therefore no signaling would occur. Lastly, the structure of host DNA could prevent sensing of host DNA by a DNA sensor. This would easily allow the cell to distinguish between host and non-host DNA based off of the associated proteins.

sense DNA where there should not be any DNA. Mitochondria also have DNA enclosed in them, but the DNA is not sensed due to a similar mechanism as discussed previously.

Generally mitochondrial DNA is not sensed by the cell due to the fact that the DNA resides in the mitochondria and cannot be accessed by any DNA sensors (Zierhut and Funabiki, 2020). However, mitochondrial DNA can be sensed in two ways: during a process called mitophagy, or when mitochondrial damage occurs due to a viral infection (Zierhut and Funabiki, 2020). Mitophagy is when damaged mitochondria are cleared from the cell and the parts are recycled. However, this process can have malfunctions where sometimes the DNA from the mitochondria can spill into the cytoplasm, where it can then be sensed by cGAS and cause interferon production (Sliter et al., 2018). Next, dengue and other flaviviruses damage the mitochondria during replication and can lead to the leak of mitochondrial DNA into the cytoplasm, which is then sensed by cGAS (Aguirre et al., 2017). This sensing of mitochondrial DNA is not bad and helps the cell to combat these viruses so well, that the virus has proteins to specifically degrade cGAS in order to be more viable.

While this mechanism for preventing the sensing of DNA due to subcellular location seems to work for endosomal DNA sensors and sensing of mitochondrial DNA, this mechanism does not work for DNA sensors that have been shown to reside in both the cytoplasm and the nucleus. When the field of DNA sensing was just starting, it was believed that the nucleus of the cell was “immune privileged” and the host DNA was not sensed due to the lack of DNA sensors in the nucleus (Paludan and Bowie, 2013). However, as discussed previously, this is not the case for cGAS and IFI16 which have both been shown to reside in both the cytoplasm and the nucleus (Dell'Oste *et al.*, 2014; Liu *et al.*, 2018; Volkman *et al.*, 2019). Another problem that arise if the nucleus was considered “immune privileged” is that several viruses, notably human simplex

virus (HSV), do not ever expose their DNA to the cytosol of a cell (Burdick et al., 2020; Kukhanova et al., 2014). After entering the cell, the genetic material of both these viruses is transported to the nucleus while still encapsulated in the capsid. This means the DNA genome of HSV never is exposed to the cytosolic DNA sensors and therefore no interferon response would be created to fight off HSV. This would lead to uninterrupted replication of HSV and potentially extreme disease in the patient.

Another virus that also does not expose its genome to the cytoplasm is human immunodeficiency virus (HIV). While HIV as a RNA genome, the genome gets reverse transcribed to DNA before it is integrated into the host's genome (Burdick *et al.*, 2020). Originally, the HIV genome was thought to have uncoated from its capsid and reverse transcribe in the cytoplasm of the cell, however, recent evidence has shown that the capsid delivers the HIV genome to the nucleus (Burdick *et al.*, 2020). This would again lead to awful effect of unregulated viral replication for the host organism due to no interferon response. HIV would be able to replicate uninterrupted and cause havoc on the organism by creating tons of virus and infecting many cells. These two examples of HSV and HIV show the importance of having DNA sensors in the nucleus and dismantles the theory that the nucleus is "immune privileged".

The last mechanism that could be involved in the discrimination of host versus non-host nucleic acid could be the structure of the nucleic acid. For RNA, structure plays a critical role in the discrimination of host versus non-host RNA (Bartok and Hartmann, 2020). Host mRNA molecules are not sensed by RNA sensors despite there are many of them floating around in a cell at any given time and this is due to the cap that is placed on the mRNA to mark it as a self RNA (Ramanathan et al., 2016). This cap is extremely important for the cell to distinguish between host and non-host RNA and protects the cell from accidentally sensing self RNA and

producing erroneous interferon. Another way that self RNA avoids sensing is through modification of the bases in the RNA which is a structural change of the RNA (Schlee and Hartmann, 2016). Modifications, mostly methylation of bases, have been shown to play a crucial role in not detecting tRNAs and rRNA (Schlee and Hartmann, 2016). Both of these RNAs are host RNAs that the cells need and both reside in the cytoplasm but are not detected due to the methyl groups added on to these RNAs. This is yet another method that the cell uses in order to avoid detecting self-nucleic acids based on structural changes.

Due to the massive amount of DNA in eukaryotic cells, estimated to be over 2 meters in length, the cell must find a way to structure and organize the DNA to be able to access key part of the DNA for gene expression, DNA replication, and other important processes (McGinty and Tan, 2015). The answer to the problem of organization is a circular, octameric protein called a histone (Taverna et al., 2007). The DNA is wrapped around the histone octamer 1.7 times; containing 147 base pairs of DNA (Mariño-Ramírez et al., 2005). Each one of these histone octamers wrapped with DNA is called a nucleosome and most of the DNA is wrapped around these histones except the linker pieces of DNA that link the nucleosomes together. Many nucleosomes come together to form chromatin, which is a complex and compact structure made of protein and DNA that helps to organize the DNA further (McGinty and Tan, 2015). In order to control the how compact the chromatin is, the cell uses protein called chromatin remodeling and histone modifying enzymes to either allow for more or less space in the chromatin. The cell must carefully regulate how densely the DNA packed in order to regulate the expression of genes in that area (Moosavi and Motevalizadeh Ardekani, 2016). In short, more tightly packed regions called heterochromatin are expressed less than less tightly packed regions called euchromatin. Chromatin structure plays major roles in cellular gene expression based on how tightly the

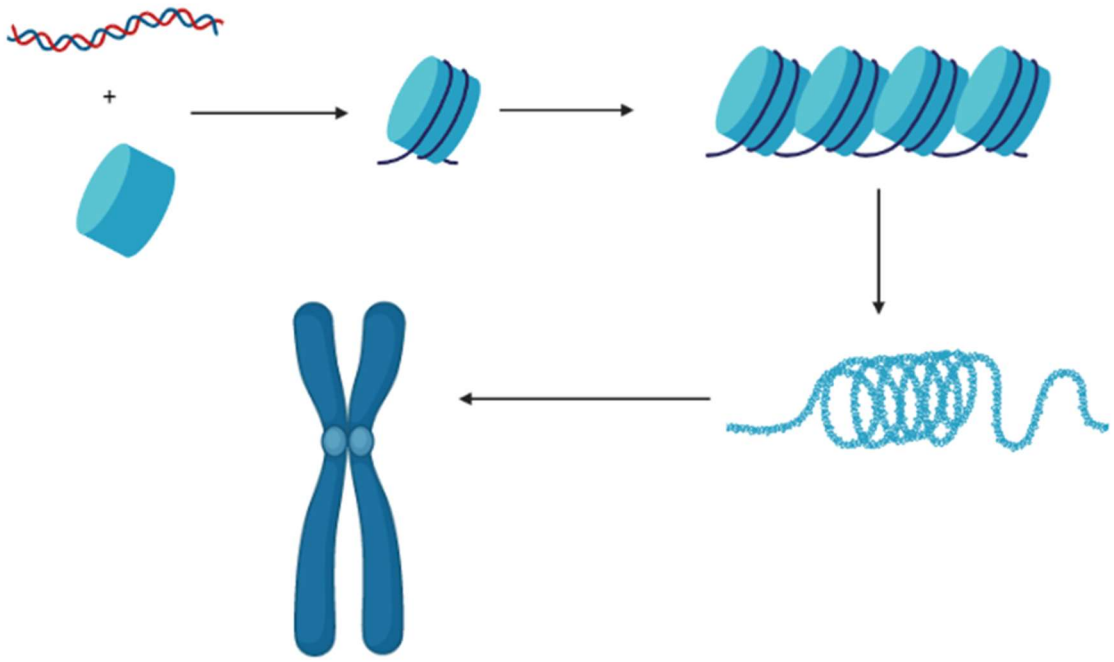

Figure 3: Details for the different levels of DNA organization for the cell.

The first level of organization of DNA in the cell consists of wrapping the DNA around histone octamers to form a nucleosome. The cell loads many histones on to the DNA and also adds chromatin binding proteins to form chromatin. Chromatin is a highly organized form of both DNA and protein in order to keep the DNA in the cell compact and organized.

nucleosome are packed together. Since all host DNA is in a nucleosome, this could point to a possible way that the cell can discriminate between host and non-host DNA.

If the nucleosome is an important factor in distinguishing between host and non-host DNA, there must be some mechanism that explains this effect. Perhaps the nucleosome could be inhibiting the DNA sensor and this would allow for a great way to distinguish between host and non-host DNA. In one paper, the authors show that cGAS was not activated by nucleosomes but was activated by the DNA after the histone was digested *in vitro* (Lahaye et al., 2018). This seems to point to an important role of the histone octamer in nucleosome by preventing the DNA from being sensed and therefore providing a way to distinguish between self and non-self DNA. However, there could be two ways that a nucleosome could not allow a DNA sensor to sense DNA: one way is that the histone octamer in the nucleosome blocks the DNA sensor from sensing the DNA due to steric issues, or perhaps the histone acts as an inhibitor to the DNA sensor and blocks its function that way. Again the literature seems to have started to dig into this topic with a paper showing that cGAS binds to the histone in a nucleosome (Boyer et al., 2020). That was not the only interesting discovery in this paper; the authors also show that cGAS not only binds the histone, but binds the histone in DNA binding site B of cGAS, which is the site with the greatest affinity for DNA. When cGAS binds the histone in site B, this does not allow cGAS to undergo a conformational change that would make it catalytically active to make 2'3' cGAMP when binding DNA in sites A or C. Therefore, STING is never activated and the pathway is stopped and does not lead to the production of interferon. However, this study was done *in vitro* and might not reflect what truly goes on inside the cell when looking at how cGAS could be inhibited by nucleosomes. This study also does not address IFI16 which opens more questions about how IFI16 could be regulated.

This project investigates whether nucleosomes will produce a type I interferon response when transfected into cells and start to decipher how the nucleosome is blocking DNA sensors. In order to do this, we transfected THP-1 cells, an immortalized monocytic cell line, with nucleosomes and compared this to nucleosomal DNA, which is DNA from nucleosome that was treated with a protease to remove the histone. We measured the amount of *ISG56* transcription, which is a gene that is stimulated by interferon, by doing a quantitative polymerase chain reaction (qPCR). If the data show that there is less expression of *ISG56* in the nucleosome condition versus the nucleosomal DNA, this would suggest that the nucleosome is causing the DNA to not be sensed. We hypothesize that the nucleosomes will lead to a smaller interferon response than nucleosomal DNA will due to the fact that the literature has shown that histones seem to play a crucial effect of regulating DNA sensing *in vitro* (Boyer *et al.*, 2020; Lahaye *et al.*, 2018)

Next, this study will also start to investigate how nucleosomes block DNA sensors. To accomplish this goal, we will again transfect THP-1 cells and use either plasmid and histone, plasmid or just histone. The key to his experiment is that the plasmid will not be wrapped around the histone to form a nucleosome. If the data shows that the plasmid and histone condition has less expression of *ISG56* than the plasmid condition, this will support that the histone has a more active role in inhibiting DNA sensors because the DNA will not have to be wrapped around the histone to cause inhibition. We hypothesis that the plasmid plus histone will lead to a smaller interferon response then just plasmid will due to the fact the literature has shown cGAS bind to histones which leads to the inhibition of cGAS (Boyer *et al.*, 2020).

## Methods

*Wrapping DNA around histones*

Eighty micrograms of DNA (obtained from Integrated DNA Technologies) was mixed with solid NaCl to make a solution with a final concentration of NaCl of 2M and 200 uL. Mixed in 0.127 ug of histone octamer (obtained from BPS Bioscience) at a 2.5:1 histone octamer to DNA ratio. Protease inhibitor (100x) was added at a volume of 20 uL to prevent degradation. The entire mixture was added to a mini dialysis bag (obtained from Thermo Scientific, molecular cutoff 10,000 kDa) according to the manufactures protocol. The dialysis bag was then placed into the dialysis solution (1.5M NaCl, 20 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 1mM DTT). It was left for 3 hours at 4$^{o}$ C before being moved to another dialysis solution (1.0M NaCl, 20 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 1mM DTT) for another 3 hours at 4$^{o}$ C. The dialysis bag was move again to another solution (0.6M NaCl, 20 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 1mM DTT) for 3 hours at 4$^{o}$ C. Lastly, the dialysis bag was moved to the final solution (0.25M NaCl, 20 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 1mM DTT) to sit overnight at 4$^{o}$ C. The contents of the bag were removed according to manufactures protocol and stored at 4$^{o}$ C. To verify the wrapping worked, a gel shift was run on a 5% dsDNA gel on ice. Each lane was loaded with the sample and 2 uL of 100% glycerol.

*Cell culture and stimulation*

THP-1 cells (obtained from ATCC), an immortalized monocytic cell line, were cultured in R10 medium containing RPMI media (obtained from Invitrogen), fetal bovine serum (obtained from Invitrogen, 10%), β- mercaptoethanol (obtained from Invitrogen, 50nM), non-essential amino acids (obtained from Invitrogen, 1mL/100mL), Normocin (obtained from Invitrogen 0.5mg/mL), and penicillin-streptomycin-glutamine (obtained from Invitrogen, 5mL/500mL). To mature these cells into macrophages, the cells will be treated with 5ng/mL of

phorbol 12-myristate 13 acetate (PMA) (Yoh et al., 2015). The cell will be matured for 72 hours before stimulation with the stimuli described below (Yoh *et al.*, 2015). The cells will be kept in a humidified incubator at 37° C at 5% $CO_2$.

Before stimulation, the cells will be counted and 2 x $10^6$ cell will be added to a 6-well plate in 3 mL of R10 medium. For part one, the nucleosomes were purchased from Millipore Sigma and the nucleosomal DNA was isolated from them by digestion of the histone octamer with proteinase K. Proteinase K (obtained from New England Biolabs) was added to the sample at a final concentration of 10 mg/mL (Doenecke, 1978). The nucleosomal DNA was added to the cells at a concentration of 4 ug/well as per the standard in the field (Shannon et al., 2018). To ensure 4 ug/well was added to the cells, we only did the digestion on the amount of nucleosome that would yield 4ug of nucleosomal DNA. According to Active Motif, a human nucleosome weighs 108 kDa and according to Integrated DNA Technologies, each base pair of DNA weighs about 650 Da. Multiplying 650 by the number of base pairs in a nucleosome gave us a weight of about 95.55 kDa. To find the percentage mass of DNA or protein, the mass of each was divided by the total mas of 203.55 kDa. This lead to the percent composition of a nucleosome to be 47% DNA and 53% protein. Using these percentages, we were able to find the amount of nucleosome to be digested which was 8.46 ug. The nucleosomes were added to the cells at a concentration of 8.46 ug/well in order to keep the amount of DNA the same in all wells.

The stimuli were delivered through transfection using OPTI-MEM (obtained from Invitrogen) and Lipofectamine 2000 (obtained from Invitrogen). In order to do the transfection, we mixed our stimulus into OPTI-MEM to a final volume of 250 uL. At the same time another mixture was made with 240 uL of OPTI-MEM and 10 uL of Liopfectamine 2000. Both of these were incubated at room temperature for 5 minutes then mixed together and incubated for another

20 minutes. After the 20-minute incubation, the 500 uL mixture was added to the matured THP-1 cells. The cells were incubated in a humidified incubator at 37° C at 5% $CO_2$ for 24 hours. After 24 hours, cells were harvested. We also set up a mock condition delivering OPTI-MEM and the Lipofectamine only and harvested at 24 hours as a negative control along with a time 0 condition, where the cells were harvested at stimulation. We decided to use the mock condition as our negative control to see what effect the Lipofectamine and OPTI-MEM would have alone since there were no stimuli. This also gives us a great condition to normalize our fold change off when we interpreted the data given from the qPCR that will be discussed later. No Lipofectamine or OPTI-MEM were added to the time 0. We used transfection to stimulate our cells because this allowed our stimulus to enter the cell and be delivered directly to the cytoplasm of the cell. Transfection can do this because it forms a lipid micelle around the stimuli which then can merge with the cell membrane and deliver the stimuli to the cytoplasm. Due to the large size of our stimuli, the stimuli would not be able to cross the membrane and would end up being stuck outside the cell if transfection was not used.

For part two, the cells were plated in the same way as in the previous part. The histones were purchased from BPS Bioscience and added to cell at a concentration of 4.46 ug/well. This concentration was used to keep the amount of histone added equal based of the amount added in the nucleosomes. We used the percentage of protein calculated above in order to find this concentration. The *LacZ* plasmid DNA was added to the cells at a concentration of 4 ug/well. We used this plasmid because it will not have an effect on our pathway and should only act as an exogenous source of DNA. The dose of 4 ug/well was used because it is a standard in the field (Shannon *et al.*, 2018). The stimuli were delivered the same way as previous using OPTI-MEM and Lipofectamine 2000 to do a transfection. The negative control and time 0 were also done as

well to act as control in the same way as discussed above and were set up as previously described.

*RNA extraction, cDNA synthesis, and qPCR*

After the stimulation for the times listed above, the cells were harvested, pelleted, supernatants collected. The cell pellets were used in the RNA extraction discussed next and the supernatants were saved for future experimentation. The RNA was extracted from the cells using the Zymo Research Quick-RNA MiniPrep Kit and the manufacturer's protocol including the optional DNase treatment step and was eluted with 50 uL of nuclease free water. The concentration of RNA was measured using a Thermo Fisher Nanodrop 2000 then stored at -80 °C. Using these concentrations and given that we wanted to use 1ug of RNA, we determined how much RNA is needed to make cDNA, then we used a ProtoScript II First Strand cDNA Synthesis Kit (obtained for New England BioLabs) to make cDNA.

qPCR was preformed using 10 uL of iTaq SYBR Green Super Mix (obtained from BioRad), 1 uL of each forward and reverse primer at a stock concentration of 10 uM, (obtained from Integrated DNA Technologies) and 2 uL of cDNA. The volume was brought up to 20 uL with nuclease free water. We used the primers to measure *ISG56* and *RPL37A* transcription. *ISG56* indicates how strongly the cells' interferon signaling pathway is turned on and *RPL37A* is a housekeeping gene used as a control. The sequences of the forward and reverse primers are listed below:

*ISG56* Forward: 5'-CCTCCTTGGGTTCGTCTACA-3'

*ISG56* Reverse: 5'-GGCTGATATCTGGGTGCCTA-3'

*RPL37A* Forward: 5'-ATTGAAATCAGCCAGCACGC-3'

*RPL37A* Reverse: 5'-AGGAACCACAGTGCCAGATCC-3'

The *ISG56* primers were designed based on Jakobsen et al 2013 and the *RPL37A* primers were designed based on Maess et al 2010.

The reactions were run in a BioRad CFX96 Real-Time PCR Machine with a 3 minute 95° C denaturation step and then 40 cycles of 10 seconds at 65° C to anneal, 30 seconds of extension at 60° C and reading of the SYBR Green dye at the end of each cycle.

In order to make understand the data collected from the qPCR, we converted the threshold cycle values (Cq) for *ISG56* to fold change using the *RPL37A* Cq values (Livak and Schmittgen, 2001). *RPL37A* encodes for a protein of the ribosomal subunit which makes it a perfect transcript to normalize the expression of *ISG56* to. The first step of this is to find the change (Δ) in Cq values by using the equation below:

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

To normalize the expression to background expression in the cells, the Cq values of the controls were used. We did this by finding the average ΔCq of the mock sample and then using this value to find the ΔΔCq value using the following equation:

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

Lastly, we used the ΔΔCq to find the fold change by using the equation below:

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

Our qPCR data is shown on graphs using the average fold change of each condition and with error bars indicating the standard deviation in the conditions.

In order to assess the quality of our qPCR, we examined the melt curves and the separation between the Cq values. The melt curve showed that there was only one product produced from the qPCR which is what was expected from a qPCR reaction. The Cq values were grouped extremely well based off condition and the spread between values from each condition was below the generally accepted spread of 0.3 between Cq values in each condition. The melt data and the Cq values showed that our qPCR was high quality and gave us great data that would be able to answer our questions.

## Results

We hypothesized that nucleosomes would lead to a smaller interferon response than nucleosomal DNA. We also hypothesized that plasmid plus histone will lead to a smaller interferon response than just plasmid. To test this hypothesis, we transfected THP-1 cells with the stated stimuli and then measured the expression of *ISG56* through qPCR. The value given from the qPCR was then turned into a fold change which was graphed. This setup allowed us to directly test out hypothesis since we will be transfecting our given stimuli and then measuring expression of *ISG56* which shows the amount of interferon and the interferon response that the cells made.

We did each of our experiments twice and each experiment is shown on a separate graph (Figure 4 and Figure 5). The T=0 condition showed the amount of basal expression of *ISG56* in our THP-1 cells and the mock condition showed the response that the cells had to the Lipofectamine and the OPTI-MEM used in the transfection. We see both of these conditions show low fold change meaning that there seems to be no effect of our transfection agents on our experiment (Figure 4 and Figure 5).

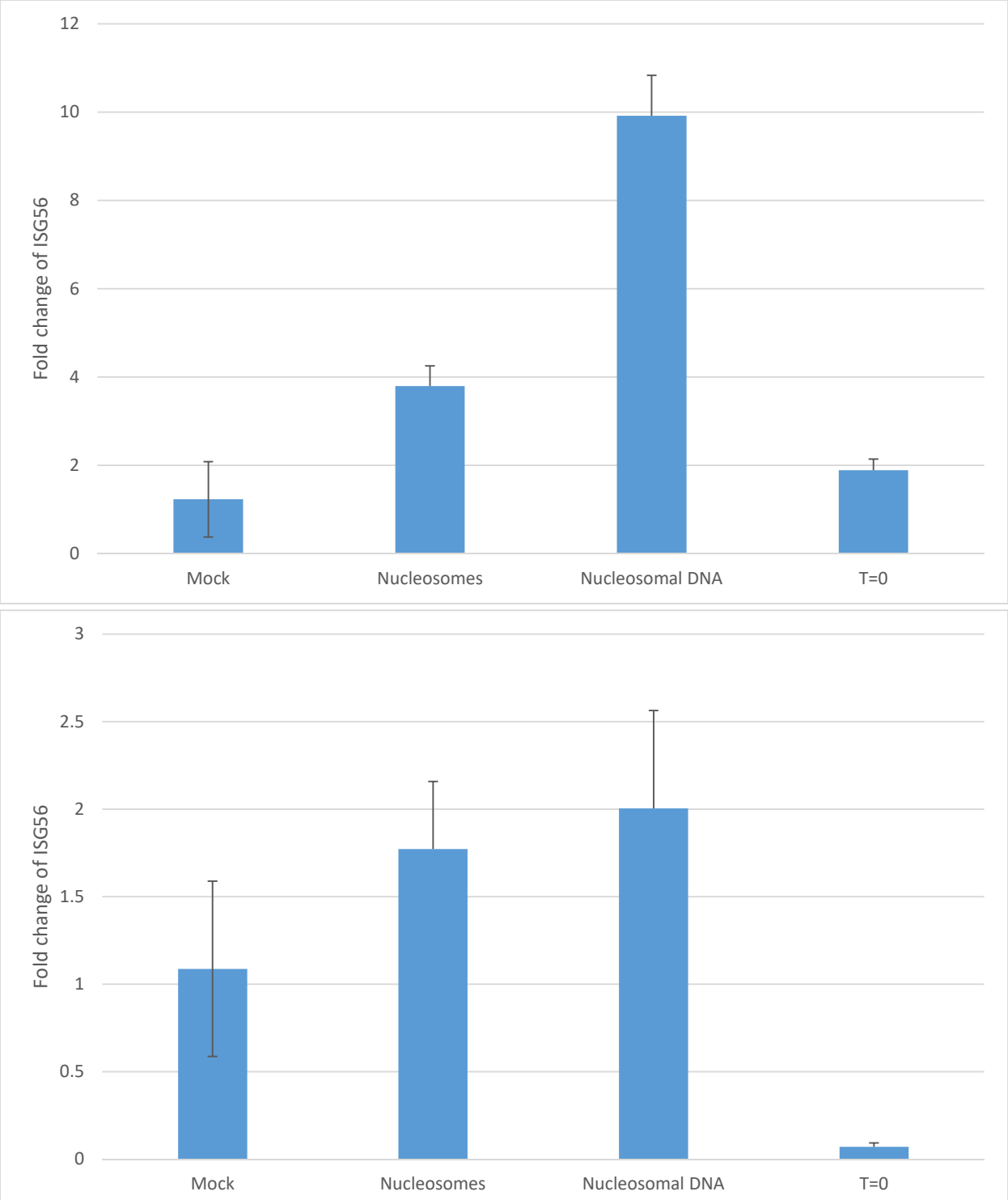


Figure 4- Measure of fold change when cells are stimulated with nucleosomes or nucleosomal DNA

THP-1 cells were matured into macrophages using PMA the were transfected with nothing, nucleosomes or nucleosomal DNA. T=0 condition was harvested at the same time the other cells were being stimulated.

In part 1, we transfected nucleosome or nucleosomal DNA into THP-1 cells in order to discover if nucleosomes can reduce the expression of *ISG56* using qPCR to measure the transcripts. We saw that nucleosomal DNA induced a higher level of expression of *ISG56* than the nucleosome (Figure 4). This indicates that DNA wrapped around histones to form nucleosomes seems to lead to DNA sensors not being able to either bind the DNA either because of the sterics that the histone causes or that the histone is inhibiting our DNA sensors. This gives a good mechanism that allows DNA sensors to distinguish from self and non-self DNA even though the DNA sensors might be able access the self DNA.

This result led us to part 2 of our experiments. We designed this experiment in order to test whether the histone is causing this reduction in *ISG56* expression or whether this reaction is due to the DNA wrapping. In order to test this, we transfected in either plasmid, plasmid plus histones that did not form a nucleosome or just histones and then we measured the expression if *ISG56* through qPCR. We used plasmid plus histones because since the plasmid would not wrap around the histone on its own and therefore would allow us to determine if the wrapping played a large role in the lower interferon response. If we were to see that the plasmid plus histone condition had the same *ISG56* expression as the just plasmid condition, we would conclude that the DNA must be wrapped around the histone in order to have less interferon production. However, if we see that there is less *ISG56* expression in the plasmid plus histone condition than the plasmid condition, then we can conclude that the histone is inhibiting the DNA sensor without requiring the DNA to be wrapped around the histone.

We found that the plasmid plus histone condition has less *ISG56* expression than the plasmid condition (Figure 5). This indicated that the histone inhibited the DNA sensor even when the DNA was not in complex with the histone to form a nucleosome. This supports that the

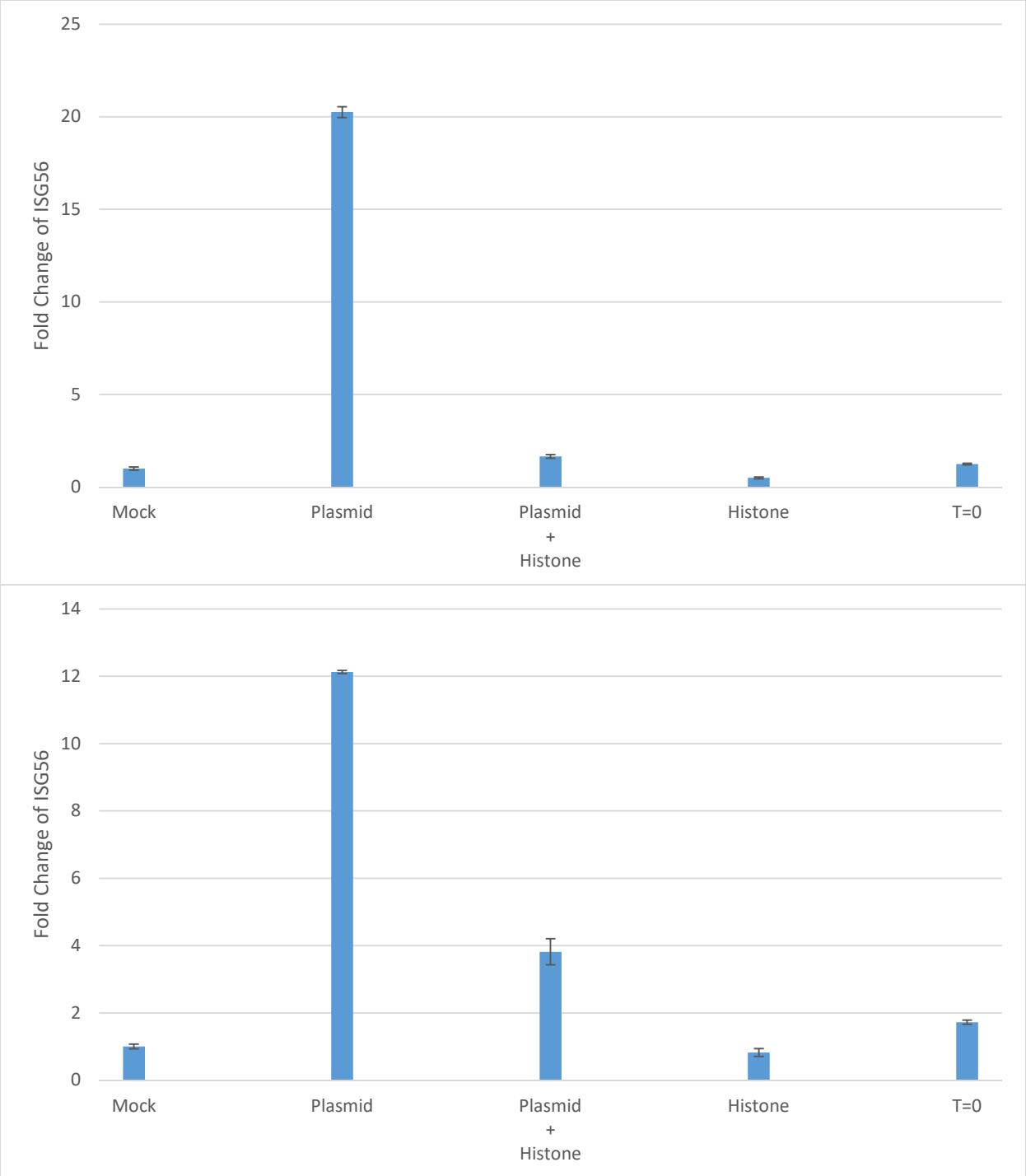


Figure 5- Measure of fold change when cells are stimulated with plasmid plus histone or plasmid

THP-1 cells were matured into macrophages using PMA the were transfected with nothing, plasmid DNA, plasmid DNA + histone or histone alone. T=0 condition was harvested at the same time the other cells were being stimulated.

histone is actively inhibiting our DNA sensor because we have clear evidence that the histone does not have to be in a nucleosome to reduce expression of *ISG56*.

## Discussion

The aim of this study was to elucidate how self and non-self DNA can be distinguished in the cell from DNA sensing perspective. We hypothesized that the structure of the host DNA, specifically DNA wrapped on histones to form nucleosomes, is responsible for determination between self and non-self DNA and nucleosomes would lead to less a smaller interferon response than nucleosomal DNA. We also hypothesized that the histone would actively inhibit the DNA sensor and would not require wrapping of the DNA to cause a smaller interferon response when the cells are transfected with plasmid plus histone than with just plamid.

Our findings here show the beginning of what could be an extremely interesting discovery and could help decipher how cells can distinguish between host and non-host DNA. We demonstrated that nucleosomal DNA separate from the nucleosomal proteins induces a much larger *ISG56* response than full nucleosomes which clearly points to the histone playing a crucial role of blocking the sensing of transfected DNA (Figure 4). The next question that now remains is how the histone leads to this lower expression of *ISG56*. We hypothesize two possible ways that the histone can prevent a DNA sensor from bind to the DNA; one possibility is that the histone, when in complex with DNA to form a nucleosome, simply sterically blocks the DNA sensor from properly binding to the DNA and then signaling. The other possibility is that the histone actively inhibits the activity of our DNA sensor thus not allowing the DNA sensor to either bind the DNA or signal after binding. Our data suggests that the latter it true and histones prevent a interferon response being generated.

There seems to be growing evidence that histones can competitively inhibit cGAS and binds to the same site where DNA binds on cGAS (Boyer *et al.*, 2020). However, the study mentioned did these studies with purified proteins and not in actual cells. We wanted to test if this same inhibition was found when the entire cell was used. Our results supported what was seen using purified proteins and indicate that the histone actively represses the DNA sensor. The DNA sensor that is being inhibited is not clear but other work indicates that it would be cGAS (Boyer *et al.*, 2020). However, more experiments could be done to continue this work.

Further experiments could reveal more and perhaps could lead to even more question of how cells can distinguish between host and non-host DNA. One possible could be to look at what DNA sensors are being inhibited by the histone in the cell. This could be done through looking at proximity of these DNA sensors to the transfected histone through either an immunoprecipitation and a western blot or through fluoresce microscopy. This would allow for us to know what DNA sensor is being bound by the histone or if the histone is binding multiple of the DNA sensors. If we see another DNA sensor than cGAS being bound, we could do similar experiment to Boyer et al. in order to determine if the DNA sensor is just binding to the histone or it is actually being inhibited. Specifically, what happens to IFI16 during this inhibition is extremely interesting sense it is a DNA sensor and would be expected to have some sort of preventive mechanism from sensing self DNA. Lastly, we could also use out CRISPR THP-1 cell lines that are missing different DNA sensors and see which missing DNA sensor abolishes the effects we have outlined here.

Another interesting experiments that could be done are to investigate the differences in using a plasmid versus using a linear piece of DNA when co-transfecting with histones. The literature shows that there seems to be 2 ways that DNA can be sensed: either via sensing of the

back bone of DNA, as has been described for cGAS, or via sensing of the free ends of the DNA, as has been described for a DNA binding protein DNA-PK. DNA-PK was recently shown to participate in a STING-independent DNA sensing pathway (Burleigh et al., 2020). Both of these pathways lead to the production of interferon but seem to detect different structures of DNA in the cell. Perhaps a difference could be seen if the plasmid DNA, which only allows for backbone sensing, was cut and now had free end that could be sensed by a separate pathway.

This same paper seemed to show a time difference between these two pathways suggesting that the cGAS-STING pathway peaks in its interferon production earlier than the DNA-PK pathway. This could suggest that moving our 24-hour time point earlier or taking multiple time points could lead to a better understanding of which pathway is being inhibited and could lead to more questions about how the histone interacts with each pathway.

Lastly, we could also use the supernatants taken from the cells before harvesting and either run a ELISA or a HEK-BLUE assay in order to test for the amount of interferon produced by the cells. This will help support our data and will show in a different manner than these stimuli decrease the amount of interferon directly, rather than looking at transcription of an interferon stimulated gene.

The data presented here introduces a mechanism of how cell can distinguish between host and non-host DNA and lead to selective interferon production based off the structure of the DNA. This mechanism solves the question of how DNA sensors are regulated in the nucleus to prevent mass sensing of host DNA which in turn can prevent the development of some interferon based autoimmune disease in humans. This information advances the knowledge in the field by discovering an important regulation of DNA sensors. This new information could lead to more

questions investigating the role of dysfunction of critical pathway and what the results could be for the organism and for the cell.

In conclusion, we present data that supports our hypothesis that nucleosomes inhibit *ISG56* production and show that this is a method that cells use to distinguish between host and non-host DNA. We then show that the histone actively inhibits the DNA sensor and works more than a steric blockage. Overall, our data our data shows that the histones play a critical role in host versus non-host DNA sensing and should be studied more in order to learn more about the roles of histones in viral infection.

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