DNA Sensing in Myeloid Cells

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1. Abstract

Mycobacterium tuberculosis remains a top threat to public health globally. Current research has been focused on creating a protein subunit vaccine that is capable of providing enhanced protection against the pathogen, as well as protection for immunocompromised individuals. Our lab previously showed that cyclic dinucleotides (CDNs) were an effective adjuvant for a TB protein subunit vaccine. We sought to examine whether CDNs were capable of eliciting cytokine signaling that favored a Th17 response upon transfection of different myeloid cells, and whether this cytokine signaling differed based on which CDN was used. To see whether cyclic dinucleotides could successfully induce a cytokine response in myeloid cells, we transfected bone marrow derived macrophages, bone marrow derived dendritic cells, and human CD14+ macrophages with CDNs, and analyzed which cytokines were induced via qPCR. Our results suggested that structurally unique cyclic dinucleotides produce differential cytokine signaling, highlighting a potential role for CDNs downstream of the STING pathway.

We also were interested in pathogen DNA sensing in neutrophils during infection. We transfected STING-deficient and Sox2-deficient ER-HoxB8 neutrophils with *L. monocytogenes* genomic DNA to see how the removal of these genes affected cytokine signaling. Our results demonstrated that STING is necessary for proper cytosolic DNA sensing in neutrophils

2. Introduction

2.1. Tuberculosis

Tuberculosis is one of the top 10 causes of death worldwide, and the leading cause of death for HIV positive individuals[1]. Tuberculosis is caused by an infection of the bacterium *Mycobacterium tuberculosis* in the lungs, and is transferred between hosts through the air. If left untreated, this infection can become fatal[2]. Currently, the Bacille Calmette-Guerín (BCG) vaccine is used globally to provide protection against *M. tuberculosis*, but this vaccine has highly variable efficacy[3]. In addition, as a live-attenuated vaccine, it is considered unsafe for immunocompromised individuals to receive the vaccine because of the possibility for it to produce an infection, leaving HIV positive children particularly vulnerable[25]. As one of the greatest threats to public health globally, current research is focused on prophylactic interventions and treatments, such as a new vaccine that is more effective and able to protect the most vulnerable. Researchers have been focused on developing a protein subunit vaccine that can provide better protection against *M. tuberculosis* and much needed protection for immunocompromised individuals.

2.2. Adjuvants

In order for vaccines to successfully build immunity, the vaccine must stimulate both the innate and adaptive immune systems. Live-attenuated vaccines can achieve both because they contain weakened pathogens that can immediately activate the innate immune system and later induce adaptive immunity[4]. Protein subunit vaccines are inactivated vaccines, meaning they are free of live components of pathogens[5]. In order to properly activate the innate immune system, these vaccines usually require an immunostimulating adjuvant[6].

Adjuvants have many different mechanisms of action when delivered in a vaccine. They can elicit an immune response by causing prolonged antigen release at the site of injection, up-regulating cytokine production, recruiting immune cells to the site of injection, increasing antigen uptake and presentation to antigen-presenting cells, activating antigen-presenting cells, or activating inflammasomes[7].

2.3. Cyclic Dinucleotides as Adjuvants

Cyclic dinucleotides (CDNs) were first discovered to play an important role as secondary signaling molecules in bacteria, and now mammalian cells[8]. It was originally thought that CDNs only functioned as bacterial pathogen-associated molecular patterns (PAMPs)[8], which are able to be recognized by pattern recognition receptors of an infected host. However, it is now known that CDNs also play an important role in cytosolic DNA sensing in mammalian immune cells.

Stimulator of interferon genes (STING) is a signaling molecule that mediates cytosolic DNA sensing. STING is known to be important for the immune response, cancer, and senescence in cells[9]. The insertion of double stranded DNA from viruses or bacteria or bacterial cyclic dinucleotides sets off an emergency cascade meant to alert the cell of intracellular infection and begin the innate immune process. Double stranded DNA can bind to cyclic adenosine monophosphate synthase (cGAS), producing a conformational change in the enzyme. This allows it to convert guanosine triphosphate (GTP) and adenosine triphosphate (ATP) into cyclic guanosine monophosphate-adenosine monophosphate (cGAMP),

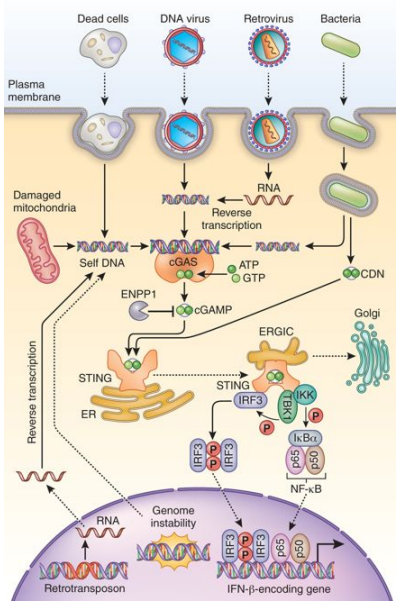


Fig I-1 DNA/CDN sensing in the STING pathway

an endogenous cyclic dinucleotide. cGAMP can then bind to STING, which activates TANK-binding kinase 1 (TBK-1), IFN regulatory factor 3 (IRF3), and NF-**κ**B[9] (Figure I-1)[22]. These subsequently induce the transcription of Type I interferons and other cytokines that are vital to immune cell recruitment during infection.

Van Dis et al. showed that cyclic dinucleotides were effective acting as an adjuvant for a protein subunit vaccine consisting of five *M. tuberculosis* proteins, as well as acting as a booster for the BCG vaccine[10]. The cyclic dinucleotides used in this experiment were RR-CDG and ML-RR-cGAMP. RR-CDG is known to activate murine STING, but not all human STING alleles, whereas ML-RR-cGAMP has been found to activate both mouse and human STING alleles[24].

In addition, the group found that the adjuvants tested elicited an antigen-specific Th1 and Th17 response. Th1 cells have long been thought to be the most important T cells in fighting infection by *M. tuberculosis*. Th1 cells are induced by IL-12 and produce IFN-γ upon infection, which in turn activate macrophages, helping to control infection[11]. Studies have shown that IL-12-deficient mice infected with *M. tuberculosis* were unable to properly control bacterial growth upon infection, indicating that Th1 cells as well as macrophage activation are paramount to host defense during infection by *M. tuberculosis*[12]. Th17 cells have also been proven to be critical during infection. Differentiation of Th17 cells depends on IL-6 and TGFβ, with IL-23, IL-1β, and TNFα in charge of maintaining these cells. Induction of Th17 cells leads to the production of IL-17, which causes the activation and recruitment of macrophages, helping to generate inflammation during infection[11]. The BCG vaccine provides protection by inducing Th1 and Th17 responses, however the Th17 response during infection is not well understood[23]. Surprisingly, Van Dis et al. found that vaccine-induced Th17 and not Th1 responses correlated

with protection against *M. tuberculosis*, suggesting that CDN adjuvants may be particularly useful for activating Th17 cells.

2.4. Neutrophils in TB Infection

Neutrophils, the most abundant type of white blood cells, are the first-responders in the body and play a significant role in innate immunity. However, in a TB infection, neutrophils have been correlated with higher bacterial burdens and worse outcomes for humans and mice. Neutrophils seem to be able to engulf, but not kill the bacteria[15]. In mice genetically susceptible to TB, researchers found prolonged accumulation of neutrophils in the lungs and that they might actually attack host lung tissue thereby aiding infection[16]. Berry et al. discovered that active TB-infected patients display a Type I IFN inducible transcriptional signature, indicating that Type I IFN(α/β) signaling is correlated with active disease[13]. Intriguingly, this response is driven by neutrophils. Sox2 is a transcription factor primarily known for its role in early embryogenesis and cell fate determination[18]. Xia et al. reported that Sox2 acts as a double stranded DNA sensor of *Listeria monocytogenes* genomic DNA in neutrophils, rather than STING[19] as in macrophages (Figure I-2)[17]. They also reported that Type I IFN signaling is not induced by pathogen DNA in their mouse-derived neutrophils.

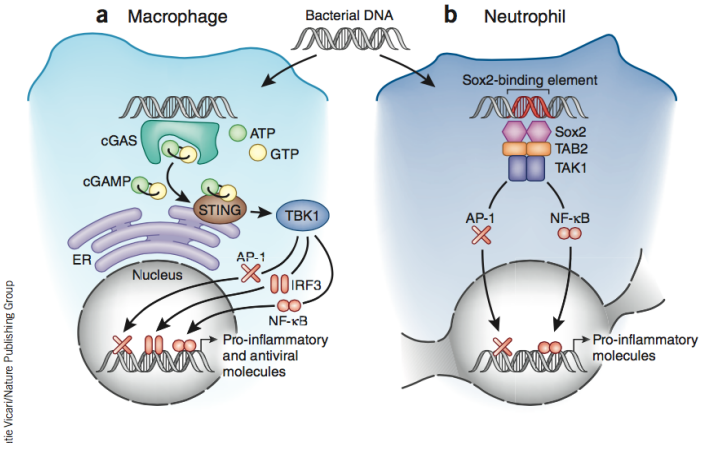


Fig I-2 Double stranded DNA sensing in macrophages and neutrophils

2.5. *Research Question*

With current research focused on the delivery of a new, safer vaccine for *M. tuberculosis*, it is important to discover a suitable adjuvant that can elicit an immune response capable of

fighting off infection and providing long-term protection. We were interested if different cyclic dinucleotides could successfully induce cytokine production in myeloid cells *in vitro*. We were also interested in how this cytokine production varied across adjuvants and different immune cells, and whether cytokines important for Th17 immune responses were induced.

While the cGAS/STING pathway has been studied extensively in macrophages and monocytes, much less is known about its role in neutrophils. We aimed to investigate whether neutrophils use the same double stranded DNA sensing mechanism (STING) as in other myeloid cells, and whether STING or Sox2 signaling leads to Type I IFN induction in ER-HoxB8 progenitor-derived neutrophils.

3. Methods

3.1. Materials

Structurally unique cyclic dinucleotides were obtained from *Aduro Biotech* (Berkeley, CA). Bone marrow derived macrophages and dendritic cells were harvested from the tibia and femur of a B6 mice, which are housed in Li Ka Shing under the supervision of OLAC. Human macrophages were isolated from fresh buffy coat, and positively selected for CD14+ cells. ER-HoxB8 neutrophil progenitors were obtained from mice expressing Cas9 by the Barton Lab at UC Berkeley.

3.2. Mouse Macrophage Culture and Transfection

After harvesting and freezing bone marrow derived macrophages (BMDMs), macrophages were thawed, placed into media (10% fbs, 10% M-CSF in DMEM), and plated into 8 wells of a 12 well plate at 6e5 cells/mL, with 1mL of cells in each well. Cells were kept at 37℃ in a humidified incubator for 48h until transfection. Media was changed after 24 hours. After 48h, cells were transfected with either OptiMEM (control), PAM (50ng/mL), cyclic-di-AMP (CDA) (4µg/mL), ADU V16 (4µg/mL), ADU V17 (4µg/mL), ADU V18 (4µg/mL), or ADU V19 (4µg/mL). ADU V16 (RR-CDG) and ADU V17 (ML-RR-cGAMP) were used as positive controls. Cyclic dinucleotides were diluted to desired concentrations from 10mg/mL stock solutions into PBS, and 4.4µL of these diluted samples were added to 50µL of OptiMEM. Lipofectamine2000, a common transfection reagent, was added into another 50µL of OptiMEM. As a positive control, 3µL of 50µg/mL PAM was added to 300µL of OptiMEM, without lipofectamine. Both mixtures of OptiMEM with LF2000 and DNA were added together, incubated at room temperature for 20 minutes, and then added to the cells. The plate was returned to the 37℃ incubator until the 6hr harvest time point.

3.3. Mouse Dendritic Cell Culture and Transfection

After harvesting bone marrow derived dendritic cells (BMDCs) from a B6 mouse, cells were plated at 5e6 cells/ 10cm tissue-cultured treated dish in 10mL of of medium (10% FBS, 1x HEPES, 1x NaPyr, 1x Glutamax, 1x Pen/Strep, 55uM BMe, 20ng/mL GM-CSF, 20ng/mL IL-4, and cRPMI up to desired volume). Cells were given more media on day 3 of differentiation. On day 7 of differentiation, the floating fraction of cells was harvested and enriched for dendritic cells CD11c+ beads using the MACS protocol. Cells were plated at 1.2e6 cells/mL/well in 2x12 plates, for 15 wells total. Plates were returned to the 37℃ incubator until 2hr and 24hr harvest time points.

Cells were transfected 2 days after plating, and followed the same protocol as the bone marrow derived macrophages. The 6hr samples were transfected with either untreated (OptiMEM), PAM, CDA, ADU V15, ADU V16, ADU V17, ADU V18, or ADU V19, whereas the 24hr samples received everything but untreated.

3.4. Human PBMC CD14+ Cell Culture and Transfection

The PBMC layer from fresh buffy coat was isolated and brought to 50mL with PBS. We then centrifuged at 350g for 10 minutes, aspirated the supernatant, resuspended in 30mL PBS, and repeated. After the second aspiration, cells were washed with 20mL of serum-free RPMI, centrifuged again for 7 minutes, and resuspended in 20mL serum-free RPMI. CD14+ macrophages were then selected for by following the MACS Miltenyi CD14+ selection protocol. CD14+ cells were then aspirated, resuspended in RPMI, and added to plates coated with Poly-D Lysine at 3e5 cells/well to collect for 2hr and 6hr time-points. After an hour, RPMI and non-adherent cells were removed and replaced with 200µL of fresh PBMC media (0.6% NEAA,

0.6% Qmax, 0.6% NaPyruvate, 40% Human Serum, and RPMI 1640 up to desired volume), with 10ng/mL GM-CSF and kanomycin.

Half media changes occurred every 2 days. After two weeks of differentiation, the media was completely changed without GM-CSF or kanomycin, to a total of 500µL/well. We then stimulated PBMCs with untreated, CDA, ADU V15, ADU V16, ADU V17, ADU V18, or ADU V19. We created stimulation concentrations of 10µM of each cyclic dinucleotide in 1200µL of PBMC medium. The 500µL in each well was removed and replaced with 500µL of the one of the CDN mixes for transfection. The untreated sample received 500µL of straight PBMC media. Samples were returned to the 37℃ incubator until 2hr and 6hr harvest time points.

3.5. *Neutrophil Cell Culture and Transfection*

Graduate student Robyn Jong made CRISPR knockouts of Sox2 and STING neutrophil progenitors that were frozen down. Another cell line was transduced with a non-targeting control sgRNA. In summary, the cell lines used were control (non-targeting sgRNA 1), Sox2 KO (sgRNA 1), and STING KO (sgRNA 4). 1mL vials of cells at 1e6 cells/mL were thawed 6 days prior to transfection, placed in 9mL of progenitor medium (10% fbs, 100x Qmax, 1% SCF conditioned medium, 30µM BMe, and OptiMEM up to desired volume). 1µL 10,000x **β**-estradiol was added to medium for each 10mL of medium. 4 days prior to infection, estrogen was withdrawn from cells by washing with PBS twice to begin differentiation. Cells were plated at 2.5e6 cells/non-tissue culture treated T25 flask without estrogen in 10mL of medium. On the day before transfection, fully differentiated cells were harvested and plated on 2 tissue culture treated 12-well plates at 1.25e6 cells/mL. Each cell type was plated into 5 wells, with 1mL in each well.

For transfection, cells types were transfected with either untransfected, CDA, dsDNA (45bp ISD), or L. monocytogenes genomic DNA (gDNA). CDA, dsDNA, and gDNA were transfected

at a final concentration of 1µg/mL in 1mL of medium per well. Calculated volumes of each transfection condition stock solutions were added to 450µL of OptiMEM with 4.5µL of Lipofectamine2000. They were incubated at room temperature for 20 minutes, added to the RNA wells, and plates were returned to the 37℃ incubator. The plate designated for protein harvest was harvested at this step (*see 3.8)*.

3.6. *RNA Harvest and Isolation*

For macrophages and dendritic cells, supernatants were aspirated, and plates were washed with 1mL of PBS. This was then aspirated and replaced with 500µL Trizol. We then pipetted up and down and transferred lysates to an eppendorf tube. For PBMC macrophages, a similar protocol was followed but without the PBS wash.

For ER-HoxB8 neutrophils, a similar protocol was also followed for harvesting RNA. Supernatants with the floating fraction of cells for each sample were transferred to eppendorf tubes and centrifuged at 12,000xg for 5 minutes to collect the cell pellet. The supernatant was then discarded. 1mL of Trizol was added to each well to collect adherent cells, pipetted up and down, and then pooled with the respective cell pellet.

3.7. *Reverse Transcription and RT-qPCR*

Each cell line followed the same RNA isolation procedure using the Trizol/RNeasy hybrid protocol. Chloroform was added to each sample (100µL for 500µL of Trizol, 200µL for 1mL of Trizol). Samples were placed into phase-lock tubes, vigorously shaken, and centrifuged at 12,000xg for 15 minutes. The aqueous phase containing the RNA was placed into fresh tubes, and an equal volume of RNase free ethanol was added to each tube. The RNeasy Mini Kit was then used, using instructions provided by the manufacturer *Qiagen*. RNA concentration was determined using OD 260/280 ratio.

RNA was then converted to cDNA. 1000ng of RNA for BMDM, BMDC, and PBMC macrophages, or 700ng of RNA for ER-HoxB8 neutrophils, was then treated with DNase using the Invitrogen DNase I protocol, followed by reverse transcription using Invitrogen's SuperScript® III First Strand Synthesis System. 20µL of cDNA from each sample was placed in 180µL of dH2O, followed by RT-qPCR with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and run in a CFX96 machine. Target genes for BMDMs, BMDCs, and PBMC macrophages included **β**-actin, 18s rRNA, IFN**β**, TGF**β**, IL12 p40, IL6, IL23 p19, IL12 p35, and TNF**α**. Target genes for neutrophils included **β**-actin, STING, 2 different Sox2 primers, pan IFN**α**, IFN**β**, cGAS, TNF**α**, and IL6. Gene expression levels were normalized to **β**-actin or 18s rRNA. A detailed list of primers used can be found in the supplementary section. BMDMs, BMDCs, and PBMC macrophages were tested using triplicates, whereas ER-HoxB8 samples were tested with duplicates.

3.8. Neutrophil Western Blot

The second plate of ER-HoxB8 neutrophils, with 1 well per cell line, was not stimulated with anything, and instead was harvested for protein. Supernatants were harvested and spun down to a pellet. The plate was placed on ice with 400µL of 1x SDS added to each well. After pipetting up and down, the mixture was used to resuspend their corresponding pellets. Lysates were boiled for 10 minutes and stored at -20℃ until performing a Western blot.

3.9. *Statistical Analysis*

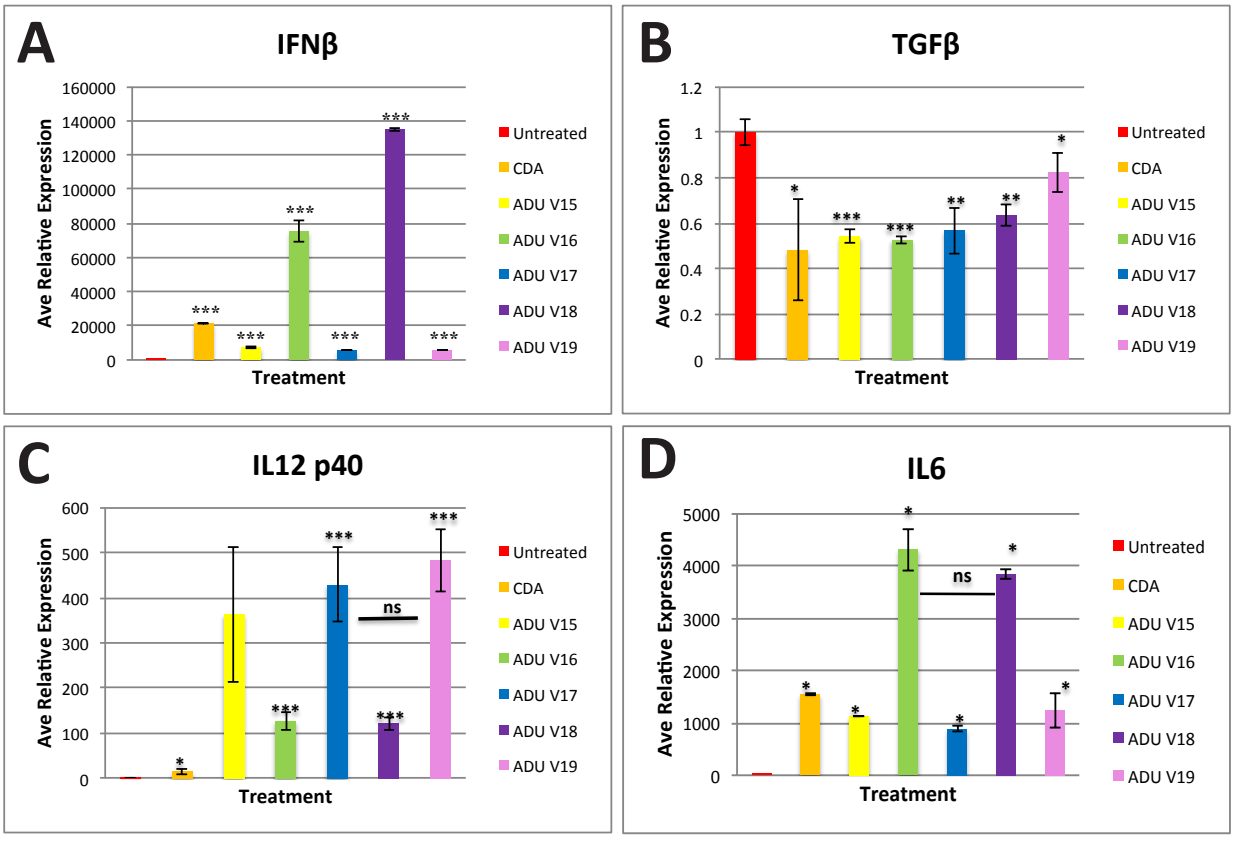
qPCR cycle threshold (Ct) values were normalized to **β**-actin for BMDMs, BMDCs, and ER-HoxB8 neutrophils, and 18s rRNA for PBMCs, both common housekeeping genes. All qPCR graphs were made in Excel (version 16.16.4) and display average normalized expression with error bars representing the standard deviation of these normalized expression values. The data

were statistically analyzed in Excel using a two-tailed Student's t-test, comparing untreated samples with all conditions (unless otherwise noted). P-values < .05 were considered significant and denoted with 1 asterisk; p-values < .01 were denoted with 2 asterisks; p-values< .001 were denoted with 3 asterisks.

4. Results

4.1. *BMDM Cytokine Production*

To determine if cyclic dinucleotides could successfully induce cytokine production, we first looked at mouse bone marrow derived macrophages (BMDMs). We stimulated tissue cultured macrophages with structurally unique CDNs (ADU V15-V19), with CDA, ADU V16 and V17 are positive controls. We analyzed the resulting cytokine response via RNA harvest and RT-qPCR. Type I interferon (IFNβ) induction was an expected result of STING activation. IL-6, TGFβ, IL-23 (consisting of subunits IL12 p40 and IL23 p19), and TNFα expression were also studied for their known roles in Th17 response activation and maintenance *in vivo*.



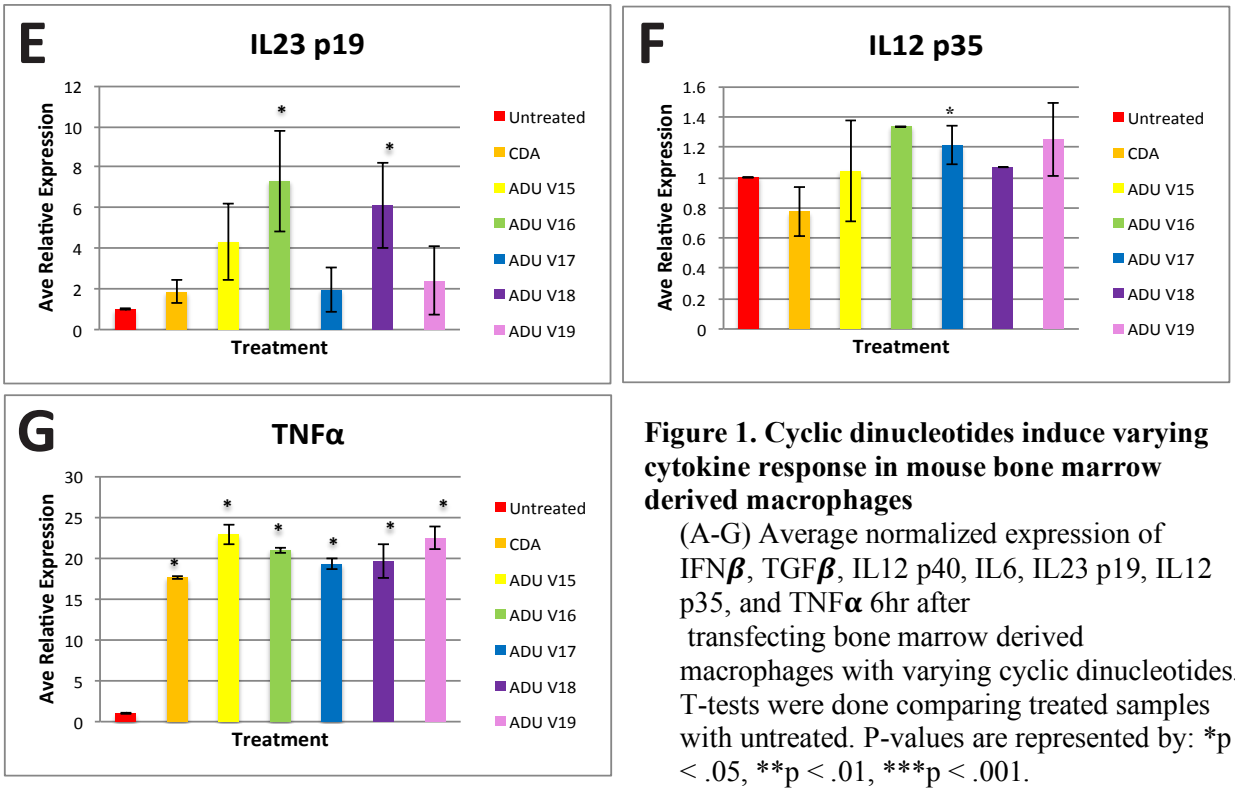


Figure 1. Cyclic dinucleotides induce varying cytokine response in mouse bone marrow derived macrophages

(A-G) Average normalized expression of IFN***β***, TGF***β***, IL12 p40, IL6, IL23 p19, IL12 p35, and TNF**α** 6hr after transfecting bone marrow derived macrophages with varying cyclic dinucleotides. T-tests were done comparing treated samples with untreated. P-values are represented by: *p < .05, **p < .01, ***p < .001.

Figure 1 shows that CDNs can not only elicit cytokine production in BMDMs, but also this cytokine production varies between CDNs. ADU V16 and V18 appeared to be the most potent inducers of IFNβ, IL23 p19, and IL6, whereas ADU V17 and ADU V19 seem to be the most potent inducer of IL12 p40. Other cytokine production varied less between CDNs stimulation. All CDNs tested saw lower relative expression of TGFβ compared to untreated and higher relative expression of TNFα compared to untreated. There was no expression of IL-12 p35 across all treatments.

4.2. BMDC Cytokine Production

Dendritic cells serve as messengers between the innate and adaptive immune systems. As antigen-presenting cells (APCs), dendritic cells are key initiators of the T-cell response[20]. Therefore, we were curious to see whether CDNs could induce cytokine signaling in bone marrow derived dendritic cells (BMDCs), and if this signaling varied among CDNs.

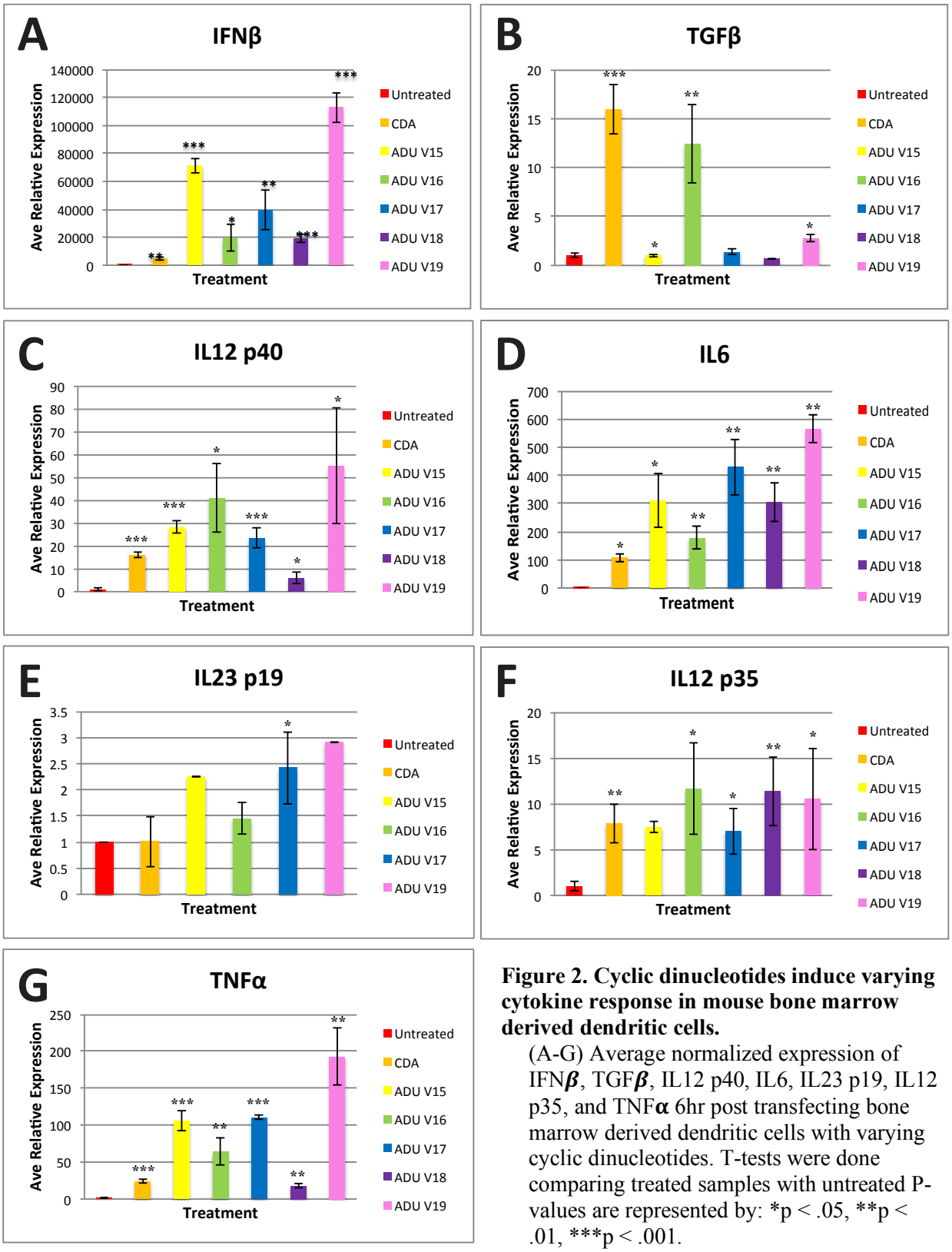


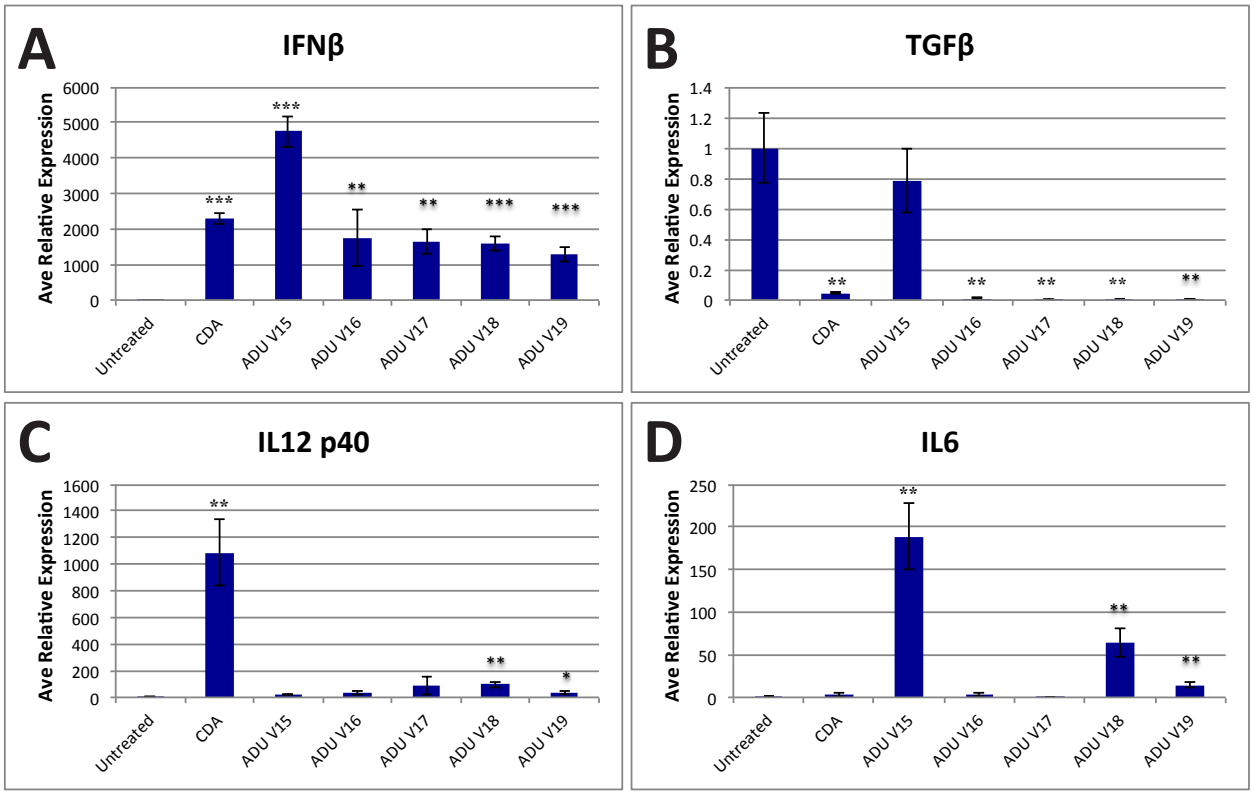
Figure 2. Cyclic dinucleotides induce varying cytokine response in mouse bone marrow derived dendritic cells.

(A-G) Average normalized expression of IFN***β***, TGF***β***, IL12 p40, IL6, IL23 p19, IL12 p35, and TNF**α** 6hr post transfecting bone marrow derived dendritic cells with varying cyclic dinucleotides. T-tests were done comparing treated samples with untreated P-values are represented by: *p < .05, **p < .01, ***p < .001.

Figure 2 shows that, as in macrophages, CDNs can elicit cytokine production in dendritic cells. In addition, this cytokine production varied based on which CDN was used. ADU V19 induced the greatest expression change in IFNβ, IL12 p40, IL6, IL23 p19, and TNFα, and also induced moderate levels of IL12 p40. However, ADU V16 and CDA induced the strongest TGFβ response. It should be noted that this experiment showed more variation within triplicates of each sample, explaining some of the large error bars.

4.3. Human PBMC CD14+ Macrophage Cytokine Production

After testing mouse cell lines and discovering differential cytokine production, we then turned to human macrophages to see if the same patterns were shown.



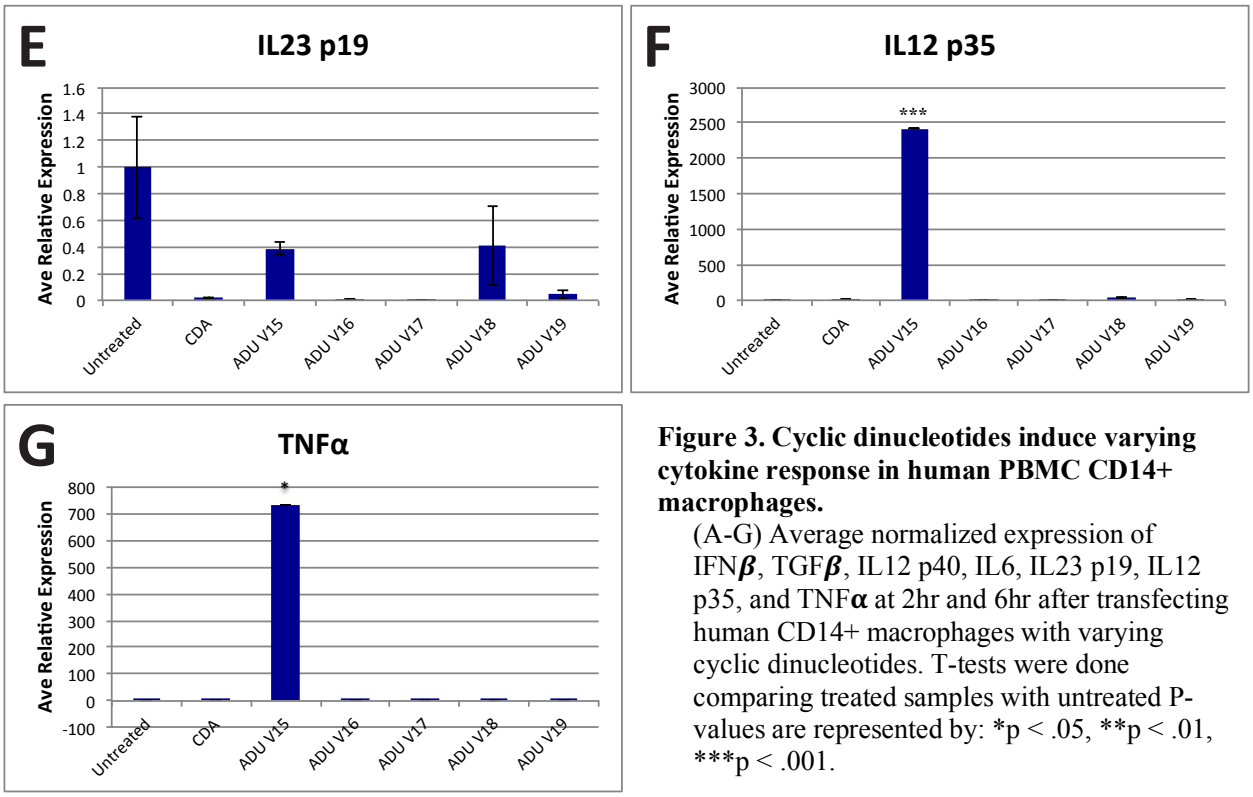


Figure 3. Cyclic dinucleotides induce varying cytokine response in human PBMC CD14+ macrophages.

(A-G) Average normalized expression of IFN***β***, TGF***β***, IL12 p40, IL6, IL23 p19, IL12 p35, and TNF**α** at 2hr and 6hr after transfecting human CD14+ macrophages with varying cyclic dinucleotides. T-tests were done comparing treated samples with untreated P-values are represented by: *p < .05, **p < .01, ***p < .001.

Figure 3 shows that, as in mouse macrophages and dendritic cells, cyclic dinucleotides are able to produce varying cytokine responses. All CDNs tested seemed capable of eliciting an IFN*β* response. TGF*β* and IL-23 p19 expression was not induced by any CDN. IL-6, IL-23 p35, and TNF**α** had the highest relative expression with ADU V15.

4.4. ER-HoxB8 Neutrophils, Cytokine Production, and STING

With the role of neutrophils in TB infection still unclear, we investigated how intracellular bacteria may be detected in infected neutrophils. We focused on how pathogen double stranded DNA is sensed in the cytosol of ER-HoxB8 progenitor neutrophils. As stated earlier, we created STING and Sox2 knockouts of these neutrophils, transfected with CDA, cGAS-activating dsDNA (45bp ISD), or *L. monocytogenes* genomic DNA, and analyzed the expression of target genes via qPCR.

Figure 4 shows that the absence of Sox2 seemed to have no effect on expression of pan IFN**α**, IFN***β***, or TNF**α** upon transfection with *L. monocytogenes* gDNA compared to controls. However, the absence of STING appeared to have a significant effect on cytokine expression. We observed a statistically significant decrease in expression of pan IFN**α**, IFN***β***, TNF**α**, and IL-6 in STING KO neutrophils compared to controls. For IL6, we observed a statistically significant decrease in IL-6 expression upon removing Sox2, but an even more dramatic decrease

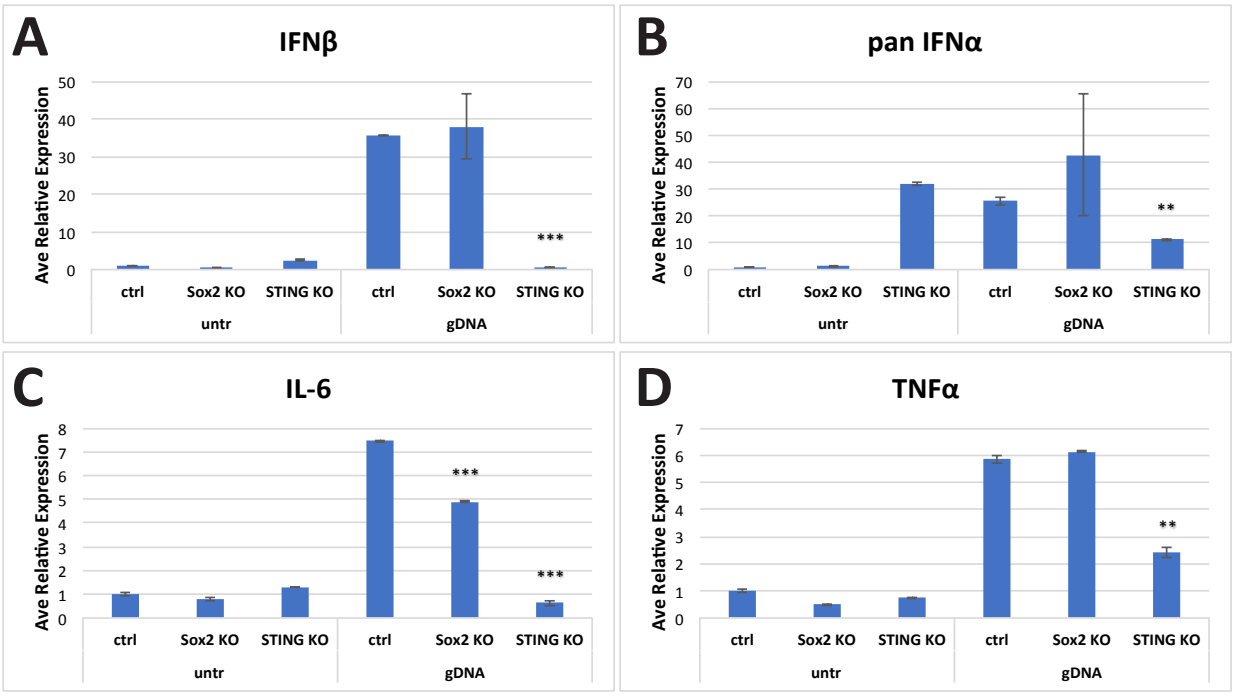


Figure 4. The loss of STING in ER-HoxB8 neutrophils impairs some cytokine signaling. (A-D) Average normalized expression of pan IFN**α**, IFN***β***, IL6, and TNF**α** 6hr after transfecting ER-HoxB8 neutrophils with either untreated or *Listeria monocytogenes* genomic DNA. T-tests were done comparing knockouts with control. P-values are represented by: *p < .05, **p < .01, ***p < .001.

in expression was observed upon removal of STING. No expression of Sox2 was detected by qPCR, but low levels of STING were detected (data not shown).

To further test for STING expression in ER-HoxB8 neutrophils, we performed a western blotting analysis of lysates from untransduced, control (non-targeted gene KO), and STING KO cells.

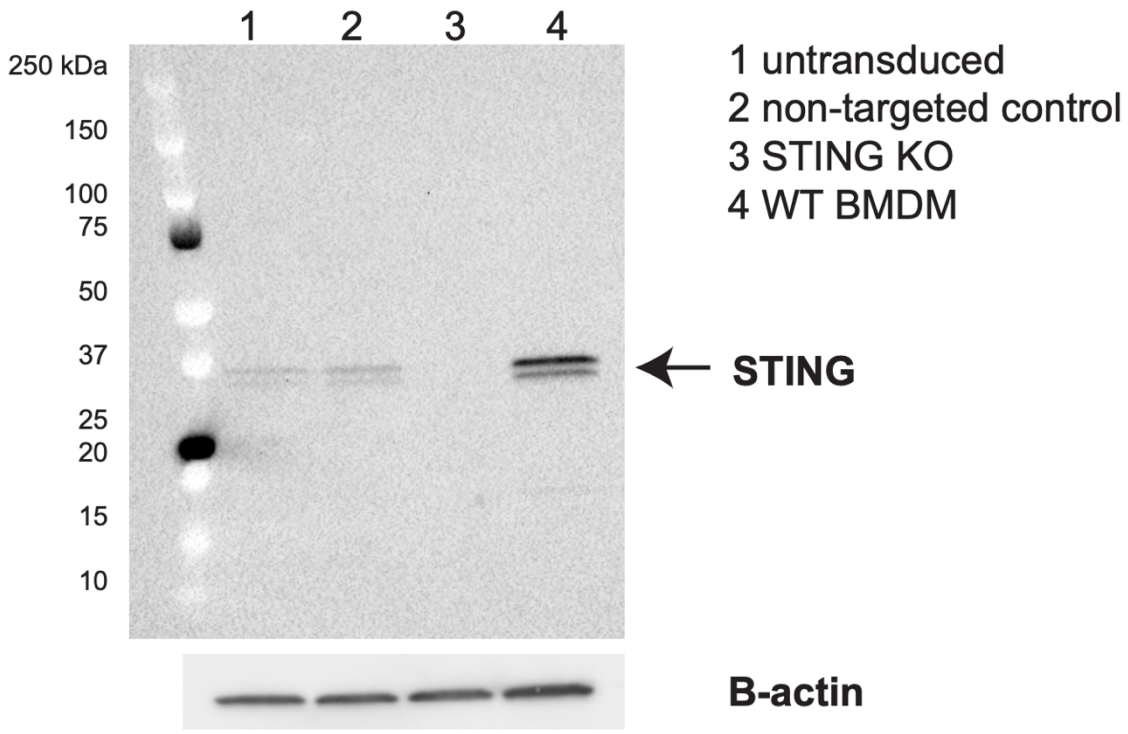


Figure 5. ER-HoxB8 neutrophils express STING.
Western blotting analysis of lysates from (1) untransduced, (2) control, (3) STING KO, and (4) WT bone marrow derived macrophages with an anti-STING antibody. WT BMDM served as a positive control. Results were normalized to B-actin.

Figure 5 shows that STING is expressed in ER-HoxB8 neutrophils, as indicated by the faint bands under wells 1 and 2, between 25-37 kDa. As expected, we saw no STING expression in the STING KO well. Compared to our positive control (wild-type bone marrow derived macrophages), the 2 bands were much fainter, suggesting that STING is not as highly expressed in neutrophils as in macrophages. However, we did not quantify protein levels from our western blot. Future experiments should quantify these levels in order to get an accurate comparison of STING expression in macrophages and neutrophils.

5. Discussion

The data above show that cyclic dinucleotides are effective inducers of cytokine production, but this cytokine production is not uniform across all CDNs. In bone marrow derived macrophages, we discovered a pair of potent inducers (ADU V16 and V18) of IFNβ, IL-6, and IL-23 p19 (Figure 1-A, D, and E). On the other hand, bone marrow derived dendritic cells saw high levels of expression of IFNβ, IL-12 p40, IL-6, IL-23 p19, and TNFα upon stimulation with ADU V19 (Figure 2-A, C, D, E, and G). In human CD14+ macrophages, no clear pattern could be established. All CDNs were capable of eliciting IFNβ signaling, however some CDNs were capable of inducing expression of only one cytokine (Figure 3). This variability in cytokine production suggests that cyclic dinucleotides can elicit diverse responses in myeloid cells, perhaps due to variation in cell type-specific mediators downstream of the STING pathway.

As previously mentioned, dendritic cells serve as key recruiters of T-cells[20], and can specifically induce Th17 cells[21] that are critical in some models of vaccine-mediated protection against TB. In bone marrow derived dendritic cells, ADU V19 was able to induce strong expression of IL-6, which is necessary for Th17 differentiation, and IL-23 p19 and TNFα, which are necessary for Th17s to be maintained[11]. ADU V19 also elicited a strong and statistically significant IL-12 p40 response, which is important for induction of Th1 cells that help control a TB infection[11]. It should be noted that ADU V19's induced expression of certain cytokines were stronger than those induced by ADU V16 (RR-CDG) and V17 (ML-RR-cGAMP), which are known activators of mouse and human STING. These data were complimented by *in vivo* experiments, which suggested ADU V19 elicited a more inflammatory Th17 response (data not shown). Therefore, ADU V19 seems like a very strong adjuvant candidate for TB protein subunit vaccines.

However, our human *in vitro* data tells a different story. For these macrophages, ADU V19 was only a potent stimulator of IFNβ. Most of the target cytokines appeared to have different CDNs as their most potent inducers, which differed from the mouse bone marrow derived macrophages and dendritic cells. More experiments should be carried out in human cell lines to better inform the development of CDN-adjuvanted vaccines for clinical applications.

Furthermore, our data show that STING, not Sox2, may be necessary for the sensing of pathogenic double stranded DNA in an infected neutrophil. Upon transfection with *L. monocytogenes*, expression of pan IFNα, IFNβ, or TNFα did not significantly change by removing Sox2. However, expression of pan IFNα, IFNβ, TNFα, and IL-6 did significantly decrease when STING was removed (Figure 4). This suggests that STING is important for both the sensing of pathogenic DNA as well as subsequent cytokine signaling in neutrophils. We found STING to be expressed in our ER-HoxB8 neutrophils (Figure 5), and could not detect Sox2 expression by qPCR. This further indicates that STING is both present and active in neutrophils, whereas Sox2 may not be present or expressed. In addition, since pan IFNα and IFNβ signaling was impaired in STING deficient cells, this suggests that the STING pathway may be important for the neutrophil-driven, Type I IFN inducible transcriptional signature[13] observed in humans.

Our findings are at odds with those in Xia et al, which suggest that Sox2 directly recognizes microbial DNA, thereby activating the innate immune system[19]. They found that their neutrophils had low expression of STING, whereas we found moderate expression of STING (Figure 5). In addition, they found that upon transfection of *L. monocytogenes* gDNA, STING-deficient neutrophils and wild-type neutrophils expressed comparable levels of TNF, IL-6, and IL-1β. We found a statistically significant decrease in TNFα and IL-6 expression in STING

deficient neutrophils compared to controls. One factor that could explain this oppositional data is the type of neutrophil tested. Our ER-HoxB8 neutrophils came from a Cas9 mouse and could be inherently different than their neutrophils derived from bone marrow and peripheral blood. No other publications have shown a role for Sox2 in neutrophil DNA sensing. These results underline the importance of further characterizing cytosolic DNA detection in neutrophils.

5.1. Limitations

The major limitation of this study was that these findings are from *in vitro* data only, specifically from mouse cells. The patterns displayed might greatly differ in an *in vivo* mouse model, and even in humans. Another limitation is in the PBMC CD14+ macrophage data. 18s rRNA Ct values varied greatly between samples, thereby skewing normalized expression results. In addition, triplicates for these qPCR data were not as precise, producing great variation in our expression results. This could potentially explain the erratic cytokine expression that we observe.

5.2. Future Directions

Due to the irregular Ct values from the PBMC CD14+ samples, this experiment should be repeated on this cell line to elucidate clearer results. In addition, now that a mouse *in vitro* model has been established, the experiment should be conducted in other human cell lines to see if the same patterns in mouse myeloid cells exist in those of humans.

The STING pathway might require some more investigation, as the whole story seems to be incomplete. The differential cytokine expression elicited by different cyclic dinucleotides suggests that these molecules may be acting on something else other than STING, perhaps downstream in the transcriptional process. More research into this mechanism might shed light on an unknown role that CDNs play in cytokine signaling in the immune system. In a similar vein, this varying cytokine response gives reason to compare the efficacy of a protein subunit

vaccine with these varying CDNs, and to see which provides better protection against *M. tuberculosis* challenge.

Furthermore, neutrophils need to be more closely examined. As one of the most important immune cells in a TB infection, it is vital that their role is thoroughly understood. This experiment should be replicated in other neutrophils to see if STING is expressed and active. If neutrophils secrete Type I IFN through STING as our data suggests, and considering Type I IFN signaling correlated with active disease[13], researchers should examine whether STING in neutrophils is important for control of TB by challenging wild-type and STING KO neutrophils with *M. tuberculosis*.

5.3. Conclusion

Our research shows that cyclic dinucleotides are capable of eliciting a cytokine response *in vitro*, with different cyclic dinucleotides inducing differential cytokine expression. This was seen in mouse macrophages, mouse dendritic cells, as well as human CD14+ macrophages. We discovered that certain CDNs were capable of producing a skewed immune response that is beneficial during a TB infection. However, the mechanism behind CDNs ability to induce this differential expression is still unknown. Furthermore, our research suggests that STING is critical for pathogen DNA sensing in an infected neutrophil, and responsible for the production of the Type I IFN response seen during active infection. Further research should examine the STING pathway and its role in *M. tuberculosis* infection.

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