Spring 2023

Influence of Cell Cycle on AIM2 Inflammasome Activation

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INFLUENCE OF CELL CYCLE ON AIM2 INFLAMMASOME ACTIVATION

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Ashok Kumar Dubey

August, 2023

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Biology

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ABSTRACT

A cell cycle's progression has a significant role in the development of a disease. All the signaling pathways and inflammatory reactions are maintained in a healthy mode if cell cycle checkpoints are precisely regulated and kept in homeostasis. AIM2 inflammasome is a cytoplasmic double-stranded DNA sensor. Upon activation, it elicits an inflammatory reaction with the release of pro-inflammatory cytokines such as IL-1β and IL-18 and they are cleaved by the proteases, Caspase-1 from their inactive form. Also, the AIM2 inflammasome helps in regulating cell division and inhibits cell proliferation; thus, leading to a cell death called pyroptosis. Here, I examined the effects of inhibiting the cell cycle on the AIM2 inflammasome. Furthermore, I investigated whether AIM2 gets inactivated or shut down during mitosis. By using immortalized murine macrophages and treating these cells with LPS, DNA, and etoposide, I found that there was a change in AIM2 gene expression. However, there was no significant change in AIM2 protein level or IL-1β levels. My data indicates that mRNA expression is not solely responsible for controlling AIM2 expression and inflammasome activation but also there might be some internal activation such as phosphorylation or post-translational modifications going on.

KEYWORDS: inflammasome, AIM2, caspase-1, cytokine, etoposide, IL-1β, IL-18, LPS

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ACKNOWLEDGEMENTS

I would like to first express my gratitude to the Almighty who has allowed me to pursue higher education. I am thankful to God that I got full funding to pursue my higher education in the US.

I would like to express my sincere gratitude to my advisor, Dr. Christopher Lupfer, who helped me in this entire process of research and thesis writing. He showed immense faith in me and my work. He believed in my abilities as a student and told me to never quit. I will never forget his mentorship and the patience that he provided. In addition, I would like to thank my committee members Dr. Babur Mirza and Dr. Laszlo Kovacs for supporting me through this project by providing insightful ideas and motivation.

I want to thank Missouri State's Department of Biology and the Graduate College for funding and supplying endless resources that were vital to my portion of this project. I also want to thank Cathy as she was always there in case I needed any help.

I would like to thank my parents for their belief and support this whole time. I would also like to thank my dear friends Mr. Achyut Duwadi, Mr. Aakash Adhikari, Ms. Rakshya Bhatta, Mr. Adarsha Kiran Khadka, and Mr. Suman Neupane for their support and encouragement.

I dedicate this thesis to each graduate student who is already exhilarated and exhausted from being inculcated into the discipline and from all the learning, yet now finds him/herself ready to prepare for publication by polishing the thesis, the culminations of years of work.

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INTRODUCTION

We are cognizant of the fact that there is always combat between host and pathogen. This has resulted in many dreadful consequences like epidemics and pandemics. However, mankind has made many achievements and advancements in subjugating them including, decoding their intricate signaling pathways, discovering their toxins, and preventing or inhibiting their infections with vaccines and antimicrobial drugs. However, this battle is a never-ending one as the pathogens either mutate themselves or modify their way of infecting the host. The body's immune system is the natural defense against pathogens, yet how the immune system distinguishes between pathogen and self is an ongoing area of research. This thesis examines the relationship between the immune system and the cell cycle, and how they affect the response to infection.

When there is inflammation in the body, either from stimuli signaling damage or infection, it is often linked with pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are present on pathogen cell walls, carbohydrates, DNA, lipoproteins, or other structures. One example is lipopolysaccharide (LPS) which is found on the external cell wall of gram-negative bacteria. On the other hand, DAMPs are found in host cells; for instance, tumor cells and dead or dying cells release ATP and HMGB1 (Newton & Dixit, 2012).

Pattern Recognition Receptors

PAMPs and DAMPs get bound to pattern recognition receptors (PRRs), such as Toll-Like receptors (TLRs), intracellular retinoic acid-inducible gene-I-like receptors (RLRs),

cytoplasmic NOD-like receptors (NLRs), C-type receptors (CLRs) and absent in melanoma 2 like receptors (AIM2). Usually, the cells that have PRRs are immune cells like monocytes, macrophages, dendritic cells, and mast cells; however, non-immune cells such as fibroblasts and epithelial cells can also express PRRs. Binding of these receptors with specific ligands results in transcriptional changes and post-translational modifications that trigger immune responses (Mogensen, 2009).

Inflammasome

The inflammasome is a complex of proteins that plays a critical role in the innate immune response to pathogens and cellular stress. It gets activated by specific signals, such as the presence of PAMPs or DAMPs, and leads to the production of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and interleukin-18 (IL-18), and the initiation of a type of programmed cell death called pyroptosis (Molla et al., 2020). Several types of inflammasomes have been identified, including the NLRP3, AIM2, and NLRC4 inflammasomes. Different stimuli are responsible for activating inflammasomes and lead to the production of different inflammatory mediators (Guo et al., 2015).

Recent research has revealed the significance of inflammasomes in the host defense against a variety of pathogens, including bacteria, viruses, and parasites, along with their potential role in the development of certain autoimmune and inflammatory disorders. Dysregulation of inflammasome activation has been linked to several diseases, such as gout, Alzheimer's disease, and cancer (Cheon et al., 2020). Overall, the inflammasome is a major component of the innate immune system that plays a significant role in the host's defense against

pathogens and cellular stress, and its proper regulation is indispensable for maintaining overall health.

AIM2 Inflammasome

AIM2 stands for "Absent in melanoma 2". The AIM2 inflammasome is one of several inflammasomes that have been identified, and it is activated by the presence of double-stranded DNA in the cytoplasm. There are four members of AIM2 found in humans and 13 reported in mice so far. Once activated, the AIM2 inflammasome triggers the production of pro-inflammatory cytokines and leads to pyroptosis. AIM2 directly binds to double-stranded DNA in the cytoplasm and then combines with apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1 to assemble a multi-protein complex called the AIM2 inflammasome (Wang & Yin, 2017).

AIM 2 is a member of the Interferon- inducible HIN-200 protein family. Structural analysis shows that the positively charged HIN-200 domain contains the double-stranded DNA binding motif, while the pyrin domain (PYD) recruits ASC for caspase-1 activation. ASC is a bipartite PYD-CARD-containing protein. To create the basic structural elements of the AIM2 inflammasome, the CARD domain of ASC helps to recruit procaspase-1 to the complex. Caspase-1, which is a cysteine protease, cleaves the immature pro-forms of IL-1β and IL-18 and causes pyroptotic cell death that permits mature IL-1β and IL-18 to be released from the cell. The AIM2 inflammasome is also activated by self-DNA deposited in keratinocytes to release IL-1β in lesions of psoriatic patients providing evidence suggesting that AIM2 might respond to self-DNA produced during the damage of a cell to drive chronic inflammatory diseases (Dombrowski et al., 2011).

Unlike other PRRs, which only recognize molecules from pathogens, once dsDNA enters the cytoplasm, the HIN200 structure of AIM2 opens up and is exposed. Following that, the HIN200 domain directly interacts with DNA through mainly electrostatic interaction (Figure 1). Here, on the DNA backbone, a positively charged amino acid residue binds with phosphates and sugar moieties. This means that AIM2 can recognize DNA from any sequence or any source, including self-DNA. The only requirement is that the DNA is 80 bp in length or longer (Jin et al., 2013).

Other studies indicated the role of AIM2 in inflammation and cancer. Furthermore, AIM2 decreases the cell proliferation of breast cancer and mammary tumor growth in a mouse model and exhibited more frameshift mutations in colorectal cancers. In addition to this, analysis of the cell cycle of colon cancer cells showed AIM2- mediated inhibition of cell proliferation is linked to the accumulation of cells at the late S-phase, which results in G2/M arrest (Patsos et al., 2010).It is clear that AIM2 can affect the cell cycle, but it is not clear if the reverse is true, and the cell cycle can affect AIM2.

Cell Cycle Arrest

Cell cycle arrest is the temporary stoppage of cell division at a certain stage of the cell cycle. This process can occur at different stages of the cell cycle, including the G1, G2, and M phases. The cell replicates DNA in the S phase while during mitosis (M phase), replicated DNA in the form of sister chromatids is sorted into two daughter cells. G1 spans from mitosis to the next round of DNA replication. Similarly, G2 extends from S to the next M phase (Morgan, 1995). Cell cycle arrest can be triggered by a variety of internal and external signals, including

DNA damage, nutrient deprivation, and exposure to certain chemicals or drugs (Huang & Zhou, 2020).

Being a crucial mechanism, cell cycle arrest maintains genomic stability and prevents the proliferation of damaged or abnormal cells. In normal cells, the cell cycle is tightly monitored by a complex network of signaling pathways and regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs). Cell cycle arrest can occur by different mechanisms, such as the activity of p21, p16INK4a, and p27. It seems that p53 is vital for the induction of p21 after DNA damage. Moreover, p21 can be controlled separately from p53 in a number of circumstances, such as normal tissue development, after serum stimulation, and during cellular differentiation (Abbas & Dutta, 2009). These proteins can inhibit the activity of CDKs, preventing the cell from progressing through the cell cycle (Kumari & Jat, 2021).

Cell cycle arrest can also occur through the activation of the tumor suppressor protein p53, which can trigger cell death or inhibit cell proliferation by inducing cell cycle arrest. The p53 protein can activate genes that promote cell cycle arrest, such as p21 and p27, or trigger cell death through the activation of other genes such as BAX and PUMA (Chen, 2016). Cell cycle arrest plays a critical role in preventing a cell with damaged genome from entering S phase, thereby preventing the development of cancer and other diseases. Drugs that target cell cycle regulators are used in cancer therapy to stop the proliferation of cancer cells (Otto & Sicinski, 2017).

The cell cycle is a cell division regulating process. To better understand how it works and how it can go wrong in cancer, scientists have designed a computational model for the group of proteins called cyclin-dependent kinases (CDKs) that control the cell cycle in mammals. This

model aids in comprehending the balance between cell cycle arrest (when cells stop dividing) and cell proliferation (when cells keep dividing)(Gérard & Goldbeter, 2014).

In such a model, the balance might be maintained due to growth factors or hormones that influence cells to divide by activators (oncogenes) or inhibitors (tumor suppressors) controlling the cell cycle progression. If these factors exceed a certain threshold, the model predicts a change in the behavior of the CDK network. This switch can result in either a stable state where the cell cycle is arrested or sustained oscillations of the cyclin/CDK complexes, leading to cell proliferation (Morgan, 1995).

Etoposide

Etoposide is an anti-cancer chemotherapeutic drug. Etoposide has been found to induce apoptosis in cancer cells by activating caspases and by releasing cytochrome c from the mitochondria (Jamil et al., 2015). It's also worth noting that while etoposide is an effective cancer treatment, it also has toxic effects on normal dividing cells, such as those in the bone marrow and gastrointestinal tract, leading to side effects such as anemia, leukopenia, and nausea. The way etoposide works is that it inhibits DNA replication via making a complex with Topoisomerase II and DNA. This complex causes breaks in double stranded DNA and stops repair by Topoisomerase II. These accumulated breaks in DNA prevent it from entering the mitotic phase of cell division and the progression of cells through the cell cycle ultimately resulting in apoptosis. This is achieved through the activation of the tumor suppressor protein p53 and the induction of the CDK inhibitor p21. Additionally, it can activate DNA damage response pathways, such as ATM and ATR, which can lead to the activation of p53 and p21 (Rezonja et al., 2013).

LITERATURE REVIEW

Recent literature showed that AIM2 inhibits the proliferation of the intestinal stem cell population. In contrast to its role in the protection of the host from infection, AIM2 safeguards against intestinal epithelial cell proliferation through an inflammasome independent mechanism (Man et al., 2015). Another study suggested that the AIM2 inflammasome plays a role in regulating cell division. Importantly, the AIM2 inflammasome induces cell cycle arrest at the G1 phase through inhibition of cell proliferation, via the regulation of cyclin-dependent kinase inhibitors (CDKIs) such as p21 and p27 (Peng et al., 2013). Research has also revealed that the AIM2 inflammasome can activate the tumor suppressor protein p53, which can trigger cell death or inhibit cell proliferation by inducing cell cycle arrest. In addition to its inhibitory effects on cell division, the AIM2 inflammasome appears to play a role in promoting cell death through a process called pyroptosis. This type of cell death is characterized by the release of inflammatory mediators, such as IL-1β and IL-18, and is mediated by the caspase-1 enzyme (Sagulenko et al., 2013).

In contrast to the role of NLRP6 in targeting the secretion of goblet cell mucin, as reported by Wlodarska et al., 2014, the proliferation and expansion of intestinal stem cells is mainly regulated by AIM2. The significant number of cells extracted from $Prom1^{+}$ cells that line the intestinal crypt in mice deficient in AIM2 following aberrant induction of β-catenin showed the potency of these highly proliferative cells. In vitro studies point out that AIM2's ectopic expression might be useful for enhancing cell-cycle arrest and inhibition of cell proliferation (Patsos et al., 2010). It has also been suggested that the AIM2 inflammasome can contribute to cancer development by promoting cell proliferation and resistance to cell death. Studies have shown that the AIM2 inflammasome is activated in several types of cancer such as breast cancer,

melanoma, and lung cancer and that its activation can promote cancer cell proliferation and survival. Overall, the literature suggests that the AIM2 inflammasome plays a complex role in regulating cell division and cell death, and its regulation may have important implications for the development of cancer and other diseases (Sharma et al., 2019).

In addition to regulating the cell cycle and tumorigenesis, a primary function of AIM2 is in the immune response. A study carried out by Saiga et al., 2012 showed that macrophage in mice that were lacking AIM2 and infected with *Mycobacterium tuberculosis* displayed seriously dysregulated secretion of IL-1β and IL-18. Another study showed that macrophages that lack AIM2 are incapable of caspase-1 activation, IL-1β secretion, and cell death in response to cytosolic DNA, or even when there is infection with *Francisella tularensis*. Even though the transcriptional type I interferon response to *F. tularensis* infection is not influenced by the AIM2 deficiency, AIM2-deficient mice are more susceptible to the harmful effect of *F. tularensis* than wild-type mice. Therefore, they suggested that the type I interferon response is not enough on its own for protection against *F. tularensis* infection and that the AIM2 inflammasome activity is needed for complete innate immunity against this pathogen (Fernandes-Alnemri et al., 2009).

Although research suggests that AIM2 can affect the cell cycle, after going through some published research papers and their literature review, I was eager to see the impact of the cell cycle on AIM2 inflammasome activation. More specifically, does it get inactivated or shut down during mitosis (M phase) in order to prevent inflammasome activation when self-DNA is in the cytoplasm. Therefore, I hypothesized that there should be changes in the gene expression or protein expression of AIM2 if Etoposide is added to inhibit the cell cycle or induce cell cycle arrest.

MATERIALS AND METHODS

Animal Welfare

All the experiments were performed in the Blunt Hall 232 at Missouri State University following all the required guidelines. The research did not include any humans, animals, or radioactive substances. Therefore, approval for Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB) was not required. However, Institutional Biosafety Committee (IBC) approval was taken (Appendix).

Examination of cell cycle arrest and Serum Starvation

Immortalized *Nlrp12*$^{-/-}$ macrophages were grown in T75 flasks in 10 ml of Dulbecco's Modified Eagle Medium (DMEM) and 5 ml of Bone Marrow-Derived Macrophage medium containing G-CSF (BMDM) (Table 1). When the cells reached their confluency of 70 %, the cells were scraped and transferred to a 15 ml vial. 10 µL was used on a hemocytometer to count the number of cells. Following that, 1 X 10^6 cells were plated into 6 wells of a 12-well plate in 1 ml of medium. See (Figure 2) for more information.

On the next day, media was removed from all wells. Following that, 1 ml PBS was added to all the wells. The cells were scraped with a pipette tip and 1 ml of the scraped cells were kept in 6 Eppendorf tubes and centrifuged for 4 minutes at 2000 rpm. After that, the supernatant was removed. Then 500 µL ice-cold 70 % ethanol was added to the pellets and pipetted to and fro to suspend the cells efficiently. The cells were incubated at 4° C for 30 minutes and centrifuged for 2 minutes at 2000 rpm henceforth. After that, the supernatant was taken off and 1 ml PBS was added and centrifuged and repeated twice. Finally, the supernatant was removed and 200 µL of PBS mixed with 5 µL of 7-AAD dye (Catalog No. 51-68981E, BD

Pharmingen) was added to each of the 6 wells. After 5 minutes, 200 µL of PBS was added and analysis of cell cycle phase distributions was done by flow cytometry on a BD Accuri C6 Plus. (Figure 3)

Treatment protocol

Initially, the Etoposide that we ordered was diluted with DMSO (dimethyl sulfoxide). 23.5 µL of diluted Etoposide and 976.5 µL DMSO were added to Eppendorf tubes to make a stock solution of 1 mM. Since the concentration of 0.1 µM was more effective, I mixed 1µM of Etoposide with 9 µL of DMSO and added 1 µL of this mixture to treated wells for 24 hours at a final concentration of 0.1 µM.

For the transfection procedure, I removed the media from all wells and added 750 µL of DMEM only. Then the transfection mixture was made by adding 100 µL DMEM, 1.5 µg DNA, and 4.5 µL PEI (polyethyleneimine). The mixture was shaken 5-10 times and incubated for 15 minutes at room temperature. Then 67 µL of the mixture was added to each well that needs to be transfected. The plate was incubated for 4 hours, and the media was replaced with a normal medium (FBS+BMDM and DMEM). The day after treatment with Etoposide and DNA, the cells were stimulated with 100 µg/ml LPS. After 4 hours, supernatants (200 µL) were saved in a 96-well plate for ELISA. The treatment scheme of the 12 well plate layout is illustrated as Figure 4.

RNA Isolation.

After treatment was complete, the rest of the media was removed from the 12 wells and 500 µL of RNAzol (Invitrogen, catalog #AM97381) was added to each well and incubated for 5

minutes at room temperature for the extraction of the total mRNA from the samples. After a minute, the cells were scraped and transferred to a nuclease-free Eppendorf tube with the help of a filtered micropipette, followed by storage in a -80 °C freezer. After thawing the samples, 200 µL of RNAse free water was added. Samples (0.5 mL) were then transferred to labeled 1.5 mL centrifuge tubes and centrifuged for 15 minutes at 12000 x g. The clear aqueous liquid on the top layer that mainly contains RNA was carefully pipetted off without disturbing the pellets and transferred to new labeled tubes. Following that, 500 µL of isopropanol was added to each sample. Tubes were shaken vigorously by hand for about 15 seconds and incubated at room temperature for 10 minutes. Then, tubes were centrifuged at 12,000 x g for 10 minutes at 4° C. Again, supernatant is removed and 500 µL of 75 % Ethanol was added to all tubes. The tubes were centrifuged at 8,000 x g for 3 minutes and the procedure was repeated twice. The supernatant was taken off and the lid was left open to let the ethanol evaporate. Then 30 µL of RNase-free water was added to each tube. The concentrations of the RNA samples were measured using an Implen Nanophotometer. All samples were normalized to 200 ng/µL by adding additional nuclease-free water to each sample.

cDNA Synthesis and qRT-PCR.

To perform qRT-PCR, the unstable RNA must be reverse-transcribed into complementary DNA (cDNA) to be used. The High Capacity cDNA Reverse Transcriptase Kit (ThermoFisher Scientific, catalog #01313646) was used to reverse-transcribe mRNA into cDNA according to the manufacturer's instructions using 1 µg total RNA. cDNA samples were diluted 1:5 (80 µL of nuclease-free water to the 20 µL of amplicon solution). 5 µL of the diluted cDNA samples and 2-fold standard curve samples were analyzed via quantitative real-time PCR (qRT-

PCR) utilizing the DyNAmo HS SYBR Green qPCR Kits (Thermo-Fisher Scientific, catalog #F.410L) according to the manufacturer's directions. Please see (Table 2) for more information. Primers (forward and reverse) for β- Actin and AIM2 were used to test gene expression (Table 3). The instrument used for data acquisition was a Quant Studio Pro 6 qPCR machine.

Enzyme-Linked Immunosorbent Assay (ELISA)

The analysis of IL-1β was done from the supernatants of control and treated cell cultures. Mouse IL-1β uncoated ELISA kits were purchased from Thermo Fisher Scientific (88-7013-88, 88-7064-88), and assays were performed following the manufacturer's recommendations except the antibodies and streptavidin-HRP were diluted to a concentration of 1:500. Then, plates were read using a BioTek ELx808 microplate reader at 450 nm.

Western Blotting

Initially, cell lysates treated with RIPA buffer and 4X SDS dye were thawed and boiled at 95° C for 20 minutes. See (Table 4) for recipe of RIPA buffer. Following that, a 15 µL volume of samples and 2 µL of the protein ladder (Accu Prestained Protein Ladder, catalog no. G02) were loaded into 12 % acrylamide gels and electrophoresis was done at 100 V for one and a half hours with the help of a running buffer (Table 5). Finally, the isolated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Immobilon-P, Merck Millipore, LOT #0000160848) at 45 V for 45 minutes. After the completion of the transfer run by using transfer buffer (Table 6), the PVDF membrane underwent blocking with 5 % skim milk for 30 minutes in 1X TBST. One of the membranes was treated in AIM2 rabbit primary antibody (Cell Signaling, Catalog no. 63660S, LOT # 1) and another one with Actin antibody solution overnight

at 4° C on a shaker. The diluted primary antibody was removed and stored. Then membranes were washed three times with roughly 10 mL of 1X TBST for 10 to 20 minutes shaking per wash at room temperature. The membranes were then treated with species specific horseradish peroxide-linked secondary antibodies (1:5,000) (Catalog AB_2721864) diluted in 5 % milk + TBST after the final wash was discarded. Membranes were gently shaken while being incubated at room temperature for about 45 minutes. The membranes were washed four times with 10 mL of 1X TBST at room temperature with vigorous shaking for around 10 to 20 minutes for each wash after the secondary antibody was removed and stored. After the final wash was discarded, each membrane was treated for a minute with 1 mL of the substrate (Azure Biosystems Radiance Q, Chemiluminescent substrate for Quantitative Westerns, LOT #220316-95) and imaged using a C300 digital imaging system from Azure Biosystems.

Statistical Analysis

For data analysis, one-way ANOVA with Tukey's post hoc analysis was performed using GraphPad Prism 6 software (GraphPad Prism 6 Software, San Diego, CA, USA). A p-value <0.05 was considered statistically significant. All the figures with visual representations illustrating immune signaling or cellular mechanism were completed with the latest version of the academic membership of Biorender.com.

RESULTS

Cell cycle arrest by Etoposide

I examined the ability of Etoposide to induce cell cycle arrest. After treating with Etoposide at concentrations of 0.1μM, 0.5 μM, 1 μM, and 2 μM, it was found that 0.1 μM was the most effective. Initially, the untreated cell population showed 47.7 % being arrested at the G1 phase while only 22.3 % in the G2/M phase (Figure 5A). On the other hand, there was a substantial increase in the G2/M population by more than two fold (52.4 %) when treated with 0.1 μM etoposide (Figure 5B), accompanied by a marked decrease in cells at the G1 phase (31.6 %) (Figure 5B). On the other hand, upon treatment with 0.5 μM Etoposide, the number of cells at G1 phase elevated (54.4 %) with a reduced cell population at G2/M phase (28.1%) (Figure 5C). Similarly, with 1 μM Etoposide, the G1 cell population peaked up (62.3%), while cells at G2/M were highly reduced (24.8 %) (Figure 5D).

Effect on gene expression

I performed qRT-PCR to observe the AIM2 expression by quantifying the levels of mRNA. In comparison to the control, there was no significant increase in the mRNA levels when cells were treated with etoposide. The relative mRNA expression was doubled when treated with Etoposide (10.04 ±21.44) as compared to the control (4.09 ± 2.52). However, with the addition of LPS along with transfection, the level of AIM2 increased by more than five-fold (24.52±47.76). Finally, when the cells undergo treatment with all three of the reagents like LPS, DNA, and Etoposide, it resulted in a substantial amount of AIM2 expression (36.16±60.47), being highest relative to the rest (Figure 6).

Pro-inflammatory IL-1 β cytokine response:

When the cells were treated with LPS and DNA, there was a significant increase in IL-1β when compared to the untreated control cells or the cells treated with only etoposide (Figure 7). The control showed baseline levels of only 103.8 pg/ml ±2.368. Similarly, the addition of etoposide did not make much difference. When the cells were treated with LPS and DNA, there was a huge uptick in the IL-1β levels resulting in a mean of 2379 pg/ml ± 90.61. However, adding etoposide to the LPS and DNA treatment did not increase IL-1β any further with a mean of 2196 pg/ml ± 115.2. Thus, it was observed that etoposide alone did not elicit significant amounts of IL-1β and Etoposide with LPS and DNA did not enhance IL-1β (Figure 7).

Expression of AIM2 protein

To understand the impact of treatment factors on the production of AIM2 protein, I performed Western blotting for AIM2 and used Actin as a control. If we observe the protein bands at AIM2 and Actin (Figure 8), we can see that the relative amount of AIM2 is slightly upregulated when treated with Etoposide which is 0.9838±0.1719 (Figure 9) as the bands are distinct in control (β-actin) and AIM2. However, when cells were treated with DNA and further with etoposide and LPS, it got downregulated and sharply reduced and is almost invisible. The relative value of AIM2 with respect to Actin was only 0.8996±0.1309 (Figure 9).

DISCUSSION

Inflammation involves a well-coordinated network of various cell types like macrophages and monocytes that respond to tissue damage and infection. It is imperative to have precise regulation and homeostasis during cell cycle progression. If not, it might lead to inflammatory reactions resulting in a catastrophe and the emergence of a plethora of diseases. Any dysregulation in the cell cycle pathways and checkpoints either by mutations or by other means contributes to uncontrolled proliferation and cancer too (Mahesh et al., 2022). AIM2 (absent in melanoma 2) is the most prominent member of the AIM2 class of receptors and forms an inflammasome by recruiting caspase-1 and ASC protein. It can recognize cytoplasmic double-stranded DNA irrespective of its origin. Upon binding with the DNA, it leads to the secretion of cytokines like IL-1β and IL-18. During the cell cycle, specifically at the M phase, the nuclear envelope breaks down, and DNA is released into the cytoplasm. In this study, I am trying to find out what happens to AIM2 then. If AIM2 is still in the cytoplasm, how does it avoid activating the inflammasome?

In my study, I used etoposide, an anti-cancer drug to induce a cell cycle arrest at the G2/M phase. Etoposide targets Topoisomerase II and inhibits DNA synthesis via making a permanent complex with Topoisomerase II and DNA. This effect triggers the accumulation of double-strand breaks in the genome after it is replicated. These accumulated breaks in DNA prevent cells from entering mitosis. It further activates G2 checkpoints, thus also producing cell cycle arrest at the G2 phase (Montecucco et al., 2015).

Previous studies showed that etoposide treatment of cell lines, such as MCF-7, caused DNA damage-induced G2/M arrest by the activation of protein kinase ataxia telangiectasia

mutated (ATM), followed by the activation of Chk2 that subsequently inactivates CDC25C, resulting in G2/M arrest (Zhang et al., 2016). Another study showed that usage of much lower doses of etoposide for 48 hours was more effective than those of higher doses at inducing cell arrest. (Higginbottom et al., 2002). This corresponds to my data, as the lowest concentration was more effective in my study too.

To test this effect, I used LPS to induce AIM2 expression and transfected DNA into the cytoplasm to activate AIM2 and thus lead to activation of Caspase 1 and release of IL-1β. I observed that there was an enormous release of IL-1β when treated with LPS and DNA together. but almost the same even with Etoposide added to LPS and DNA or Etoposide alone. It is evident from a research paper in which murine macrophages model was used and displayed the production of a high amount of IL-1β by stimulation with LPS (Xie et al., 2014). Since LPS is a strong activator that stimulates immune cells like macrophages to produce inflammatory cytokines such as IL-1β.

As mentioned in the qRT-PCR data above, it is obvious that there is elevated expression of AIM2 upon stimulation of cells with combined treatment with LPS, DNA, and etoposide. However, it is reduced by more than half when treated with Etoposide alone. I hypothesized that the cell cycle might be controlling the expression of the AIM2 gene. Even if AIM2 is supposed to be activated, it can be self-activated irrespective of the cell cycle. This further implies that there might be other ways of internal regulation. It's not only the mRNA expression controlling AIM2 expression, but many post-translational mechanisms may also come into play. For instance, phosphorylation or other post-translational modifications.

However, my mRNA and protein abundance data contradict one-another. In the case of the qRT-PCR, a significant amount of AIM2 expression was observed with etoposide treatment,

but Western blotting revealed that AIM2 protein levels were downregulated when stimulated with LPS+DNA+etoposide. Since more AIM2 gene is being expressed, more AIM2 protein ought to have been expressed as well. The question that arises here is with less AIM2 protein expressed, why would I still get the same amount of inflammasome activation and the same amount of IL-1β produced? This seems enigmatic.

I believe the reasons behind low protein expression is that the mRNA is prevented from being translated. One study showed that if the RNA binding proteins and microRNAs bind to the regulatory elements in 3' untranslated region (3' UTR) of mRNA, it might repress mRNA translation. Furthermore, the microRNAs (miRNAs) can lead to repression of mRNA translation too. Additionally, the polyadenylation level at the 3' end of the mRNA can greatly influence translation of mRNA. (Baker & Coller, 2006). Sometimes, mutational changes and availability of abundant free ribosomes might affect the mRNA translation as well.

A recent study highlighted that epigenetic factors such as DNA methylation and histone acetylation regulate NLRP3 mRNA expression in response to *Mycobacterium tuberculosis* infection. Having said that, evidence showed that post-translational modification like phosphorylation and de-ubiquitination might trigger inflammasome assembly too (Zheng et al., 2020). I assume that the AIM2 inflammasome might have a similar kind of mechanism due to the influence of post-transcriptional and translational modulations. That may be the reason behind reduced AIM2 protein levels despite higher IL-1β production and elevated AIM2 gene expression.

Similar research demonstrated that AIM2 was expressed at reduced levels in osteosarcoma cell lines. Moreover, the overexpression of AIM2 caused inhibition of cell proliferation, invasion, migration, EMT, and enhanced apoptosis in osteosarcoma cell lines. Even

though there was a significant downregulation of phosphorylated (p)-PI3K, p-AKT, and p-mTOR after the overexpression of AIM2. Thus, the PI3K/AKT/mTOR signaling pathway may be inactivated by AIM2, which further inhibited osteosarcoma progression (J. Zheng et al., 2022). The low level of AIM2 protein in my research as indicated in the western blotting might be due to the inactivation of the PI3KT/AKT/mTOR signaling pathway. This pathway controls major cellular processes such as transcription, apoptosis, cell cycle progression, and translation through its downstream substrates (Hassan et al., 2013).

A former study found that mice lacking AIM2 are saved from both subtotal body irradiation-induced gastrointestinal syndrome and entire body irradiation-induced hematopoietic failure. Ionizing or UV radiation of 40 Gy and 80 Gy were used. AIM2 mediates the caspase-1-dependent death of intestinal epithelial cells and bone marrow cells in response to dsDNA breaks caused by ionizing radiation and chemotherapeutic agents. Furthermore, they found that AIM2 detects DNA damage which is caused due to radiation in the nucleus to mediate inflammasome activation and cell death (Hu et al., 2016). Their study suggested that AIM2 might be a novel therapeutic target for ionizing radiation exposure.

There are still several mysteries in the realm of AIM2 inflammasome that need to be answered. In the future, the reasons why only a few DNA-containing pathogens activate AIM2 inflammasome even though DNA is found in every bacterial pathogen and DNA virus needs to be illuminated. Another direction of enquiry is to find out why there is a link between AIM2 expression in tumor cells and patient survival. The answers to these and many more pending questions may help to formulate new treatments that can target various components of the inflammasome for combating diseases.

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Table 1. Bone Marrow Differentiating Medium Recipe

Ingredients	Volume
DMEM	300 ml
L-929 Medium	150 ml
Heat Inactivated FBS	50 ml
Penicillin/Streptomycin (10,000 U/mL)	5 ml
Non-Essential Amino Acids (NEAA)	5 ml

Table 2. cDNA PCR Master Mix Recipe

Ingredients	Amount (w/o inhibitor)
13X RT Buffer	26 µL
13X RT Random Primers	26 µL
dNTP Mix	10 µL
Multiscribe Reverse Transcriptase	13 µL
Nuclease-free H2O	119.6 µL

Table 3. Quantitative Real Time-PCR Primer Sequences

Specific Gene	Forward Primer	Reverse Primer
β-*Actin*	5’ GGC TGT ATT CCC CTC CAT CG 3’	5’ CCA GTT GTT AAC AAT CGG ATG A 3’
Aim2	5’ GAT TCA AAG TGC AGG TGC GG 3’	5’ TCT GAG GCT TAG CTT GAG GAC 3’

Table 4. 1 X RIPA Lysis Buffer Recipe

Ingredients
10 ml RIPA buffer
1 Protease Inhibitor Tablets
100 µl of Phosphatase Inhibitor

Table 5. 10 X Running Buffer (For 1 Litre)

Ingredients	Amount
Tris base	30 g
Glycine	144 g
SDS	10 g
Deionized H_2O	1 ltr

Table 6. 1 X Transfer Buffer (1 Litre)

Ingredients	Amount
ddH20	700 ml
10 X Transfer buffer	100 ml
Methanol	200 ml

Table 7. 1 X Tris Buffered Saline Tween 20 (TBST)

Ingredients	Amount
10X TBS	100.00 mL
diH2O	Fill up to 1000 ml
Tween 20	0.50 ml

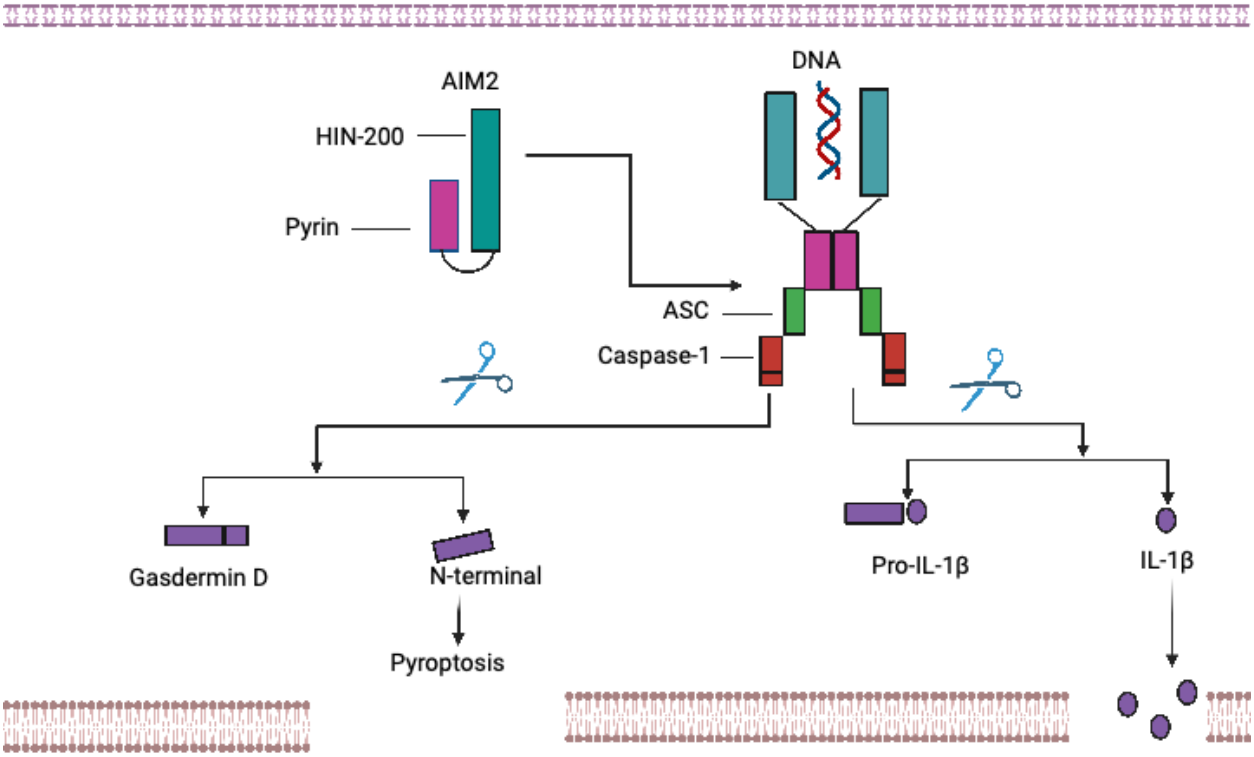

Figure 1. Illustrating the mechanism of AIM2 inflammasome assembly and its activation. Upon interaction with double-stranded DNA, AIM2 gets activated, and eventually, cleavage of pro-inflammatory cytokines like pro-IL 1β into mature IL- 1β occurs. All visuals were created on Biorender.com.

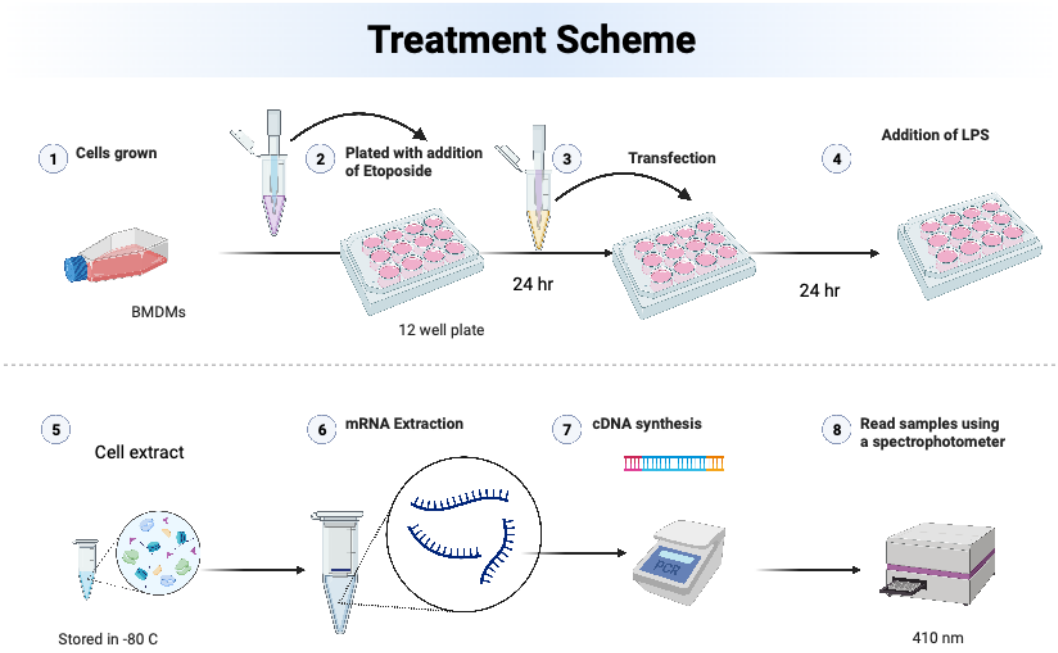

Figure 2. Representing the entire treatment plan of experiments. The stepwise scheme shows BMDM cells being grown followed by plating, drug treatment, and culminating analysis. All the visuals were created on Biorender.com.

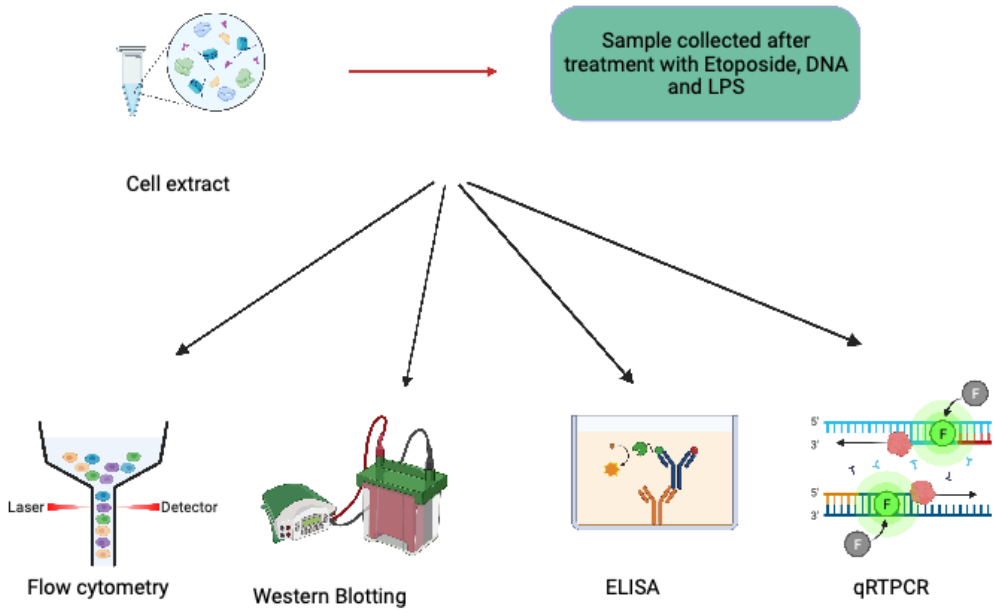

Figure 3. Schematic diagram representing the summary of all the experiments. The experiment was conducted from cell extract obtained post-treatment with Etoposide, DNA, and LPS. All the visuals were created by the latest version of Biorender.com.

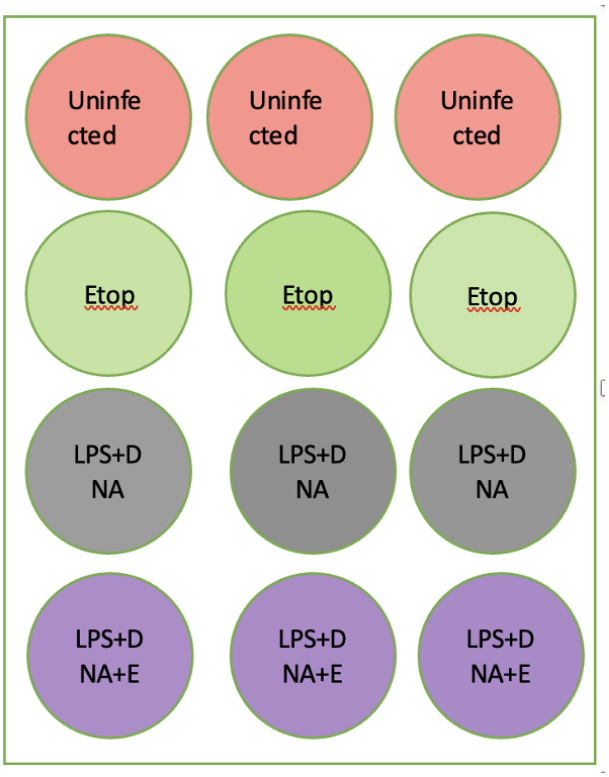

Figure 4. 12-well plate layout for treatment scheme. The layout represents the treatment applied to the cells when stimulated with uninfected (Control), etoposide, transfected uninfected and transfected cells with etoposide and lipopolysaccharide.

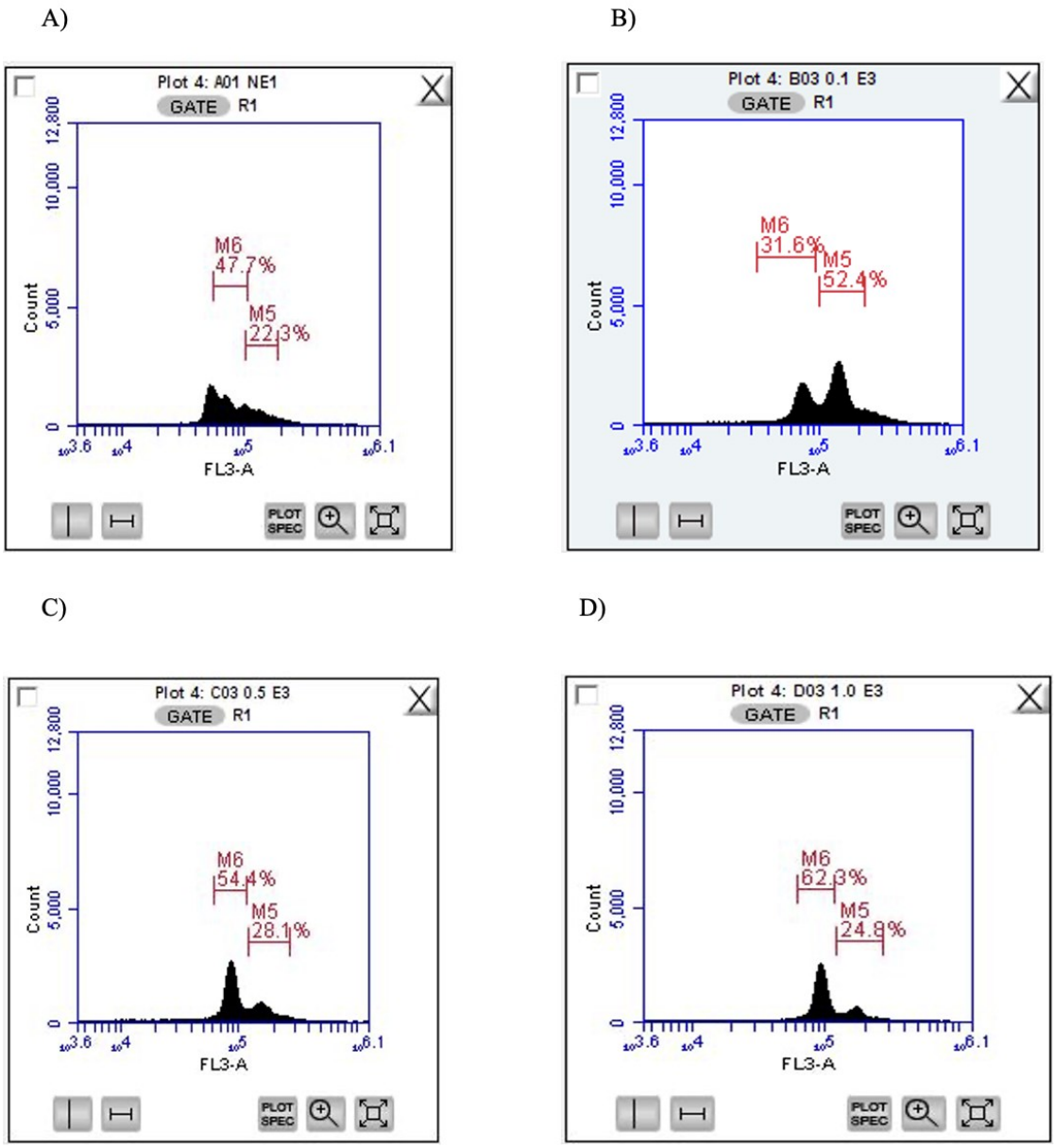

Figure 5. Cell cycle profiles under the influence of Etoposide. Treatment of Nlrp12$^{-/-}$ macrophages grown in (A) Control, (B) 0.1 μM etoposide, (C) 0.5 μM etoposide, or (D) 1 μM of etoposide and was examined by flow cytometry. Cell cycle arrest was examined by staining DNA with 7-AAD dye after 24 hours of etoposide treatment. Results are representative of four independent experiments.

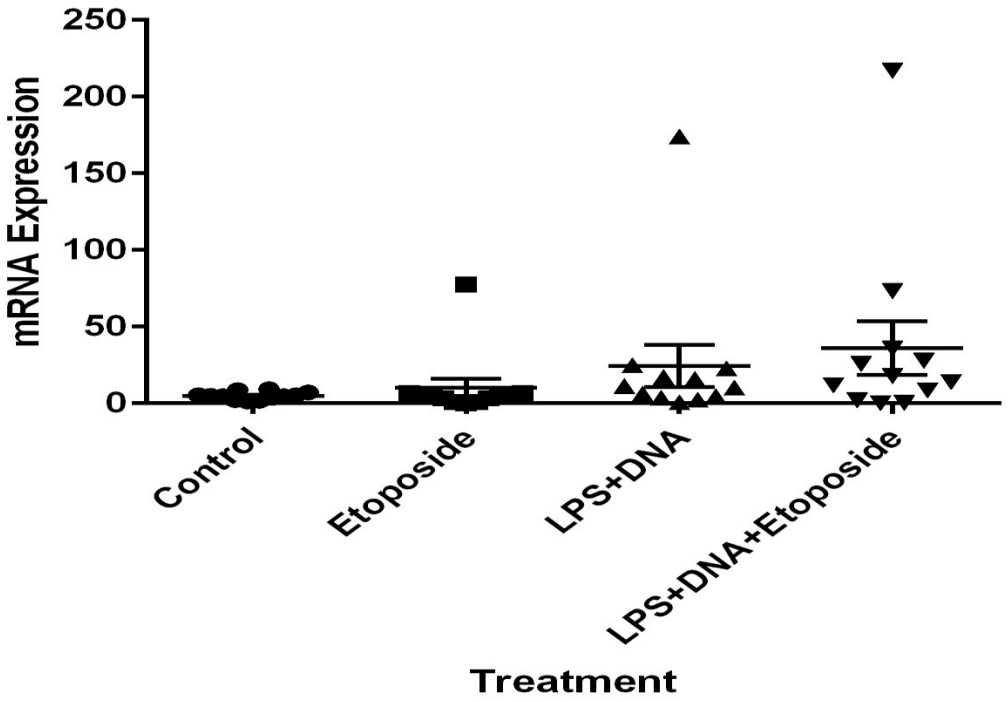

Figure 6. Quantifying mRNA expression by qRT-PCR. Detection of AIM2 expression in the Nlrp12$^{-/-}$ macrophage cell line was performed via reverse transcription-quantitative PCR (qRT-PCR). One-way ANOVA using Tukey's post hoc analysis was used for statistical comparison. AIM2 mRNA was normalized to β-Actin. ns: not significant, p-values: <0.05 (*), <0.01 (**), <0.001 (***).

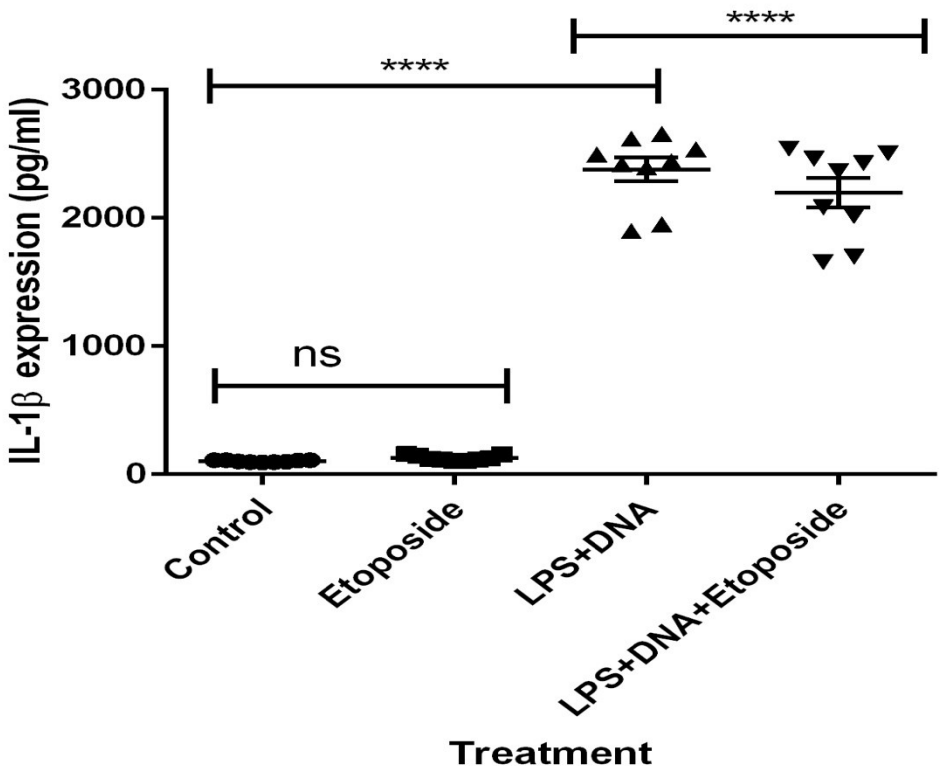

Figure 7. Quantification of Pro-inflammatory cytokine IL-1β levels. Nlrp12$^{-/-}$ macrophage cell line was treated with 0.1µL Etoposide for 24 hours, 10µl LPS and DNA for 4 hours, or LPS + DNA+ Etoposide. IL-1β expression was examined from the supernatants of cells by ELISA. Cytokine concentration was determined by standard curve generation using spectrophotometry. Experimental data are presented as the mean ± SE from three independent experiments. Statistical significance was determined using one-way ANOVA with Tukey post hoc for multiple comparisons. ns-not significant *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

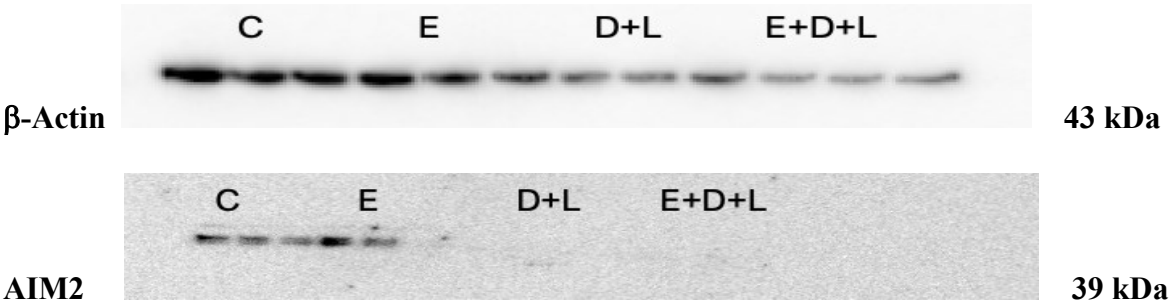

Figure 8. Western blot showing expression of β-actin and AIM2 proteins. Nlrp12$^{-/-}$ macrophage cell line was treated with 0.1µL Etoposide for 24 hours, 10µl LPS and DNA for 4 hours, or LPS + DNA+ Etoposide. β-actin and AIM2 were measured by western blotting. The results are representative of four independent experiments.

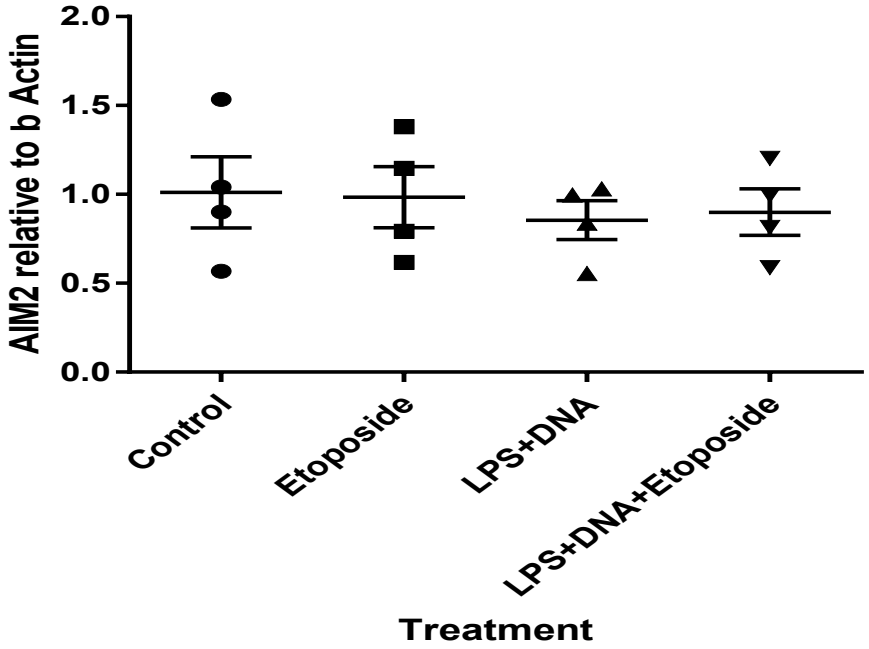

Figure 9. Level of AIM2 protein in relative to β-Actin. The AIM2 protein level from western blots was normalized relative to the control β-Actin using densitometry in Image-J. One way ANOVA post hoc analysis was used for analysis. ns-not significant *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The results are shown from 4 independent experiments and expressed in the terms of mean ± SE.

APPENDICES

Appendix

IBC approved 4/28/2022 to 4/27/2024

Missouri State University

MEMORANDUM OF UNDERSTANDING & AGREEMENT (MUA) FOR BIOHAZARDS OTHER THAN RECOMBINANT DNA EXPERIMENTS

All MUA'S can be submitted electronically to researchcompliance@missouristate.edu or submitted as a hard copy to the OSRP in Carrington 407. A signed copy must be provided. *Biosafety in Microbiological & Biomedical Laboratories (BMBL)* should be used as a reference when completing this MUA (see http://www.cdc.gov/biosafety/publications/bmbl5/).

A. *General Information*

Date: April 28th, 2022

Researcher Name: Christopher Lupfer

Researcher Title: Assistant Professor

Phone: 6-6887

Department: Biology

Office Bldg & Room #: Temple Hall, Room 254

Laboratory Bldg & Room #: Temple Hall, Room 232

Granting Agency: Planned grant applications to the National Institutes of Health, and American Lung Association

Grant Number (if applicable):

Title of Grant or Project: Inflammasome mediated hyperinflammation drives pathology during viral-bacterial coinfections.

B. *Project Information*

1. Describe the experiments involving biohazard(s). Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it.

Bacterial coinfections that occur during an influenza A virus infection are often severe and life threatening. The likelihood of death from a severe bacterial coinfection is at least twice that found in severe cases of influenza alone [Finelli et al. 2008, Pediatrics 122(4): p. 805-11]. In recent years, there has also been an increasing trend in the pediatric setting for sever coinfection with influenza and *S. aureus* [Reed et al. 2009, Pediatr Infect Dis J 28(7): p. 572-6]. IL-1β is among the cytokines that are known to be synergistically upregulated during coinfection [Smith et al. 2007, Comp Med. **57**(1): p. 82-9]. Although IL-1β is essential to a proper immune response, the uncontrolled production of this master cytokine can have detrimental effects. This is demonstrated by the fact that purified IL-1β alone is capable of inducing pneumonia in laboratory animals when administered in the absence of any infectious agent [Ganter et al. 2008, Circ Res. 102(7): p. 804-12]. However, little is known about the role of IL-1β during coinfections.

To examine the production and function of IL-1β during different coinfections, we will first examine IL-1β production in macrophages in cell culture *in vitro*. Primary murine bone marrow macrophages (BMDM) are made by extracting bone marrow from 6-12 week old C57BL6 mice and differentiating in BMDM growth medium supplemented with 20ng/ml recombinant mouse M-CSF for 5 days. In addition to primary macrophages immortalized macrophages (RAW264.7) will be used for *in vitro* studies. Macrophages will be plated in tissue culture treated plates and allowed to adhere. Macrophage cell cultures will be infected in one of three ways: (1) Wells will be infected with influenza A virus alone, bacteria alone or treated with a bacterial ligand (such as LPS) alone for 3h. (2) Wells will be infected with influenza A virus for 3h and then bacteria or a bacterial ligand is added to the infection for an additional 1h. (3) Wells will be infected with bacteria or treated with a bacterial ligand for 3h and then influenza A virus is added for an additional 1h. After the 4h process, additional growth medium is added and cells are incubated for 20 more hours. For this first round of experiments, influenza A/PR/8/34 H1N1 will be used as its ability to induce coinfections is well documented [21]. Purified bacterial or fungal ligands to be tested in this experiment will include peptidoglycan, MDP, LPS, LTA,

pneumolysin, synthetic DNA (CpG DNA or poly dA:dT), zymosan, mannen and flagellin. Bacterial strains to be used include *Streptococcus pneumoniae*, *Staphylococcus aureus,* and *Haemophilus influenzae* bacteria and *Aspergillus fumigatus* fungus. All pathogens will be provided by Drs. Paul Thomas, Thirumala-Devi Kanneganti, and Jon McCullers at St. Jude Children's Research Hospital or attained from ATCC. As a readout of synergy during these experiments, we will collect cell culture supernatants at 24h post-primary infection and use ELISAs assays to examine the level of IL-1β, IL-6, and TNF-α. We will also perform Western blots to examine cell signaling pathways and caspase-1 inflammasome activation.

Finally, alveolar macrophages will be isolated by bronchial-alveolar lavage and plated in tissue culture treated plates. Similar coinfections will be performed; however, only those experiments yielding positive results for BMDMs will be performed in alveolar macrophages due to the relatively small numbers of alveolar macrophages that can be obtained from mice. Once we have determined those pathogens or ligands that are capable of synergizing in macrophages in cell culture, we will perform coinfection experiments in mice to determine the *in vivo* relevance of the synergistic IL-1β response.

2. Provide an assessment of the physical containment required for the experiments.

The influenza A/PR/8/34 H1N1 virus and *Streprococcus pneumoniae, Staphylococcus aureus*, and *Haemophilus influenzae* bacteria and *Aspergillus fumigatus* fungus are all biosafety level 2 pathogens (BSL2). As such, the likely hood of transmission to humans is low to moderate. All pathogens proposed for use here are laboratory mouse adapted strains with lower virulence than human clinical isolates due to repeated laboratory culturing and infection of non-human laboratory animals like mice. In keeping with BSL2 guidelines, all experimental procedures should be conducted in a Class II biosafety cabinet and all personnel working with these pathogens must be wearing appropriate personal protective equipment. Proper handling of infectious cultures or samples must be observed. Proper decontamination of research equipment, and personal protective equipment must be followed. Also, proper personal hygiene in the laboratory environment must be maintained to prevent accidental contamination or infection.

3. Describe the facilities and specific procedures which will be used to provide the required levels of containment.

Temple 232 contains the space and designated equipment necessary for working with influenza A virus and *Streprococcus pneumoniae, Staphylococcus aureus*, and *Haemophilus influenzae* bacteria and *Aspergillus fumigatus* fungus. This includes a tissue incubator with sealed air-tight door, designated centrifuge, and a class II biosafety cabinet (BSC). In addition, for work involving animals, the vivarium in Temple Hall has a procedure room with a BSC and is available for work with BSL2 level pathogens.

All procedures will be performed in the BSC present in the lab in Temple 232 or in the procedure room of the vivarium. All samples collected will only be opened and handled in a BSC. The BSC will be decontaminated prior to and following all procedures using either 70% ethanol or 10% bleach, which must remain on the surface for 5 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags and autoclaved prior to being discarded. Any liquid cultures will be collected in sealed containers containing bleach at a final concentration of at least 10% to inactivate pathogens. Samples collected during experiments may be handled outside of the safety cabinet if contaminating pathogens have been killed by either incubation for a minimum of 5 minutes with formaldehyde or other fixative at a concentration > 1%, incubation in solutions containing at least 10% bleach >5 minutes can also be used to decontaminate samples. Finally, heating at >95 degrees Celsius for at least 15 minutes (for example, boiling samples in Laemmli buffer prior to SDS-PAGE analysis) may also be used to ensure pathogens are destroyed. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

All personnel working with pathogens will wear disposable latex or nitrile gloves, a laboratory coat and eye protection. These must be worn at all times while working with infectious agents or potentially contaminated samples or cultures. Long pants and close toed shoes are also required. Prior to exiting the BSC, gloves must be removed and placed in the biohazard bag or decontaminated with 70% ethanol or 10% bleach. Samples to be removed from the BSC for transport or storage must likewise be decontaminated. No eating (including chewing gum), drinking, applying cosmetics or contact lenses is allowed in the laboratory even when work with infectious cultures is not taking place.

In addition to these procedures and precautions, it is recommended that individuals working directly with these pathogens be current on all recommendations for vaccinations with seasonal influenza, pneumococcal and Hib.

4. Describe the procedures and precautions to be followed if biohazardous organisms or agents are to be transported between laboratories.

If samples need to be transported, they should first be inactivated by chemical or heat inactivation as described above. A secondary container should also be used if the sample contains any liquid (small paint can filled with paper towels etc.). If live cultures or samples containing potentially live organisms must be transported, then samples must be sealed in a shatter resistant container (such as a threaded-cap polypropylene plastic test tube) and the outside of the container decontaminated with 70% ethanol or 10% bleach. The shatter resistant container must then be placed in a leak proof secondary container with absorbent material (small paint can filled with paper towels etc. and then properly sealed).

5. Is this a select agent? If yes, contact the Office of Research Compliance (ORC).

No.

6. The undersigned agree to certify the following conditions of the proposed research:

a. The information above is accurate and complete. We agree to accept responsibility for training of all laboratory workers involved in the project. We agree to comply with the CDC requirements pertaining to shipment and of hazardous biological materials. We are familiar with and agree to abide the provisions of the Missouri State University policies and procedures applicable to experiments involving biohazards.
b. We understand that only the organisms specified are covered by this MUA, and work with other organisms or types of biohazards may require other MUAs.

Via email 4/28/2022			
Principal Investigator	Date	Department Head	Date

7. The Institutional Biosafety Committee has determined, based on information provided the principal investigator, that:

a. No special medical surveillance (other than usual University health programs) is required for the project described in this MUA

b. The following specific medical surveillance procedures must be carried out, for individuals listed by name, before commencing the project described in this MUA:

8. We certify that the Missouri State University Institutional Biosafety Committee has reviewed the proposed project and has found in to be in compliance with Missouri State University's policies and procedures applicable to experiments involving biohazards.

MSU IBC Chair or Representative _____ Date _____