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THE ROLE OF AIM2 IN INFLAMMATORY GENE EXPRESSION

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Abstract

The importance of the cytosolic receptor, absent in melanoma 2 (AIM2), has recently been discovered. Its influence on inflammatory gene expression is essential for proper inflammatory response. In this study, inflammatory gene expression in wild type macrophages was observed, revealing a key role of AIM2 in certain inflammasome gene expressions. With a combination of the data gathered and additional experimentation, the role of AIM2 in inflammatory gene expression can be appreciated.

Table of Contents

Table of Figures

Acknowledgements

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Introduction

The immune system integrates uses multiple mechanisms to regulate defensive measures. These defense mechanisms are vital for the survival of an organism. An organism's innermost protection strategies require cells to perform any and all measures. This may include even suicidal measures for the cell, known as programmed cell death or apoptosis (Voet, Voet, & Pratt, 2008).

There are two parts to the immune system; innate and adaptive, which work together to protect the organism. The differences between these two parts of the immune system are noteworthy, though. While the adaptive immune system requires some time to become activated, the innate system responds to invading microorganisms almost immediately (Franchi, Eigenbrod, Munoz-Planillo, & Nunez, 2009). While the importance of the adaptive immune system cannot be undermined, the innate immune system is central to defense of the host from infection and is the focus of this project. The innate immune system is a less specific form of defense which distinguishes between self and non-self structures (Guarda & So, 2010). This determination allows for attempts at removal and destruction of invading microbes. The receptors which recognize foreign structures, such as double stranded DNA (dsDNA) and proteins, are located on the cell surface as well as in the cytosol of the cell (Franchi et al., 2009). While the receptors are all "germline-encoded pattern recognition receptors" (Hornung et al., 2009), those on the cell surface are membrane-bound and those in the cytosol are free to move (Franchi et al., 2009). Both membrane-bound receptors, such as Toll-like receptors (TLRs), and cytosolic receptors, such as Nod-like receptors (NLRs), which recognize specific patterns of foreign microbes (Franchi et al., 2009; Hornung et al., 2009). These patterns include 'pathogen-

associated molecular patterns' (PAMPs), which may be microbial products such as peptidoglycan and lipopolysaccharide (LPS) or microbial nucleic acids (Franchi et al., 2009).

Recent study of these receptors and the responses of cells to microbial nucleic acids has led to the discovery of absent in melanoma 2 (AIM2), a cytosolic receptor, which appears to act in a manner similar to some members of the NLR family (Hornung et al., 2009). Though not part of the NLR family, the AIM2 protein does have similarities to this family. Structurally, both contain a pyrin domain (PYD), which then associates with a protein known as apoptosis-association speck-like protein containing a caspase activation and recruitment domain (ASC) (Hornung et al., 2009). The AIM2 protein associates with ASC and activates the formation of a large multiprotein, AIM2-containing inflammasome (Hornung et al., 2009). ASC, which contains a caspase-recruitment domain (CARD), plays a crucial role in the activation of caspase-1 (Franchi et al., 2009). Once stimulated, caspase-1 goes on to perform further functions, including conversion of the cytokines prointerleukin-1β (proIL-1β) and prointerleukin-18 (proIL-18) to interleukin-1β (IL-1β) and interleukin-18 (IL-18), respectively (Hornung et al., 2009). These cytokines are critical in inflammatory responses. ProIL-1β is formed by the cell in response to stimulation by LPS, unlike proIL-18, which the cell naturally contains. IL-1β has many roles in immune responses, and acts by binding to cellular receptors and activating many different cell types; it also leads to production of chemokines which recruit inflammatory cells to infection sites (Arend, Palmer, & Gabay, 2008; Franchi et al., 2009; Gu et al., 1997). IL-18, has key functions in production of interferon gamma, important in the removal of pathogens inside cells including both bacterial and viral forms (Arend et al., 2008; Gracie, Robertson, & McInnes, 2003).

These immune responses are made possible through NLR proteins as well as AIM2. Though both AIM2 and NLRs are stimulated by DNA, AIM2 is activated by transfected bacterial, viral, mammalian, or synthetic DNA, while NLRs are not (Hornung et al., 2009). Experimentally, the purposeful introduction of double stranded DNA (dsDNA) into the cell, through transfection, has revealed the binding of dsDNA to the HIN200 domain of AIM2 which initially stimulates AIM2 (Hornung et al., 2009). A schematic of the mechanism of AIM2 can be seen in Figure 1 below.

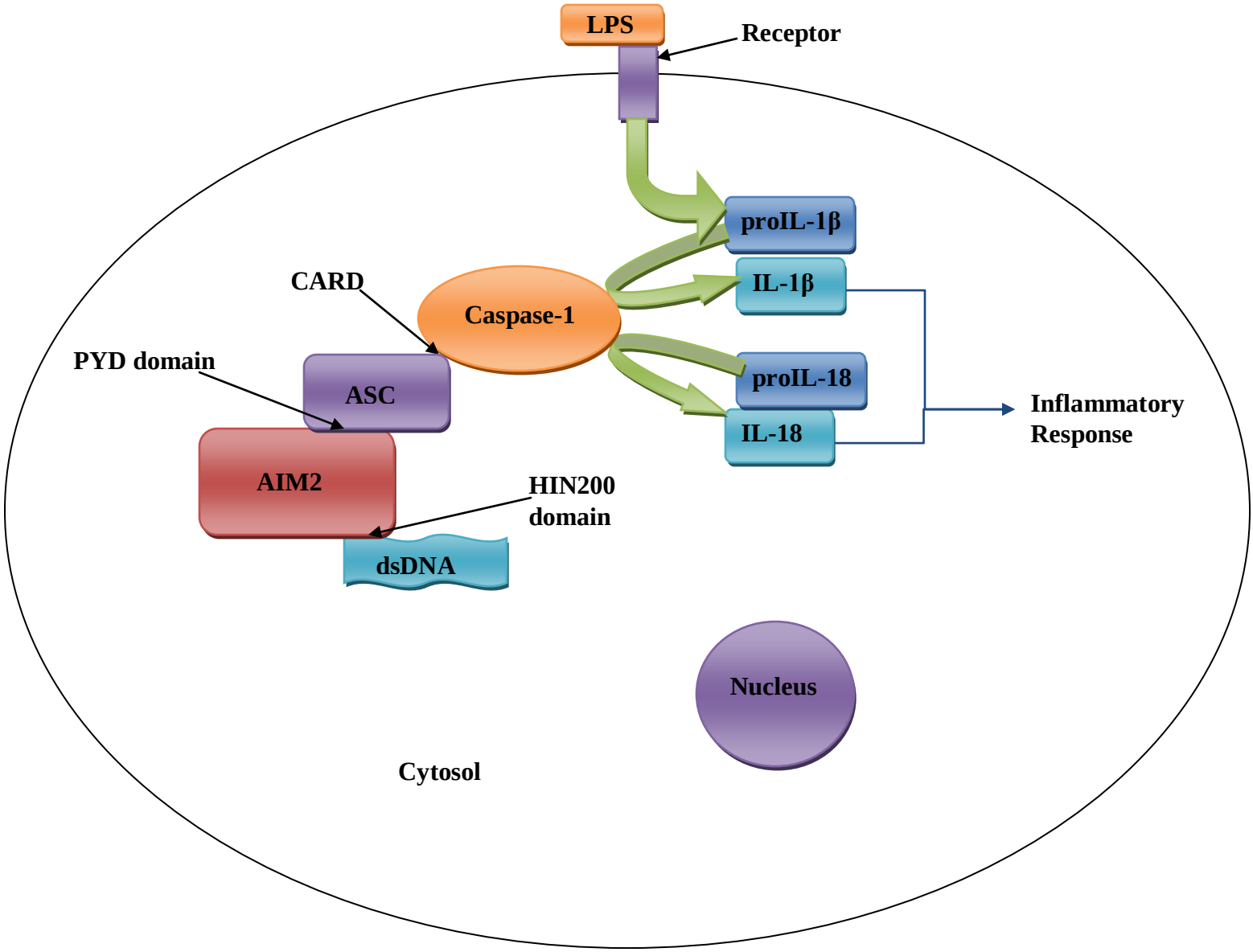

Figure 1: The dsDNA binds to the HIN200 domain of cytosolic AIM2. AIM2, through its PYD domain, then binds ASC. In turn, ASC, through the domain known as CARD, then stimulates caspase-1. Caspase-1 stimulates proIL-1β, created through LPS stimulation, to IL-1β and proIL-18 to IL-18, which then initiate their respective inflammatory responses.

Recent evidence suggests that AIM2 is vital for the control of both viral and bacterial infection (Rathinam et al., 2010). Certain disease-causing agents may lead to serious infectious diseases if there is a defective AIM2 response (Fernandes-Alnemri et al., 2010). This includes the bacterium *Francisella tularensis* which, without AIM2 response, leads to greater vulnerability to tularemia (Fernandes-Alnemri et al., 2010). Similarly, the DNA of the bacterium *Listeria monocytogenes* is recognized chiefly by AIM2 (Wu, Fernandes-Alnemri, & Alnemri, 2010). Without AIM2, the host has difficulty in defense against these bacteria (Fernandes-Alnemri et al., 2010; Wu et al., 2010). Not only is AIM2 important in protection against bacteria, but it is also critical for defense against certain viruses (Rathinam et al., 2010). These include vaccinia virus and mouse cytomegalovirus (mCMV), which have both been shown to stimulate AIM2 responses in the cell (Rathinam et al., 2010).

While it is clear that AIM2 binds to DNA and activates caspase-1 in order to cause production of the mature forms of IL-1b and IL-18, the role of AIM2 in regulating other aspects of the innate immune response is unclear. Therefore, in order to further study the role of AIM2, the following specific aims are proposed. (Fitzgerald, personal communication). These aims as well as the intended methods include:

By the use of quantitative real-time PCR, it will be determined whether the addition of DNA to a cell regulates inflammatory responses through transcriptional regulation of inflammatory genes. Wild type cells will be treated with cytosolic DNA and the expression of immune response genes will be monitored.

Ultimately, through these and further studies, including the use of knockout AIM2 cells, the role of AIM2 in regulating responses other than the caspase-1 pathway in the immune system will be determined more fully and its role in combating cell infection will hopefully lead to improved treatment or prevention of disease.

Materials and Methods

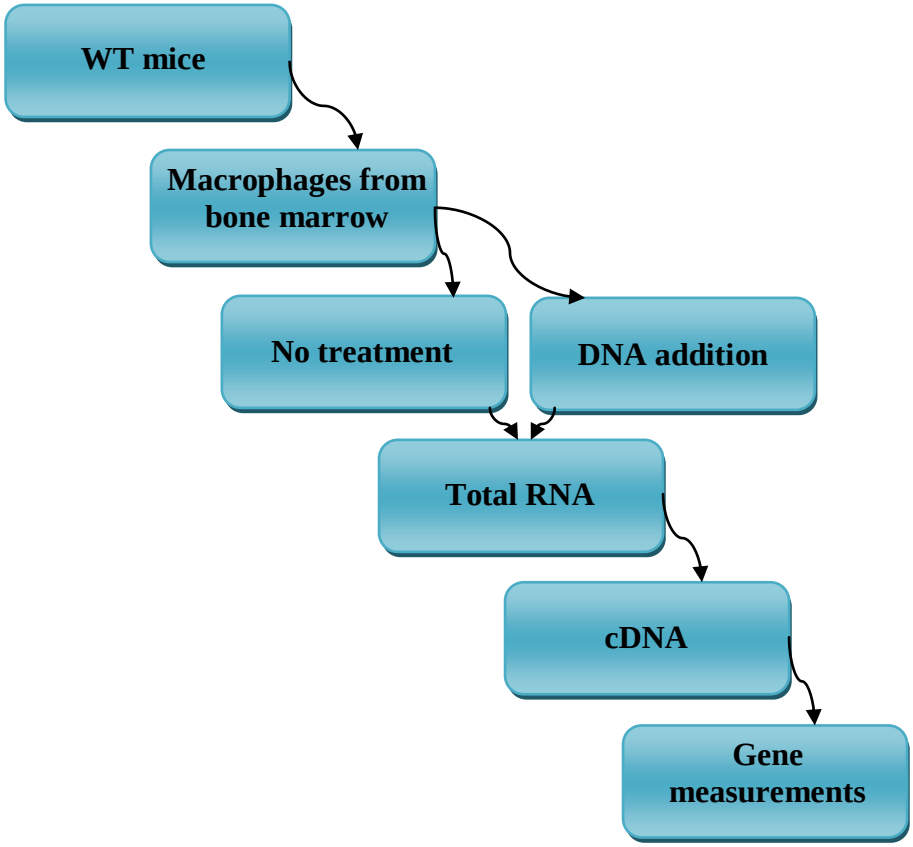

Figure 2: Flowchart of procedure

Derivation of Primary Macrophages from Bone Marrow of Wild Type Mice

Wild type mice maintained in the pathogen-free animal facility of University of Massachusetts Medical School were euthanized using CO_2. The femurs and tibiae of the mice were then removed and cleaned of muscles and tendons. These bones were then rinsed in

complete Dulbecco's Modification of Eagle's Medium (cDMEM) and the ends of the bones were cut off. Using a syringe with a needle, the marrow was flushed with cDMEM until the bone became translucent. The clusters of marrow were disintegrated gently using a pipette. The marrow was then centrifuged for five minutes at 300 x g at room temperature and the supernatant was discarded. One milliliter of red blood cell (RBC) lysis buffer was added to the pellet, which was then resuspended and let to sit for 2-3 minutes. The tube was filled with phosphate-buffered saline (PBS) lacking serum to wash the cells and centrifuged as before. Again, the supernatant was discarded, but the pellet was resuspended in cDMEM with 10% of L929 supernatant. L929 is a source of macrophage colony stimulating factor (M-CSF), which is a growth factor that leads to the differentiation of bone marrow progenitor cells to macrophages. Using two Petri dishes, 25mL of this solution, containing cells, the medium (cDMEM), and L929 supernatants, was added to each dish.

On the third day of incubation at 37°C, the macrophages have begun to differentiate and stick to the plates. On this same day, the supernatant was removed and 20mL of fresh cDMEM and 5mL of L929 was added. On the sixth day, the cells were observed and split. The cells fixed to the plate were unstuck using a cell scraper, and half of this supernatant was removed and placed on another Petri dish. Fresh cDMEM (10mL) and 2.5mL of L929 supernatant were then added to each dish. The cells were harvested on the eighth day, as they could be from the seventh to thirteenth days.

Macrophage Cell Culture

In addition to making macrophages from mouse bone marrow, the lab also has immortalized murine derived bone marrow macrophage (iMac) cell lines. These cell lines were also used in this study. The iMac cell lines were maintained in a 37°C incubator once thawed

from liquid nitrogen containers where they are aliquoted and stored. The cells were split when they became 70-80% confluent in the flask, as observed under a microscope. When this occurred, the media in which they grew (cDMEM) was changed. Complete DMEM was created using a 500mL bottle of DMEM and adding 500µL of the antibiotic used to prevent contamination, ciprofloxacin (1:1000 dilution) and 50mL of 10X fetal calf serum (fcs). This solution was used for all media replacement and experimental use.

If an experiment was to be performed, the cells were prepared using the cell-containing media before splitting. In order to remove cells at the desired concentration, cells were counted: Briefly, 10µL of the solution was placed in a slide appropriate for use in the BioRad Automated Cell Counter TC10™. This machine counts the cells, and based on this result, the appropriate amount of the solution was placed in a centrifuge tube. This amount of solution contained the desired number of cells based the number of milliliters required for the experiment. An example calculation is shown below for a sample containing fewer than $1x10^6$ cells:

Total cell count calculated by machine = $6.38x10^5$ cells/mL, which is equal to $0.638x10^6$ cells
The final concentration desired = $1x10^6$ cells/mL
The total mL required for each well to contain 200µL of solution: 10mL

Total number of cells you need: 10 x ($1x10^6$ cells/mL) = $10x10^6$ cells/mL

$$\text{so, } \frac{10x10^6}{0.638x10^6} = 15.67\text{mL from the flask}$$

Check: if 15.67mL→ $15.67x(0.638x10^6) = 10x10^6$ cells (which is 10 million).

Cell stimulation for ELISA to measure inflammatory cytokines

The macrophages, primary of immortalized cells, in cDMEM were plated in half of a 96-well plate at a concentration of 10^6 cells/mL. Half of these (3 columns) were then primed with

LPS (2μL LPS/10mL cDMEM) and incubated at 37°C for 1.5 to 2 hours. LPS is used to prime cells in IL-1β assays as LPS stimulates the production of the inactive proIL-1β form in the cell. Without proIL-1β, no inflammatory response from IL-1β would be observed. LPS priming is unnecessary for other cytokines, such as IL-18, because the inactive proIL-18 form is naturally present in the cell.

After LPS priming, in two rows of the plate, either ATP at 5mM (10μL/well) or Nigericin at 10μM (0.4μL/well) were added and incubated for one hour, before collection of the supernatant. The other rows contained a control (medium) row, two rows of a lipofectamine and optimem mix, and three of a lipofectamine, optimem, and DNA (polydA:dT) mixture. To create the mixtures, lipofectamine (12μL) was added to a transfection medium called optimem (288μL) in two microcentrifuge tubes. These sat together for 5 minutes to allow for equilibrium. DNA (polydA:dT) (6μL) was then added to one of these tubes and let sit for 15 minutes to allow for DNA binding to the transfection reagent. Both tubes were then vortexed and added to the wells in the following amounts: 10μL in row 2 and 20μL in row 3 of the lipofectamine and optimem mixture and 5μL, 10μL, 20μL in rows 4, 5, and 6 respectively of the mixture containing DNA. These incubated for 6 hours before collection of the supernatant into a new 96-well plate. All treatments were carried out in triplicate. An ELISA was then performed on these samples.

ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed using the supernatants, which contain secreted cytokines, and following the manufacturer's guidelines for IL-1β. The adjustments made to the protocol included the use of only half as much solution for each of the steps, excluding the assay diluent step. The assay diluent is used to block the vacant well sites

with protein, the amount of assay diluent should not negatively affect the results of the experiment.

Cell stimulation for Quantitative real-time PCR

The macrophages in cDMEM (1mL) were plated in a 24-well plate at a concentration of 10^6 cells/mL. These were left overnight in 37°C and stimulated the next day. The control well(s) contained solely medium. A DNA (polydA:dT)/lipofectamine mixture was also used for stimulation. This mixture contained optimem (45µL), lipofectamine (2µL, because 2µL/mL), and polydA:dT (3µL, because the stock was 0.5µg/µL and desired a 1.5µg/10^6cells). The optimem and lipofectamine were first added to a microcentrifuge tube and allowed to equilibrate for 5 minutes, then the polydA:dT was added and the mixture sat for 15 minutes. This was then added to the appropriate well(s). The third stimulation was performed with *Sendai Virus* (50µL, because desired a concentration of 200HAU/mL and the stock was 4000HAU/mL), a positive control. The final stimulation, properly performed in the primary bone marrow derived macrophage (BMDM) experiment, was with LPS (200µL/well, because desired a concentration of 200ng/mL and the stock was 1mg/mL). The experiments using the immortalized cells were not stimulated properly with LPS and therefore could not be trusted. These incubated at 37°C for two hours until RNA extraction was performed.

RNA extraction

After stimulation, the RNA was extracted from the cells using the QIAGEN RNase mini kit 50. The cells were harvested using cell scrapers and pipettes, removing as many cells as possible into microcentrifuge tubes, which were then placed on ice. Gloves and sleeves were worn from this point on to prevent RNA degradation. For cell lysing, a mixture of buffer RLT and β-Mercaptoethanol (10µL β-Mercaptoethanol/1mL RLT) was created. The mixture

(350µL/tube) was added to each tube and vortexed quickly. These tubes were then frozen in -80°C until the next day.

The samples were thawed at 37°C the next day for 15 minutes. They were then quickly vortexed and spun down. For binding, 350µL of 70% ethanol was added to each tube and mixed using the pipette. This suspension was then added to a properly labeled column and spun down for 15 seconds at 10000rpm. The catch tube was emptied after the spin.

The samples were washed by adding 350µL of buffer RW1 and centrifuged for 15 seconds at 10000rpm. A 80µL mixture of 10µL DNAse (RNAse-free) and 70µL buffer RDD was added to each column, which then incubated at room temperature for 15 minutes. After incubation, 350µL of buffer RW1 was added for further washing. The columns were spun down for 15 seconds at 10000rpm, after which the collection tube was changed. The buffer RPE (500µL) was then added and the columns were spun down as before. The previous step was repeated, but centrifuged for 2 minutes instead. Finally, to be sure the buffer was removed completely, the columns were spun down for 1 minute at 13000rpm.

The RNA was eluted by putting the column in a 1.5mL microcentrifuge tube and adding 30µL of RNAse-free water to each tube. The water was spread evenly across the membrane and centrifuged for 1 minute at 10000rpm. The RNA was then placed on ice and quantified.

RNA quantification

The RNA samples were quantifying using a NanoDrop in the UMass lab. To ensure proper results, the computer program sample type was changed to RNA. The NanoDrop was blanked using 2µL of RNAse-free water, as this was the solution in which the RNA had been placed. To remove residual solution of the samples, the machine was wiped between each sample. Each sample (2µL) was placed on the machine and measured. As nucleic acids, such as

RNA, absorb light at 260nm and proteins absorb light at 280nm, the 260/280 ratio measured by the machine should be about 2 if little protein contamination is present. Also, some of the chemicals used for RNA isolation absorb light at 230nm, so another ratio (260/230) was measured by the machine and should also be about 2, revealing the chemical contamination. The resulting amounts of RNA (ng/μL) were recorded and put into an Excel template for creation of cDNA.

Creation of complementary DNA (cDNA)

Using an Excel template provided by the Fitzgerald lab, the required number of microliters of each RNA sample and RNAse-free water was calculated. This number reflects 1μg of RNA in 15μL of solution. Under a hood specifically for this purpose, the appropriate amounts of RNA and water were added to separate polymerase chain reaction (PCR) tubes. Each tube then contained a total of 15μL of solution. To each tube 5X RT mixture (4μL) and RT enzyme (1μL) was added for a total of 20μL in each PCR tube. The samples were then put in a PCR to run a protocol for cDNA creation. The protocol was as follows: (1) no hotlid start, (2) 25°C for 10 minutes, (3) 42°C for 50 minutes, (4) 85°C for 5 minutes, and (5) 4°C hold. These samples were then used for a quantitative real-time PCR.

Quantitative real-time PCR (Q-PCR)

For a 1:3 dilution of the cDNA samples, 40μL of RNAse-free water was added to each PCR tube, for a total sample of 60μL. In a specifically designated hood, four reaction mixtures were created. The master mix (7.5μL/reaction), water (5.5μL/reaction), and appropriate primers (2μL/reaction) were added to four separate microcentrifuge tubes. The primers included β-actin, tumor necrosis factor α (TNF), interleukin 6 (IL-6), and interferon β (IFNβ). To specific wells of a QPCR plate, 15μL of the different mixtures were added. Standards, using the media sample,

were created, enough for each of the different sets of primers. The standards were created in dilutions of 1/5, 1/50, 1/500, and 1/5000. The 1/5 dilution originally contained 16µL of media and 64µL of water, this was mixed and 8µL was moved to 72µL of water. This serial dilution was performed to the 1/5000 dilution. To specific wells of the Q-PCR plate, 5µL of these standards were added in duplicate. Finally, the samples containing cDNA were then each diluted (1:10) by adding 10µL of the cDNA and 90µL of water. To proper wells of the Q-PCR plate, 5µL of the samples were added in triplicate. After everything was added appropriately, the plate was sealed.

A Q-PCR was then run on the CFX96 Real-Type System using a program in BioRad CFX Manager. The protocol run is as follows: (1) 50°C for 2 minutes, (2) 95°C for 2 minutes, (3) 95°C for 15 seconds, (4) 60°C for 30 seconds, (5) 72°C for 45 seconds, (steps 3-5 repeated 45 times), (6) 58°C for 1 second, (7) 95°C very fast, and (8) 10°C for 5 minutes. Using this computer program, as well as Microsoft Excel and GraphPad Prism, the samples were analyzed and graphs created.

Results and Discussion

Enzyme-linked immunosorbent assays (ELISAs)

In order to assess IL-1β response as a measure of inflammasome activation, enzyme-linked immunosorbent assays (ELISAs) were performed. The cells were expected to respond with the production of the active IL-1β only in specific cases. These cases include stimulations with LPS and polydA:dT, Nigericin, or ATP. As can be seen in Figure 1, the production of the inactive form of IL-1β, proIL-1β, is induced by the binding of LPS to its receptor on the cell's surface. Therefore, active IL-1β would be produced in higher amounts after treatment with

polydA:dT, Nigericin, or ATP, which induce inflammasome pathways. The ELISAs performed demonstrated responses when LPS priming occurred. Figures 3 and 4 show IL-1β ELISAs performed.

Responses were not expected in all LPS primed cells, however. Though LPS stimulates the production of the inactive proIL-1β, the ELISA measured the IL-1β secreted into the culture supernatant. Through the mechanism in Figure 1, proIL-1β is activated into IL-1β. Thus, when polydA:dT was added in increasing increments, the inflammatory response of active IL-1β secretion also increased. Figures 3 and 4 demonstrate this. A dose dependent response was expected, though Figure 3 shows a greater response in the cells stimulated with 10μL of double-stranded DNA (polydA:dT) than with 20μL. This abnormal response may be due to some cell membrane damage, which may trigger inflammasome pathways. As the polydA:dT mixture included lipofectamine, a control of lipofectamine was also performed. Lipofectamine itself does not induce the pathway seen in Figure 1, but it rather aids the polydA:dT enter the cell. It creates pores in the membrane of the cell to allow the dsDNA to enter. Therefore, it was used to show that the pathway was not induced by the lipofectamine mixture, but rather the added polydA:dT. The small responses shown in Figures 3 and 4 in response to the stimulation with lipofectamine reflect its minor ability to induce the IL-1β pathway. This ability is likely due to the fact that lipofectamine is positively charged lipid containing no charge balancing DNA, resulting in slight cell membrane damage when exposed to the cell.

The other stimulants, Nigericin and adenine triphosphate (ATP), were added to cells also as controls. These were used as positive controls, as they both stimulate the pathway seen in Figure 1 as well. Their response was expected to be as large as the dsDNA. They induce the inflammasome response via an NLRP3 pathway involving potassium efflux, while the dsDNA

induces the IL-1β response via the cytosolic receptor, AIM2, which then stimulates the activation of IL-1β. (Mariathasan & Monack, 2007). As seen in Figures 3 and 4, Nigericin and ATP both induced the production of IL-1β, but to a lesser degree than polydA:dT.

Responses in media were most likely due to nonspecific binding of the antibody in the ELISA or due to cell death. The greatest responses, seen in the cells stimulated with the polydA:dT mixture, were expected because dsDNA activates the immune response through AIM2 in the cell. Therefore, the cells contain properly functioning AIM2, respond as anticipated, and the cell line can be used for further experimentation.

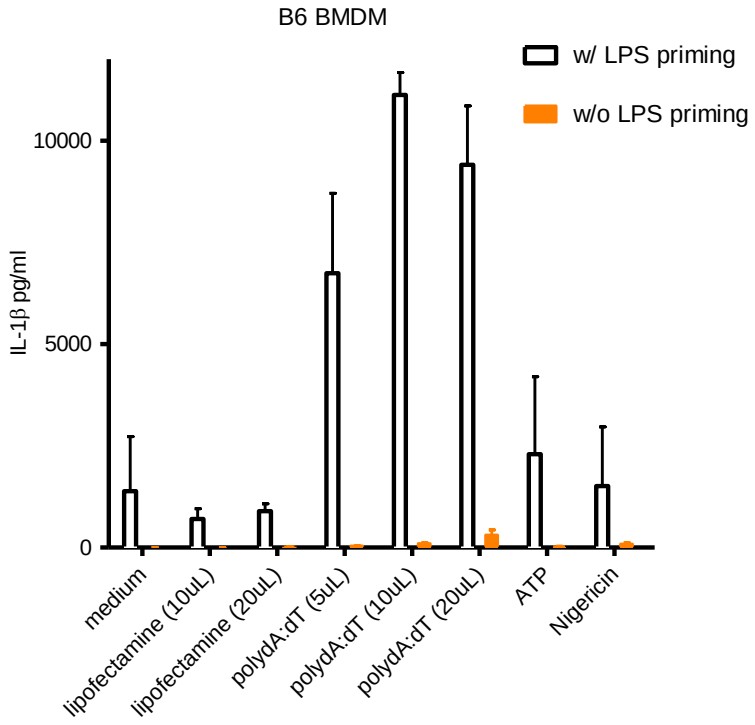

Figure 3: ELISA analysis of IL-1β production in B6 Bone Marrow Derived Macrophages

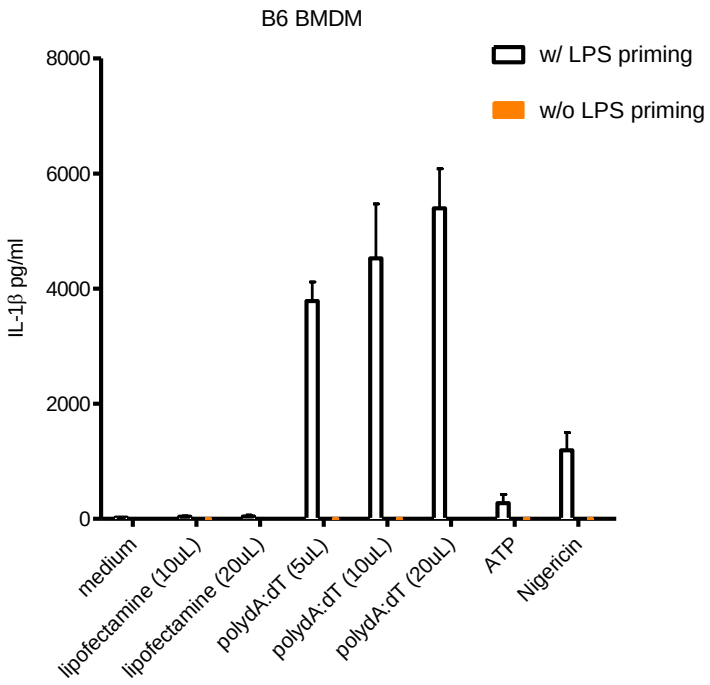

Figure 4: ELISA analysis of IL-1β production in B6 Bone Marrow Derived Macrophages

Quantitative Polymerase Chain Reactions (Q-PCRs)

Macrophage cells were induced with four separate products to determine their influence on inflammasome gene expression. The cells were treated with Lipopolysaccharide (LPS), double-stranded DNA (polydA:dT), Sendai Virus, and media for use as a control. The expression of inflammatory genes encoding for Interleukin-6 (IL-6), Interferon-β (IFNβ), and Tumor Necrosis Factor (TNF) was measured. The experiments and measurements were performed on both primary and immortalized cell lines. The process used can be seen in Figure 2. Gene expressions were measured following the procedure aforementioned on all samples, both the treated samples and the untreated media control.

Two experiments on immortalized cell lines were performed. The first experiment's data can be seen in Figures 5 through 7. The cells induced with LPS were not included in the data for Figures 5 through 7, as they were overstimulated and considered inaccurate. Figure 5 shows the

expression of IL-6 relative to the media control. As can be seen, IL-6 expression is moderately induced by the dsDNA, polydA:dT, but more so by the Sendai Virus control. Figure 6 displays the TNF expression in the cell. TNF expression was minimally induced by polydA:dT and Sendai Virus. Unlike IL-6 and TNF, the gene expression of IFNβ was relatively large, as can be seen in Figure 7. As expected, gene expression was high when the cells were infected with Sendai Virus also. The response due to polydA:dT was also reasonably high for IFNβ.

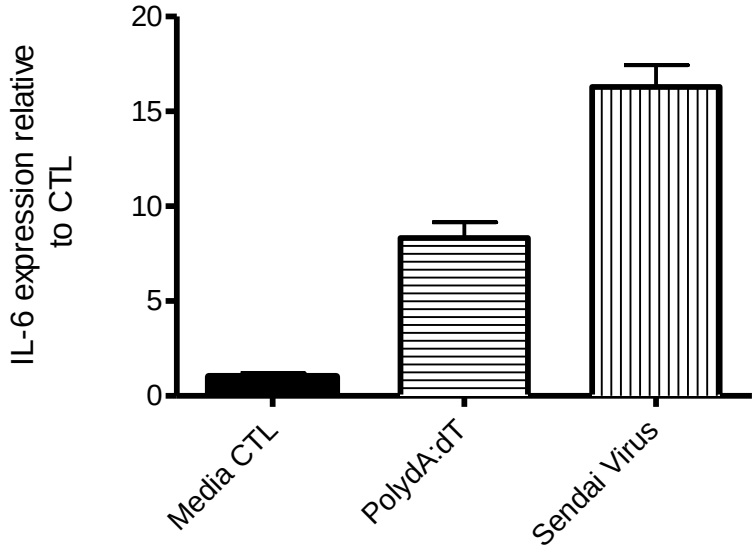

Figure 5: IL-6 expression relative to control in immortalized cell line (first trial)

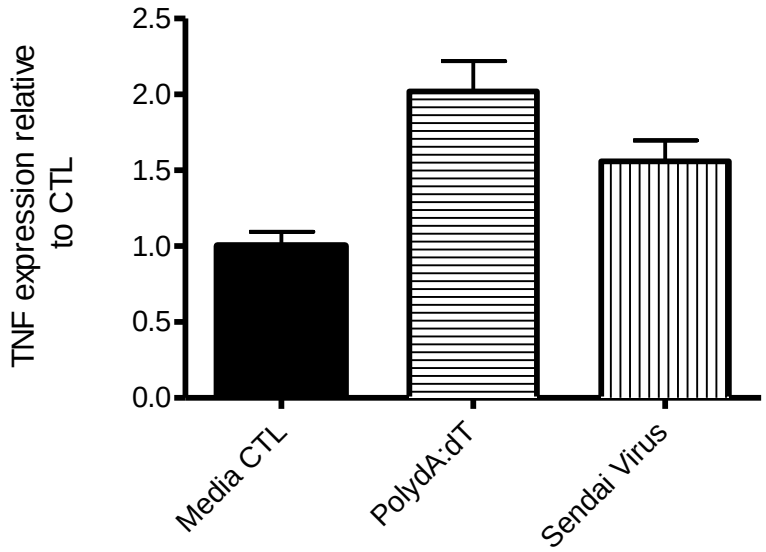

Figure 6: TNF expression relative to control in immortalized cell line (first trial)

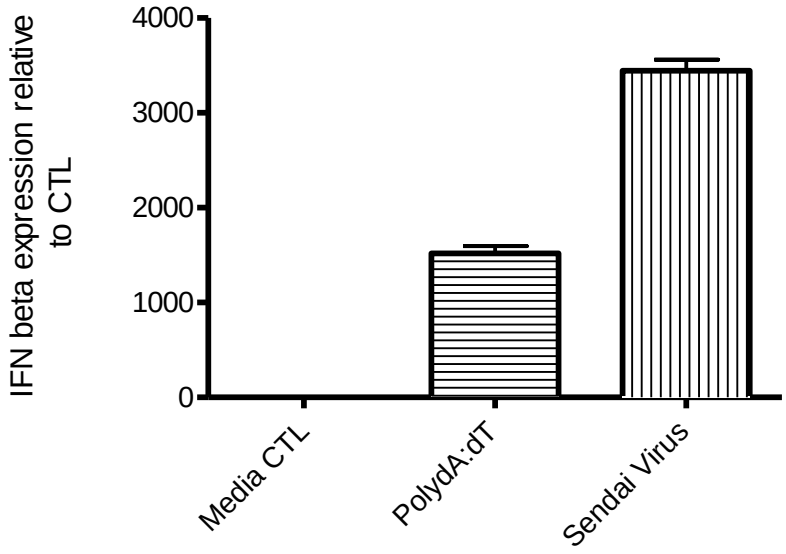

Figure 7: IFNβ expression relative to control in immortalized cell line (first trial)

For the second experiment with the immortalized cell line, two time points were chosen for each of the inflammatory genes. The data also includes LPS-induced responses. Figures 8 through 10 show Interleukin-6 expression relative to the control for each of the four stimulants. Figure 8 demonstrates the high response of IL-6 gene expression in response to stimulation by

LPS. LPS is expected to have a response as it initiates inflammatory response pathways. Also, it is not surprising that with longer exposure to LPS, greater gene expression is induced. Figure 9 shows the influence of polydA:dT on IL-6 expression. As seen Figure 5 as well, IL-6 is not greatly induced by exposure to polydA:dT. Again with more time, a greater response was observed. Finally, Figure 10 displays influence of Sendai Virus on IL-6 gene expression. As with Figure 5, the IL-6 expression is more greatly influenced by Sendai Virus exposure than with polydA:dT, but still shows a rather small response, especially in comparison to LPS exposure. As expected, with longer exposure to the virus, higher expression levels of IL-6 were observed.

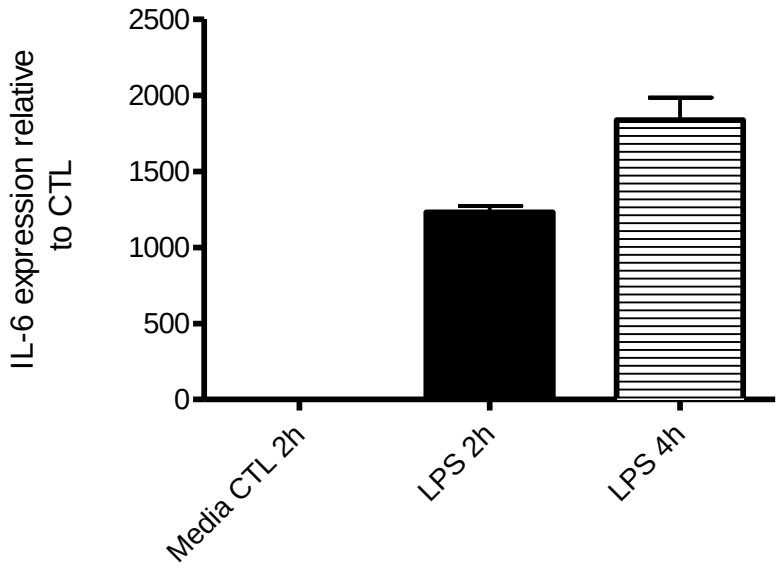

Figure 8: IL-6 expression relative to control in immortalized cell line stimulated with LPS (second trial)

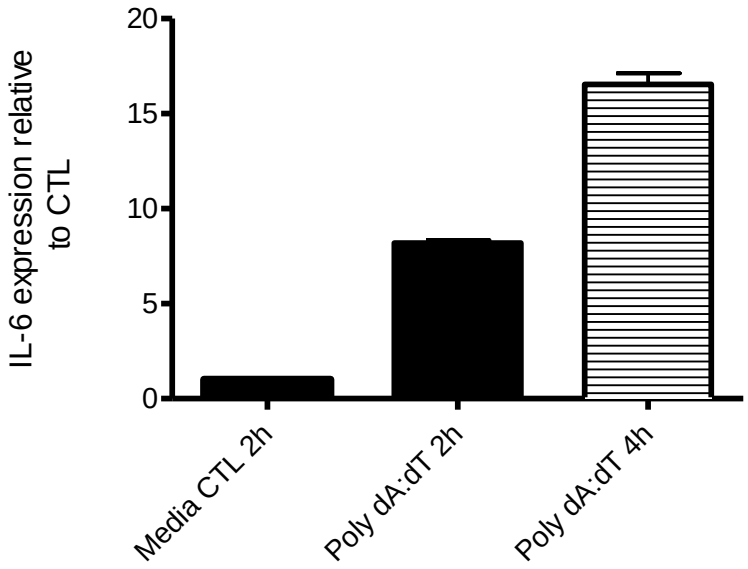

Figure 9: IL-6 expression relative to control in immortalized cell line stimulated with polydA:dT (second trial)

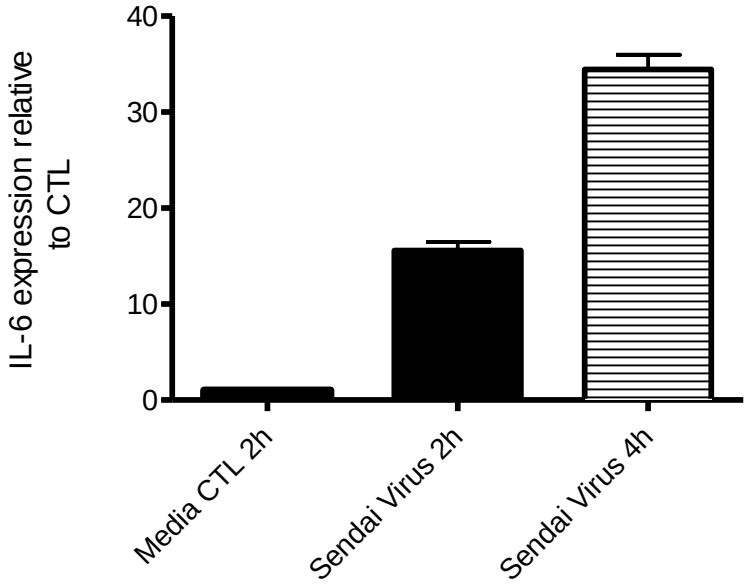

Figure 10: IL-6 expression relative to control in immortalized cell line stimulated with Sendai Virus (second trial)

The second experiment also included Tumor Necrosis Factor gene expression. Figures 11 through 13 display the data acquired. In comparison to IL-6, TNF showed a much lower gene expression response to LPS stimulation, as can be observed in Figure 11. TNF did show a time dependent response, as expected. Figure 12 shows the gene expression response of TNF to

polydA:dT. There was very little response to polydA:dT, similar to the results shown in Figure 6. Though there was not a time dependent response as expected, this is likely due to the minimal amount of gene expression exhibited by the cells. The final TNF expression experiment in immortalized cells can be seen in Figure 13. It does exhibit a time dependent response, even though the response is minimal. Similar to the data in Figure 6, TNF response to Sendai Virus is very low. It is likely that TNF gene expression is not greatly triggered by polydA:dT or Sendai Virus.

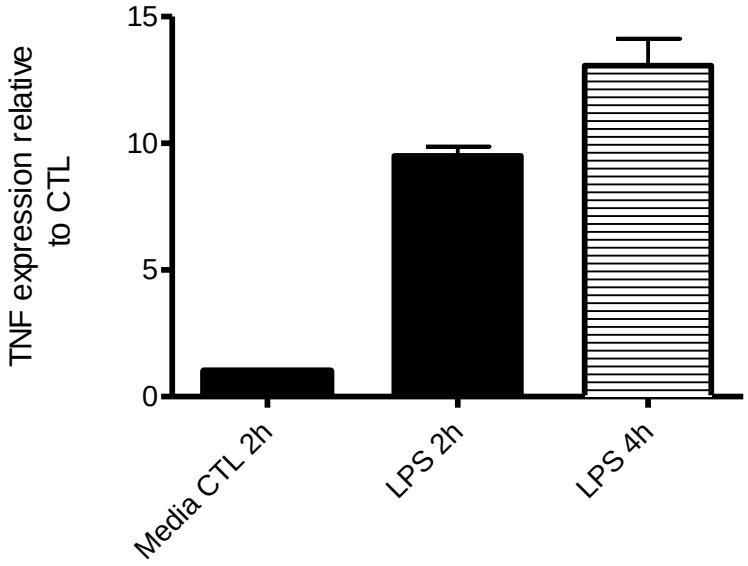

Figure 11: TNF expression relative to control in immortalized cell line stimulated with LPS (second trial)

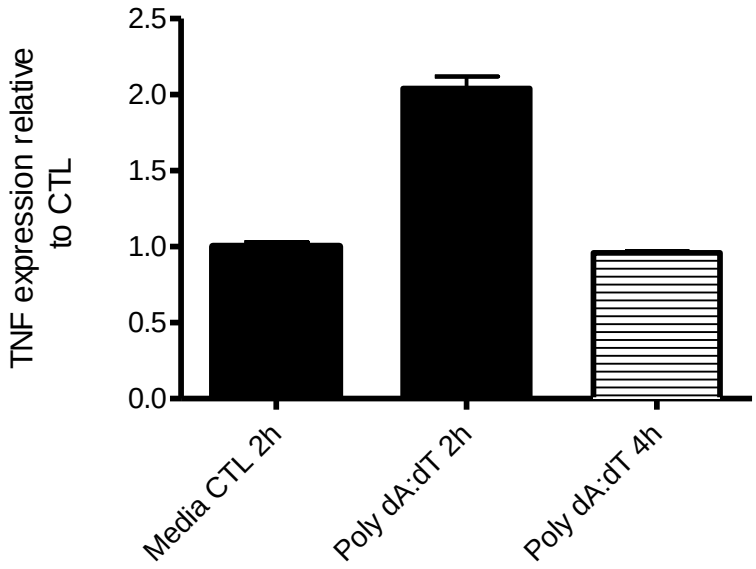

Figure 12: TNF expression relative to control in immortalized cell line stimulated with polydA:dT (second trial)

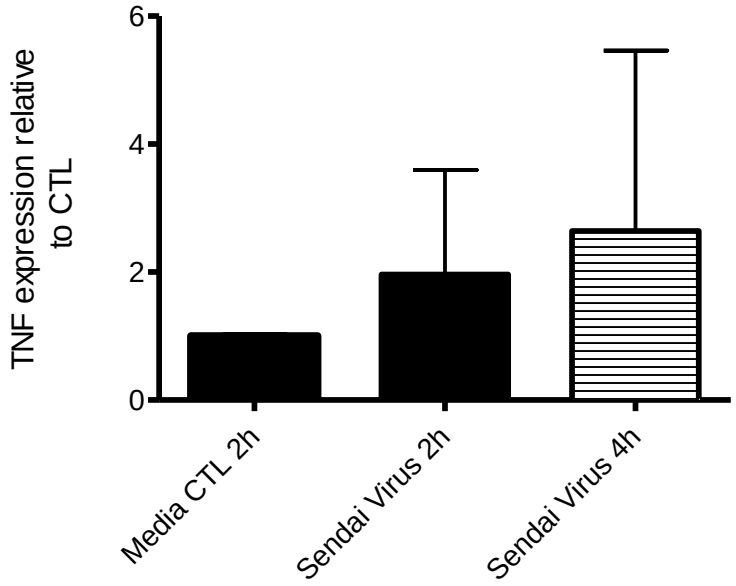

Figure 13: TNF expression relative to control in immortalized cell line stimulated with Sendai Virus (second trial)

The final gene expression experiment performed on immortalized cell line was for IFNβ. IFNβ expression was relatively large with all three stimulants. Figures 14 and 15 display these results. Figure 14 shows the high response of gene expression when stimulated with LPS. The data was not time dependent, as expected. As seen previously in Figure 7, both polydA:dT and

Sendai Virus induce reasonable gene expression of IFNβ. This is supported by the data seen in Figure 15 in which both show relatively high IFNβ gene expression in comparison to the control. Sendai Virus acted in a time dependent manner, as expected, with more gene expression after longer exposure to the virus. PolydA:dT, however, did not act in a time dependent manner, with both time points showing approximately the same gene expression of IFNβ.

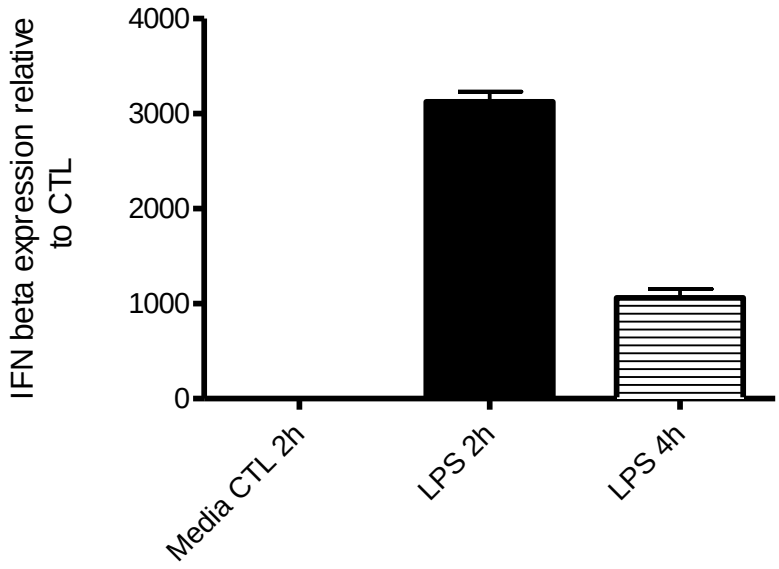

Figure 14: IFNβ expression relative to control in immortalized cell line stimulated with LPS (second trial)

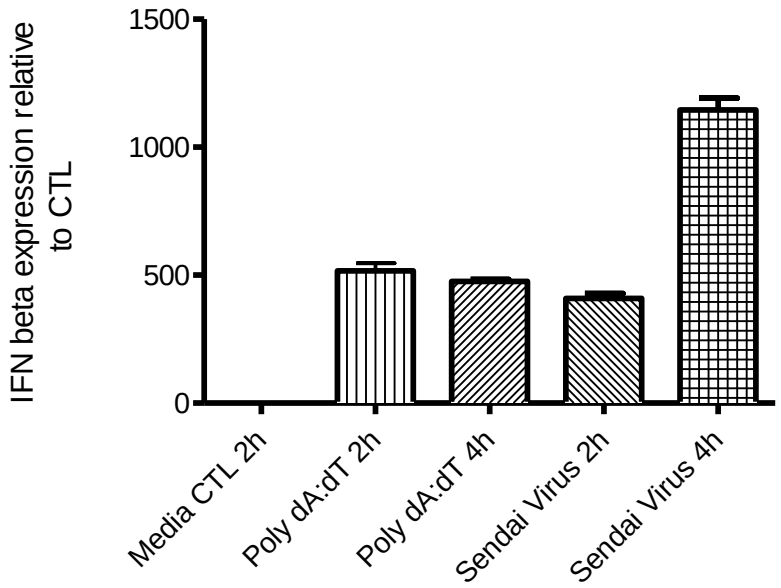

Figure 15: IFNβ expression relative to control in immortalized cell line stimulated with polydA:dT and Sendai Virus (second trial)

The immortalized cell line data implies that polydA:dT plays an important role in IFNβ gene expression. AIM2 induces the expression of inflammatory genes when it binds to dsDNA (polydA:dT) in the cytoplasm of a cell. The relatively high levels of IFNβ expression with the addition of polydA:dT imply that AIM2 is a significant player in IFNβ expression. The same experiments were also performed on primary macrophages, to insure that the immortalization of cells did not change them in a way that would influence gene expression. The primary macrophages did not seem to react in a noticeably time dependent manner, as can be seen in the following figures. These cells may be more sensitive to cell death than immortalized cells, resulting in some smaller responses over time.

The primary macrophage cell experiments can be seen in Figures 16 through 24. The expression of IL-6 can be seen in Figures 16 through 18. The expression of IL-6 is greatly influenced by LPS relative to the media control, in a time dependent manner, as seen in Figure 16. Figure 17, though, shows that IL-6 expression is only somewhat influenced by polydA:dT

exposure. While IL-6 expression is clearly expressed, the expression is quite a bit lower than LPS, for example, implying IL-6 expression is not strongly stimulated by polydA:dT. Figure 18 reflects the influence of Sendai Virus exposure on IL-6 gene expression. The data shows that IL-6 clearly is influenced by Sendai Virus exposure, though not in a time dependent manner. IL-6 seems to be influenced mainly by LPS stimulation, though it does show expression when exposed to polydA:dT and Sendai Virus.

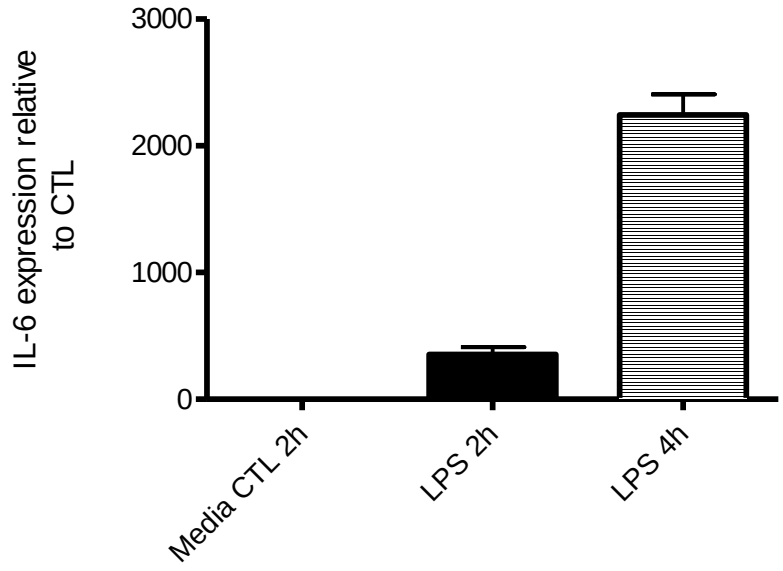

Figure 16: IL-6 expression relative to control in primary cell line stimulated with LPS

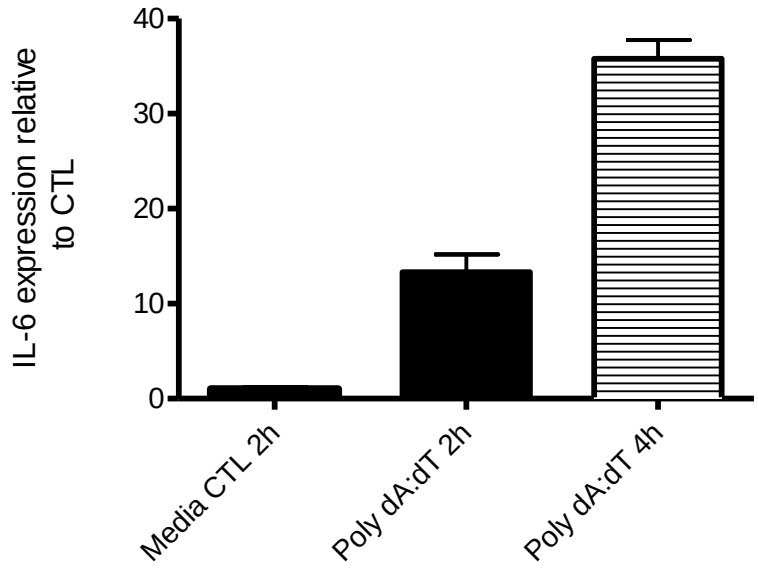

Figure 17: IL-6 expression relative to control in primary cell line stimulated with polydA:dT

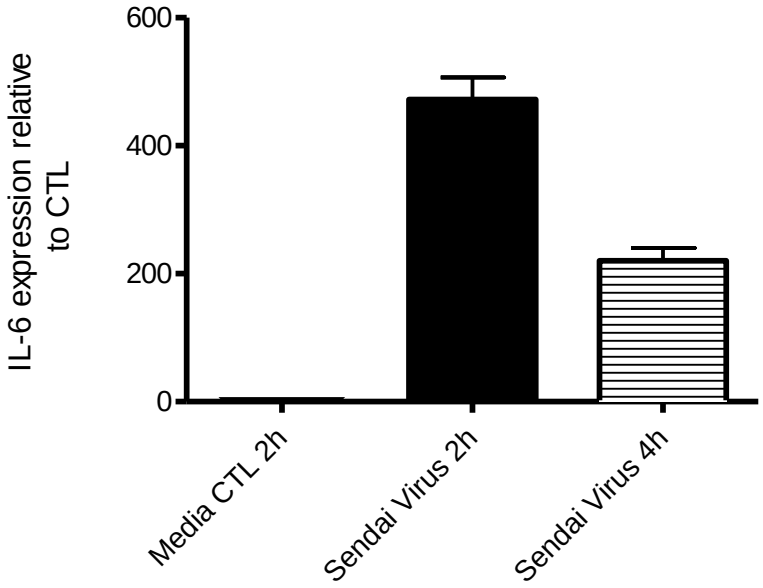

Figure 18: IL-6 expression relative to control in primary cell line stimulated with Sendai Virus

The gene expression of TNF in primary macrophage cells can be seen in Figures 19 through 21. Similar to the results in the immortalized cell lines, Figures 6 and 11 through 13,

TNF expression is considerably lower than the other two genes measured. Figure 19 reflects the influence of LPS exposure on TNF expression. TNF is somewhat induced by LPS and in a time dependent manner, but to a lesser degree than IL-6. The effect of polydA:dT stimulation on TNF expression can be seen in Figure 20 below. Though gene expression does not appear to be time dependent, the already minimal expression may have made it difficult to quantify properly. The final TNF expression experiment is shown in Figure 21. The influence of Sendai Virus exposure on TNF expression is relatively small, though it does influence expression more than polydA:dT. Sendai Virus exposure did not express TNF in a time dependent manner either.

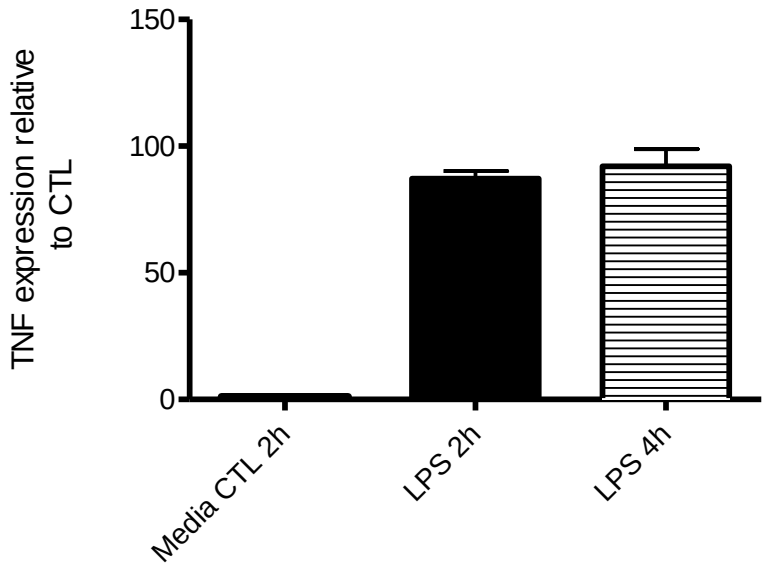

Figure 19: TNF expression relative to control in primary cell line stimulated with LPS

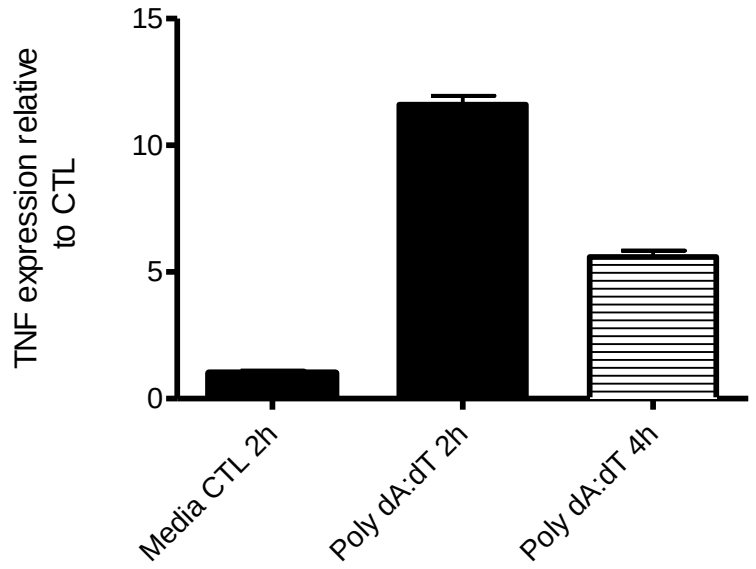

Figure 20: TNF expression relative to control in primary cell line stimulated with polydA:dT

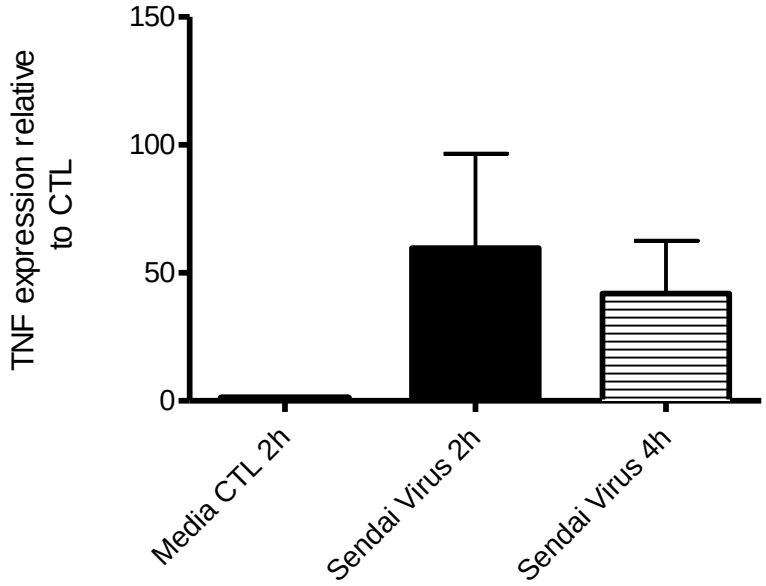

Figure 21: TNF expression relative to control in primary cell line stimulated with Sendai Virus

The final primary cell line experiment was performed with respect to IFNβ gene expression. Figures 22 through 24 show these data. Figure 22 shows the influence of LPS on IFNβ expression. Gene expression did not react in a time dependent manner, possibly due to

overstimulation of the cells at four hours. LPS, though, did stimulate some expression of IFNβ in the cells. PolydA:dT influenced IFNβ expression to a much greater degree than LPS, however. As seen in Figure 23, polydA:dT also did not act in a time dependent way, but still had a relatively high amount of IFNβ gene expression. Finally, Figure 24 shows the influence of Sendai Virus stimulation on IFNβ expression. Similar to polydA:dT, but to a much higher degree, Sendai Virus influenced IFNβ gene expression.

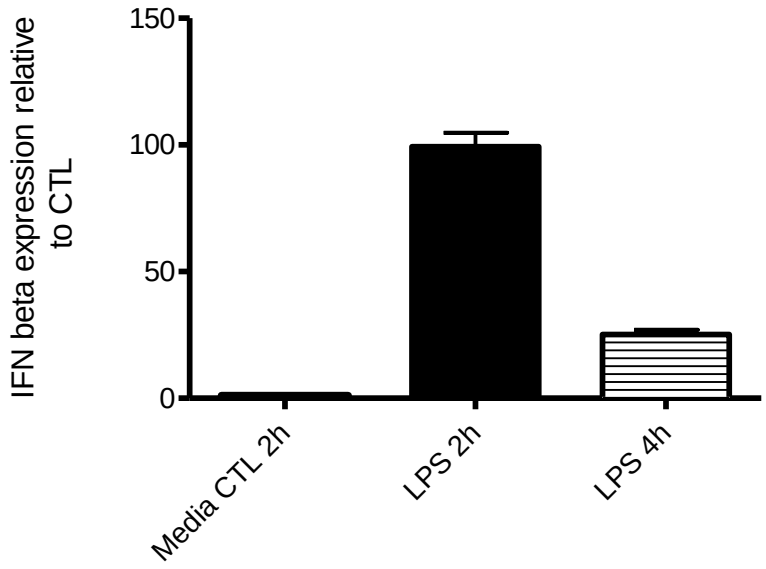

Figure 22: IFNβ expression relative to control in primary cell line stimulated with LPS

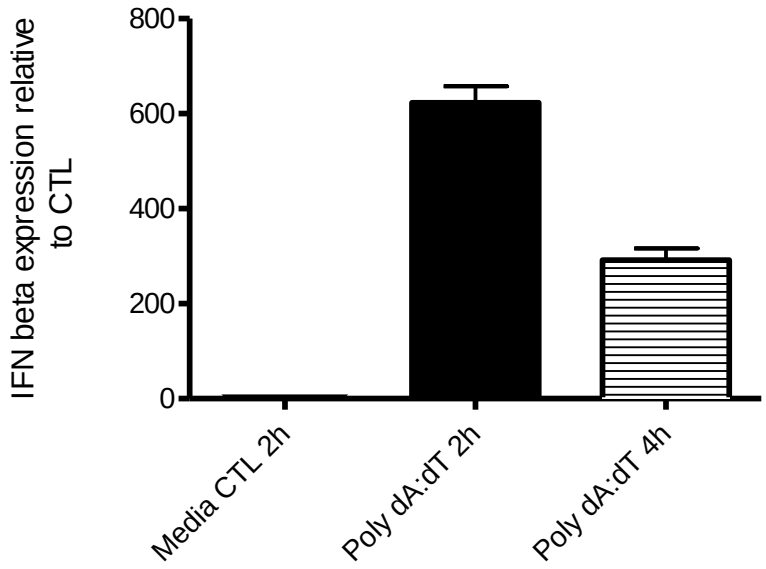

Figure 23: IFNβ expression relative to control in primary cell line stimulated with polydA:dT

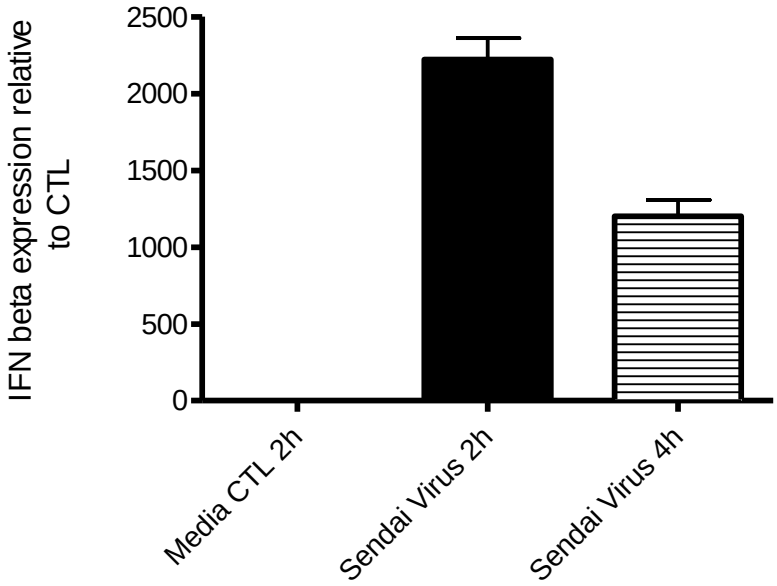

Figure 24: IFNβ expression relative to control in primary cell line stimulated with Sendai Virus

From the data of both immortalized and primary cell lines, polydA:dT stimulation seems to be the most influential in the expression of the IFNβ gene. It seems only somewhat important for IL-6 gene expression and minimally significant in TNF expression. However, further

experimentation is required to determine AIM2's role in IFNβ gene expression induced by cytosolic DNA. This can be done in several ways. For example, doing the same experiments performed here on primary and immortalized cell lines lacking the AIM2 protein. Comparing inflammatory gene expression in cells with and without AIM2 will show the influence it has on the expression of specific inflammatory genes.

Another way of determining the influence of AIM2 in inflammatory gene expression is through use of technology such as Nanostring. Nanostring has the ability to measure the expression of many more genes than performed in this experiment. Using both $AIM2^{+/+}$ and $AIM2^{-/-}$ cells, the expression of many inflammatory genes can be observed and compared. These comparisons would reflect whether AIM2 regulates the expression of inflammatory genes such as IFNβ, IL-6, and TNF in response to cytosolic DNA. With the data gathered and further experimentation, the role of AIM2 in inflammatory response can be better understood.

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