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A Study on Cellular Inflammation Response to Varied Stimuli

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Biomedical Sciences

by

Shane Erickson

December 2023

Thesis Committee:
Dr. Marcus Kaul, Chairperson
Dr. Erica Heinrich
Dr. Thomas Kuhlman
Dr. Declan McCole

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The Thesis of Shane Erickson is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE THESIS

A Study on Cellular Inflammation Response to Varied Stimuli

by

Shane Erickson

Master of Science, Graduate Program in Biomedical Sciences
University of California, Riverside, December 2023
Dr. Marcus Kaul, Chairperson

One of the master regulators of inflammation in cells is the NF-κB protein family, which regulates the NLRP3 inflammasome complex to coordinate a pro-inflammatory signaling response which can differ between cell types and from different agonists. This study observed that monocytic cell lines have greater inflammasome activity in response to common TLR4 agonists in comparison to microglial cell lines while also highlighting that primary microglia regulate transcriptional activity of NF-κB through phosphorylation of key proteins. Additionally, this study finds that microglia, in the presence of serum, have increased interferon activity and increased translocation of NF-κB to the nucleus to prime the NLRP3 inflammasome in comparison to microglia that are not in the presence of serum. Lastly, this study concludes that SARS CoV-2 spike protein causes

upregulation of NF-κB and no significant change in p38 MAPK signaling pathways in bronchiolar epithelial cells.

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Chapter 1: Monocyte and Microglial NLRP3 Inflammasome Activation by Lipopolysaccharide and Nigericin

1.1 Introduction

The immune system in humans and other mammals can be broken down into two major components, the adaptive immune system and the innate immune system. Both the innate and adaptive immune systems are crucial in protecting against infection, pathogens, and other microbial elements that can pose a risk to the health of the organism [1]. While the adaptive immune system is largely regulated by T cells and B cells, the innate immune system is composed of hematopoietic cells, such as macrophages, neutrophils, and dendritic cells, as well as nonhematopoietic cells like epithelial cells which line airways and the digestive tract.

While the adaptive immune system is very effective at dealing with specific pathogens due to the B and T cell response, the innate immune system instead works to detect a wider range of pathogenic structural motifs and initiates recruitment of other immune cells to the site of microbial detection through the production and release of various cytokines and chemokines [2]. The cells of the innate immune system contain a variety of pattern recognition receptors (PRRs) that target common microbial structures and proteins, triggering production and release of appropriate chemokines and cytokines. Among these PRRs are Toll-like receptors (TLRs), each of which recognize different

1. Turvey, S., Broide, D. (2009). Innate Immunity. *The Journal of Allergy and Clinical Immunology*, *125*(2), S24-S32.
2. Marshall, J. S., Warrington, R., Watson, W. *et al.* (2018). An Introduction to Immunology and Immunopathology. *Allergy, Asthma & Clinical Immunology*, *14*(49).

pathogen-associated molecular patterns (PAMPs) and initiates specific cell signaling pathways to recruit the correct types of immune cells that are needed [3].

TLR4 is a Toll-like receptor that can recognize several PAMPs but is most commonly associated with lipopolysaccharide (LPS), a common bacterial endotoxin that is a key and highly conserved membrane component found in Gram-negative bacteria [4]. Following TLR4 interactions with one of its agonists, such as LPS, downstream activity can occur in genes and proteins involved in controlling inflammatory response in the cell such as nuclear factor-κB (NF-κB) or interferon regulatory factor 3 (IRF3) [5].

NF-κB is a group of proteins composed of a Rel sub-group and a NF-κB sub-group involved in DNA-binding and dimerization that act as transcription factors for many genes associated with immunity and inflammation [6]. The major canonical pathway for NF-κB signal transduction involves the p65/RelA protein dimer with the p50 protein, while the non-canonical pathway instead consists of a dimer between p52 and RelB. The p50 protein comes about as a product of processing of the p105 protein while the p52 protein is a product of processing of the p100 protein. Both the p50/RelA dimer and the p52/RelB dimer act as transcription factors affecting genes that control inflammatory

3. Takeda, K., & Akira, S. (2015). Toll-Like Receptors. *Current Protocols in Immunology*, *109*(1), 14.12.11-14.12.10.
4. Vaure, C., & Liu, Y. (2014). A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Frontiers in Immunology*, 5, 316, 1-15.
5. Popli, S., Chakravarty, S., Fan, S., Glanz, A., Aras, S., Nagy, L. E., Sen, G. C., Chakravarti, R., & Chattopadhyay, S. (2022). IRF3 inhibits nuclear translocation of NF-κB to prevent viral inflammation. *Proceedings of the National Academy of Sciences of the United States of America*, 119(37)
6. Gilmore T. D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*, *25*(51), 6680–6684.

response within the cell. NF-κB is regulated by proteins involved in negative feedback loops such as the IκBα and A20. IκBα is an inhibitory protein that targets the p50/p65 dimer and prevents DNA-binding activity from the dimer. A20 has key ubiquitin-editing properties, allowing for the deubiquitination of key proteins involved in both the canonical and non-canonical NF-κB pathways, preventing DNA-binding from any of the NF-κB-associated dimers [7].

One major protein complex regulated by the NF-κB protein family is the NLRP3 inflammasome. The NLRP3 inflammasome is responsible for cleaving IL-1β and IL-18 into their active forms, so that a pro-inflammatory signaling cascade can begin, as well as contributing to cell death via pyroptosis [8]. IL-1β and IL-18 are cytokines that recruit immune cells so that a pro-inflammatory response can occur upon activation of the NLRP3 inflammasome. Pyroptosis is a result of K^+ efflux from the cell, something that can be achieved either by the cell identifying toxins such as LPS within the nucleus or via drugs or antibiotics such as the microbial antibiotic Nigericin. Nigericin has been found to cause significant K^+ efflux in cells and as such has been identified as a key activator of the NLRP3 inflammasome [9].

The goal of this study is to observe the effects of LPS and Nigericin on NF-κB and its

7. Coornaert, B., Carpentier, I., & Beyaert, R. (2009). A20: central gatekeeper in inflammation and immunity. *The Journal of biological chemistry*, *284*(13), 8217–8221.
8. Swanson, K. V., Deng, M., & Ting, J. P. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nature reviews. Immunology*, *19*(8), 477–489.
9. Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013). K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity*, *38*(6), 1142-1153.

key regulators on cells involved in innate defense of the body. A human leukemia monocytic cell line, THP-1, was selected as a candidate as they are ideal in studying cell-cell interactions and signaling pathways [10]. To look at innate immune defense in the brain, HMC3 and pHMC cell lines, human embryonic microglia and primary human microglia respectively, were selected as candidate lines as microglia are the primary immune cells in the brain and act as a second line of defense following the blood-brain barrier [11, 12].

1.2 Materials and Methods

1.2.1 Cell Culture and Treatment

THP-1, HMC3, and pHMC cells passage number 5-6 were plated in six-well plates with 250,000 cells per well. THP-1 and HMC3 cells were cultured with DMEM media with 10% Fetal Bovine Serum (FBS) and pHMC cells were cultured with Primary Human Microglia Cell Culture Media with 10% FBS.

Two days after plating, media was removed and replaced with either DMEM-10% FBS or Primary Human Microglia Cell Culture Media-10% FBS containing 0.1% DMSO and 0.1% Ethanol for four hours for vehicle treatment, 10 ug/mL LPS for four hours, or 10 ug/mL LPS for four hours followed by two hours of 25 uM Nigericin.

10. Chanput, W., Mes, J. J., & Wichers, H. J. (2014). THP-1 cell line: an in vitro cell model for immune modulation approach. *International immunopharmacology*, *23*(1), 37–45.
11. Dello Russo, C., Cappoli, N., Coletta, I. *et al.* (2018). The human microglial HMC3 cell line: where do we stand? A systematic literature review. *J Neuroinflammation 15*, 259.
12. Timmerman, R., Burm, S. M., & Bajramovic, J. J. (2018). An Overview of in vitro Methods to Study Microglia [Review]. *Frontiers in Cellular Neuroscience*, *12*.

Following treatment, each six-well plate was placed on ice and media was removed. Cells were then lysed with RIPA buffer containing protease inhibitor and phosphate inhibitor and lysate was collected in microcentrifuge tubes and sonicated four times for four seconds each. Following sonication, lysates were centrifuged for 13 minutes at 12000 rpm and the protein lysate collected in a new microcentrifuge tube. Protein levels were analyzed via BCA assay.

1.2.2 Protein Assay via Western Blot

25 ug of protein and 10 uL of SeaBlue Standard were loaded into 8-12% BisTris gel and run at 100 volts for 60 minutes and 150 volts until the protein bands reached the bottom of the gel. Gel was then transferred to a PVDF membrane that had been pre-treated in methanol for 30 seconds and ran at 75 volts for two hours.

PVDF membranes were washed with TBST and blocked with 5% Bovine Serum Albumin (BSA) in TBST for one hour. Membranes were then incubated overnight with primary antibody in 5% BSA, then incubated with secondary antibody conjugated with horseradish peroxidase in 5% BSA the following day. After exposure to West Dura/Pico, membranes were imaged and protein levels were measured through ImageJ gel analysis.

1.3 Results

The three cell types studied exhibited differing reactions to the treatment with LPS and Nigericin. Looking at protein levels of NF-κB p65 and phospho-NF-κB p65 to view the overall activity of the NLRP3 inflammasome, the only cell type that showed robust

phospho-NF-κB activity was the pHMC cell line, with the THP-1 cell line showing reduced levels of phosphorylation of the NF-κB protein (Fig. 1.1). Neither the LPS nor the LPS and Nigericin treatment had any effect on levels of phospho-NF-κB, but the standard NF-κB protein was found at significantly higher levels in THP-1 cells that had been treated with LPS and LPS with Nigericin. While not statistically significant, HMC3s and pHMCs experienced reduced levels of NF-κB after treatment (Fig. 1.2).

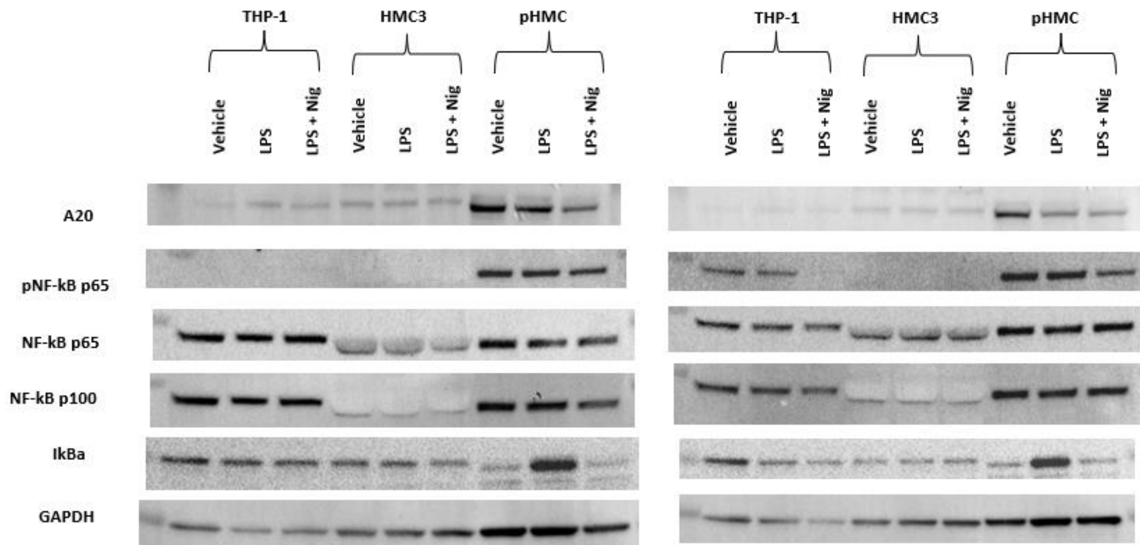


Figure 1.1 Western blot imaging of protein collected from THP-1, HMC3, and pHMC cell lines depicting vehicle, 4 hr LPS, or 4 hr LPS and 2 hr Nigericin treatments. Primary antibodies were derived from rabbit or mouse and diluted to manufacturer instructions in 5% BSA. Secondary antibodies were derived from goat and conjugated with horseradish peroxidase for imaging and diluted to manufacturer instruction in 5% BSA.

Non-canonical activation of the NLRP3 inflammasome through NF-κB p100 was seen primarily in THP-1 cells, while HMC3 and pHMC cell lines seemed to rely on canonical activation of the inflammasome.

Looking at the major regulators of NF-κB, A20 and IKBα, the THP-1 cell line showed significant upregulation of A20 as a result of treatment by LPS and LPS with Nigericin while no significant change occurred in IKBα levels from treatment (Fig. 1.2). Both the HMC3 and pHMC cell lines again saw little change in protein expression in A20 or IKBα from treatment, although the Nigericin did significantly reduce IKBα expression in the pHMC cells (Fig. 1.2).

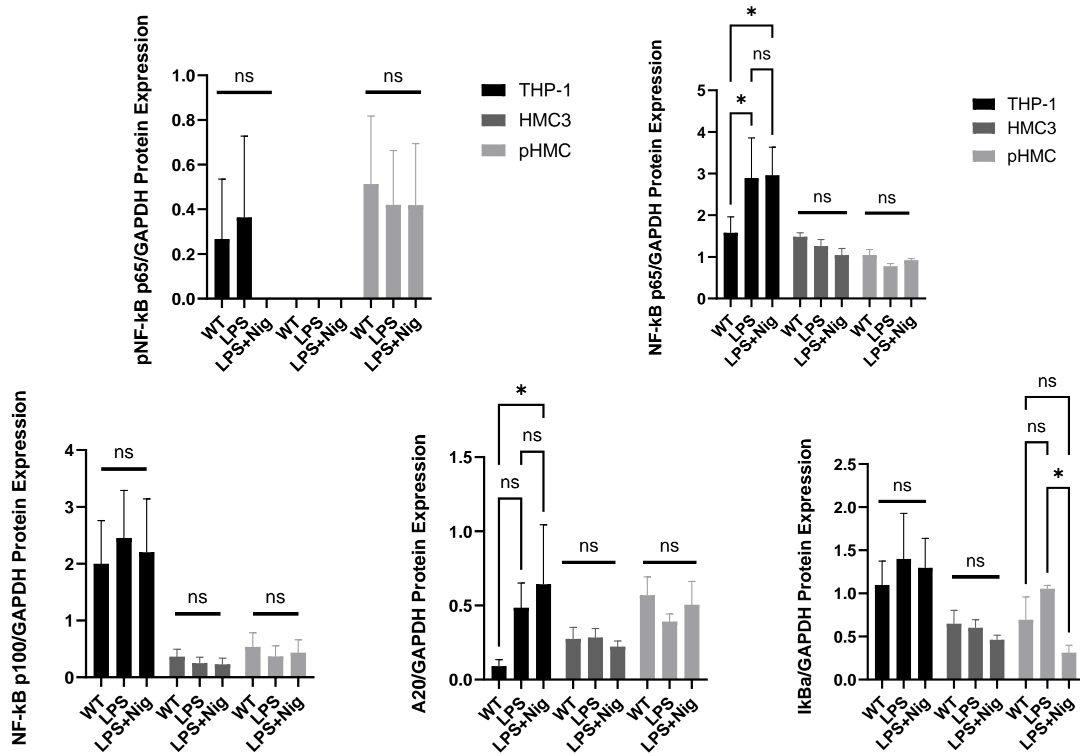


Figure 1.2 Graphs from three separate experiments of THP-1, HMC3, and pHMC cell lines depicting mean protein expression normalized to GAPDH of pNF-κB p65, NF-κB p65, NF-κB p100, A20, and IKBα. Normalized protein expression levels were analyzed via one-way ANOVA and Fisher's Least Significant Difference tests, with ns indicating a p-value greater than 0.05 and * indicating significance with a p-value less than 0.05. Error bars represent standard error of the mean.

1.4 Discussion

For canonical NLRP3 inflammasome activation via NF-κB, the THP-1 cell line was the most responsive in this study. Overall NF-κB expression was much greater in the THP-1 cells than either of the microglial cell lines across all treatment levels, indicating that monocytes rely on the NLRP3 inflammasome response to common TLR4 agonists more than microglia. However, phosphorylation of the NF-κB p65 protein was seen primarily in primary human microglia. Phosphorylation of the NF-κB proteins has been found to have a profound effect on their ability to form dimers or their affinity for the IκB family of proteins. For the p65 protein in particular, phosphorylation of the S276 site results in greater transcriptional activity [13]. While overall NF-κB p65 was lower in the pHMC cell line, the heightened levels of phospho-p65 at the S276 site indicates that it could be more efficient at activating transcription and priming the NLRP3 inflammasome than the cell lines with lower phospho-p65 expression.

For non-canonical activation of NF-κB and the NLRP3 inflammasome, the THP-1 cell line again saw the highest protein expression levels. The non-canonical NF-κB pathway is dependent on the processing of the p100 protein and can provide additional levels of cellular control of NF-κB activation [14]. From this, it can be determined that the THP-1 cell line is more versatile in the ways it can respond to TLR4 activity as proteins involved in both the canonical and non-canonical pathway are heavily upregulated in comparison to the microglial cell lines.

13. Christian, F., Smith, E. L., & Carmody, R. J. (2016). The Regulation of NF-κB Subunits by Phosphorylation. *Cells*, *5*(1), 12.
14. Sun, SC. (2011). Non-canonical NF-κB signaling pathway. *Cell Res 21*, 71–85

For the major inhibitory proteins involved in regulating NF-κB, A20 was significantly overexpressed from the treatments in the THP-1 cells, while remaining at relatively constant levels in the microglia. The IκBα, however, maintained expression levels in the THP-1 cell line, but showed significantly less expression in the pHMC cells as a result of the LPS and Nigericin treatment. This indicates that the K^+ efflux from the Nigericin treatment negates the inhibitory effects of IκBα in microglia, allowing for greater NLRP3 inflammasome activity, leading to pyroptosis. Meanwhile the monocytic cells have greater inhibitory effects from A20 activity from both treatments, combined with the higher overall levels of p65, indicating that the cells activate the NLRP3 inflammasome at a higher rate, but also exhibit greater transcriptional control over the NF-κB proteins.

1.5 Conclusion

This study into effects of known NF-κB and NLRP3 inflammasome activators on monocytes and microglia showed that the monocytic cell line exhibits greater overall inflammasome activity, greater inhibitory control over the inflammasome complex, and diversity of NF-κB activation through the non-canonical pathway. For the microglial cells, this study found that there was less overall inflammasome activity, lesser inhibitory control over the NLRP3 inflammasome, but exhibited greater control through phosphorylation of key proteins in the NF-κB family.

CHAPTER 2: Effects of Methamphetamine on Microglial Inflammasome Activity

2.1 Introduction

Drug abuse can lead to significant bodily harm and even death of the user, depending on which drug is being abused, duration of use, dosage of use, and many more factors. Some common side effects of drug abuse can include or lead to severe neurological impairment [15]. Drugs such as cocaine have been shown to impact much of the brain on a cellular level, including increased levels of inflammation in glial cells that can lead to damage in the brain [16].

Among these inflammatory pathways include the NLRP3 inflammasome activation pathway, which is known to be one of the key pathways to mediate neuroinflammation. The NLRP3 inflammasome has been shown to be a key pathway to significantly reduce microglia activity and neuroinflammation that is brought about by cocaine usage [17]. Observing the neuroprotective efforts of the NLRP3 inflammasome in regards to cocaine use raises the question of whether other drugs that bring about neuroinflammation are also mediated by NLRP3 inflammasome activity.

Methamphetamine is another drug that has been shown to have significant neurotoxic effects and be a cause of neuroinflammation. These effects are seen across several cell

15. Goforth, H. W., Murtaugh, R., & Fernandez, F. (2010). Neurologic aspects of drug abuse. *Neurologic clinics*, *28*(1), 199–215.
16. Sil, S., Niu, F., Tom, E., Liao, K., Periyasamy, P., & Buch, S. (2019). Cocaine Mediated Neuroinflammation: Role of Dysregulated Autophagy in Pericytes. *Molecular neurobiology*, *56*(5), 3576–3590.
17. Chivero, E. T., Thangaraj, A., Tripathi, A., Periyasamy, P., Guo, M. L., & Buch, S. (2021). NLRP3 Inflammasome Blockade Reduces Cocaine-Induced Microglial Activation and Neuroinflammation. *Molecular neurobiology*, *58*(5), 2215–2230.

types in the brain, such as neurons and other glial cells [18]. Additionally, breakdown of the blood-brain barrier can result in potential toxins such as LPS to enter the brain, while [19].

The goal of this study is to observe and characterize how microglia respond to methamphetamine and other NLRP3 inflammasome agonists, like LPS while also observing if other potentially introduced factors such as serum can have a neuroprotective effect.

2.2 Materials and Methods

2.2.1 Cell Culture and Treatment

Primary human microglia cells (pHMC) passage numbers 6-7 were plated on a 96-well plate at a density of 10,000 cells per well. pHMCs were cultured with Primary Human Microglia Cell Culture Media either with or without 10% FBS.

After two days, media was removed and replaced with fresh media, then either PBS for the vehicle or 100 uM methamphetamine for one hour. Following this, media was removed and replaced with fresh media and either 0.1% DMSO for vehicle or 10 ug/mL LPS for 30 minutes, one hour, four hours, or 24 hours.

Following treatment, supernatant was removed, transferred to a sealed 96-well plate and stored at -20°C for one day, then -80°C until use. Wells were then fixed with 4% PFA in

18. Yu, S., Zhu, L., Shen, Q., Bai, X., & Di, X. (2015). Recent advances in methamphetamine neurotoxicity mechanisms and its molecular pathophysiology. *Behavioural neurology*, *2015*
19. Hussain, B., Fang, C., & Chang, J. (2021). Blood–Brain Barrier Breakdown: An Emerging Biomarker of Cognitive Impairment in Normal Aging and Dementia [Review]. *Frontiers in Neuroscience*, *15*.

PBS for 25 minutes at 4°C. Then 4% PFA was removed and cells were washed with PBS and stored in PBS at 4°C.

2.2.2 Multiplex Assay

Supernatant was thawed from -80°C and run on the LegendPlex Human Type 1/2/3 Interferon Panel per manufacturer's instructions. Following LegendPlex protocol, samples were transferred to FACS tubes and run on a NovoCyte flow cytometer. Bead analysis was performed on Qognit.

2.2.3 Staining and Imaging

Cells were permeabilized with 0.2% Triton x100 in PBS for 5 minutes at room temperature then washed with PBS. 10% goat serum in PBST was added for 30 minutes at room temperature as a non-specific binding block. Primary antibody mixtures for NF-κB and Tubulin were diluted in 5% goat serum in PBST to manufacturer's instructions and incubated at 4°C overnight.

Cells were then washed with PBST, then secondary antibody mixtures diluted in 5% goat serum in PBST per manufacturer's instructions were added for 30 minutes while kept away from light. Cells were washed with PBST, then Hoechst diluted 1:150 in PBS was added for 5 minutes at room temperature away from light. Cells were again washed with PBS and wells filled with 10uL vectashield and wrapped in aluminum to be stored at 4°C until imaging.

Three images per well were taken at 40x magnification and NF-κB levels were normalized to a negative control with no added primary antibody. NF-κB levels were measured in the nucleus, cytoplasm, whole cell, and entire image.

2.3 Results

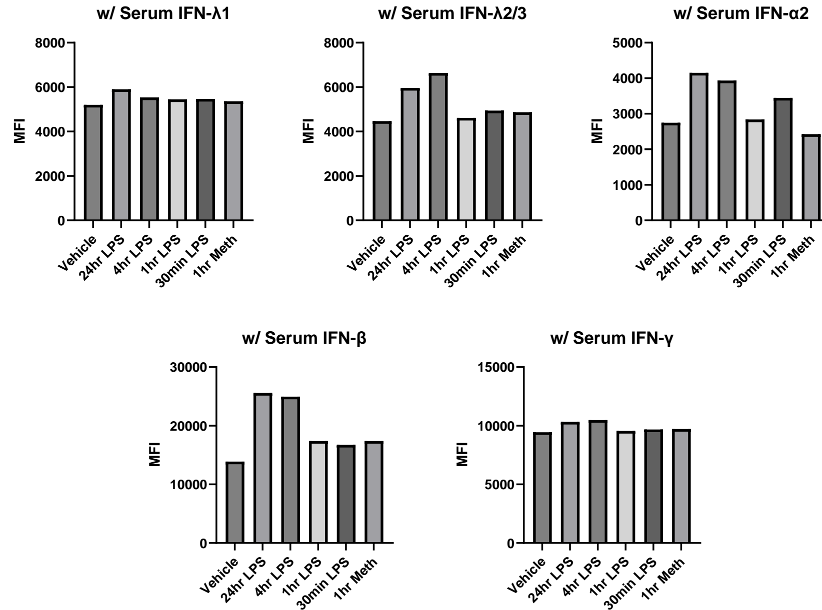
2.3.1 Multiplex Assay of Interferon Activity

Interferon levels were measured through multiplex analysis. For cells cultured with 10% FBS, higher levels of interferon activity were seen in cells with longer exposure to LPS after the methamphetamine treatment in comparison to the vehicle (Fig. 2.1a). The methamphetamine treatment alone did not have any significant effect on interferon levels for any of the five measured. For cells cultured without serum, significant decreases were seen in each of the interferons measured, with longer incubation time with LPS correlating with lower levels of interferon activity (Fig. 2.1b). As with the cells cultured with serum, the methamphetamine treatment alone did not have any significant effect on interferon levels.

2.3.2 Imaging of NF-κB

From the images taken of the pHMC cell line after treatment, NF-κB p65 was observed to translocate to the nucleus in microglia cultured with 10% FBS after prolonged exposure to LPS following the methamphetamine treatment (Fig. 2.3b). This wasn’t the case for microglia cultured without serum, which did not see heightened levels of translocation of NF-κB p65 to the nucleus (Fig. 2.3a).

a)



b)

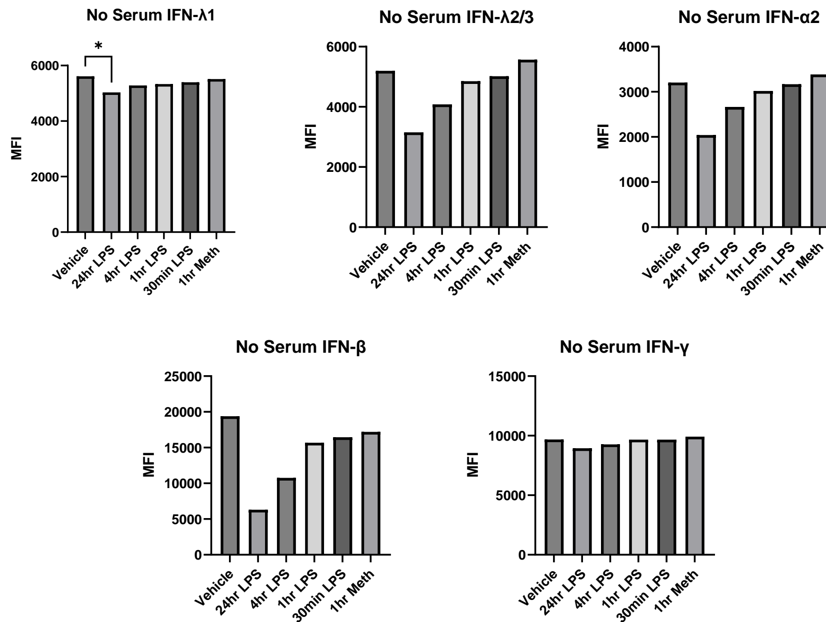
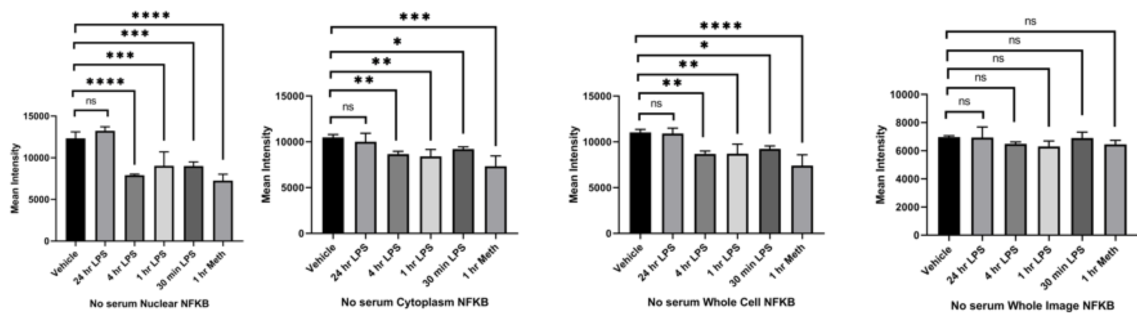


Figure 2.1 Multiplex assay analysis results, showing mean fluorescent intensity (MFI) for beads representing IFN-λ1, IFN-λ2/3, IFN-α2, IFN-β, and IFN-γ. a) cells cultured with 10% FBS and b) cells cultured without serum.

This observation is strengthened by measuring cytoplasmic NF-κB, which is significantly reduced in cells cultured with serum after methamphetamine and LPS exposure (Fig. 2.2b). For microglia cultured without serum, there was significant reduction in nuclear NF-κB in both the cytoplasm and in the nucleus (Fig. 2.2a).

a)

No Serum – Mean NFKB



b)

With Serum – Mean NFKB

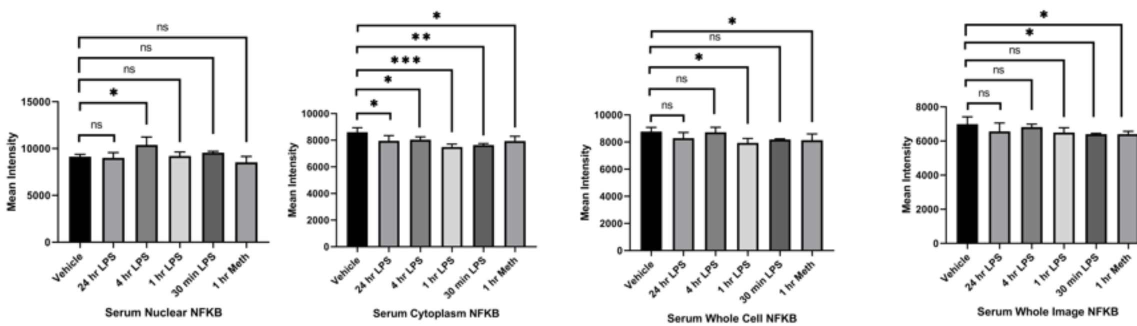


Figure 2.2 Mean fluorescent intensity of NF-κB levels in the nucleus, cytoplasm, whole cell, and whole image for pHMC cells cultured a) without serum and b) with 10% FBS

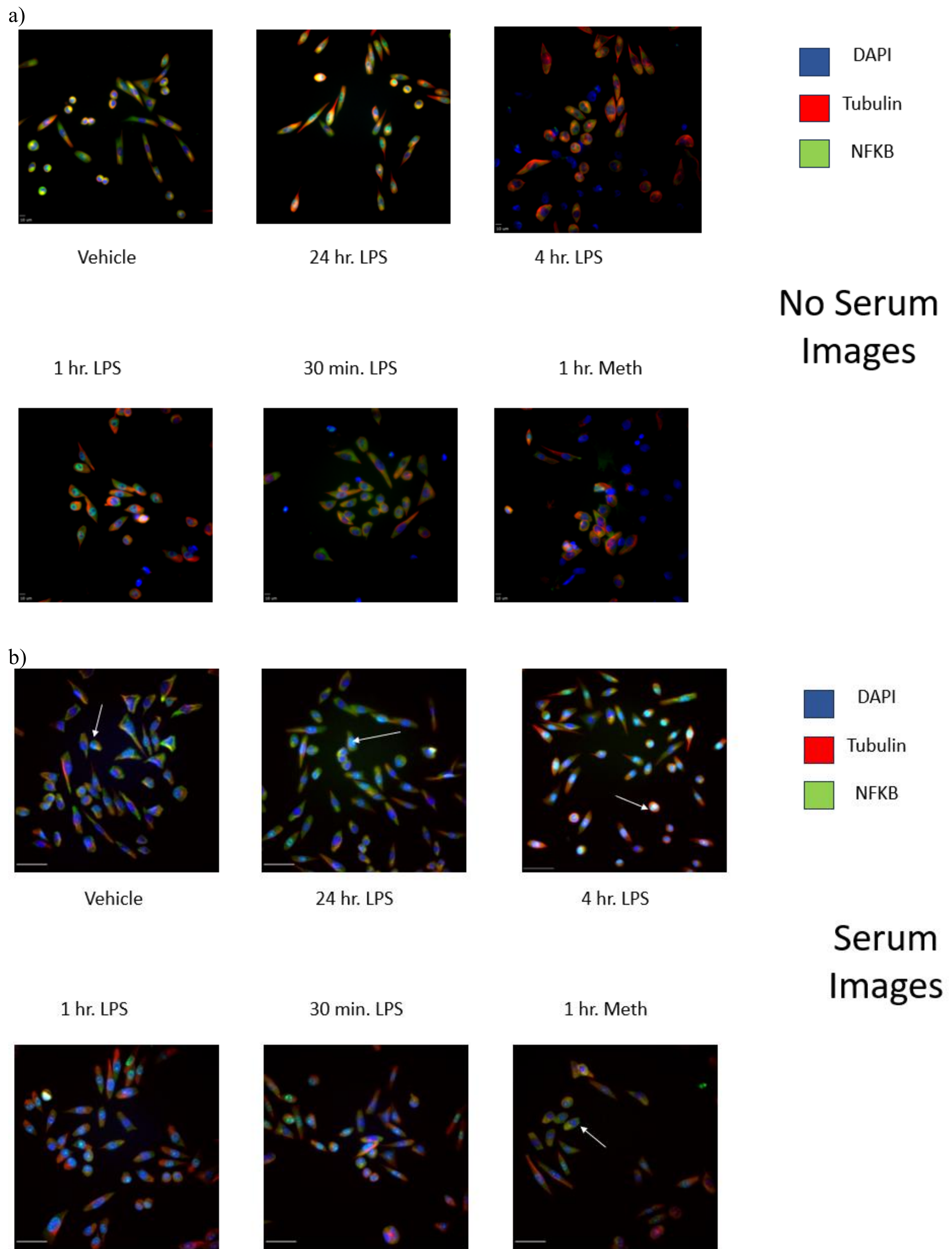


Figure 2.3 Images taken at 40x magnification of pHMC cells a) cultured without serum or b) cultured with 10% FBS. Nuclei are stained in blue, tubulin is stained in red, and NF-κB is stained in green.

2.4 Discussion

Because of the common co-morbidity of methamphetamine use and HIV infection, measuring interferon response is vital as it can identify if NF-κB expression and NLRP3 inflammasome activity can be neuroprotective against HIV as heightened interferon levels result in a reduction in viral replication [20]. When pHMCs were in the presence of serum, exposure to LPS after methamphetamine treatment led to increased interferon response, while microglia that were deprived of serum saw a decreased interferon response to LPS and methamphetamine treatment. Whether the neuroprotective antiviral response from the serum-cultured microglia is a result of NF-κB and NLRP3 inflammasome activity or a different pro-inflammatory pathway requires further inquiry. While measuring overall NF-κB in a cell can help gauge the activity level of the NLRP3 inflammasome, observing translocalization of NF-κB to the nucleus confirms that transcriptional activation is occurring as this is indicative of the priming step of the inflammasome pathway [21]. From the imaging done in this study, microglia cultured with serum showed higher co-localization of NF-κB and nuclear DAPI staining after methamphetamine and LPS treatment, particularly around the four-hour LPS time-point. This co-localization was not seen in the microglia deprived of serum, as these cells exhibited significantly lower NF-κB levels both in the nucleus and in the cytoplasm

20. Katze, M., He, Y. & Gale, M. (2002). Viruses and interferon: a fight for supremacy. *Nat Rev Immunol 2*, 675–687.
21. Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B. G., Fitzgerald, K. A., Hornung, V., & Latz, E. (2009). Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression1. *The Journal of Immunology*, *183*(2), 787-791.

following methamphetamine and LPS treatment at all time-points. This leads to the conclusion that microglia exposed to serum effectively prime the NLRP3 inflammasome through NF-κB-mediated transcriptional activation.

2.5 Conclusion

Primary human microglia exhibit stronger interferon response and undergo the complete NLRP3 inflammasome priming process as a result of methamphetamine and LPS treatment in the presence of serum. pHMCs deprived from serum fail to increase interferon response and do not exhibit translocation of NF-κB to the nucleus as a response to methamphetamine and LPS treatment.

Chapter 3: Downstream Inflammatory Response from SARS CoV-2 Spike Protein Treatment in Bronchiolar Epithelial Cells

3.1 Introduction

While the majority of cells that make up the innate immune system are of hematopoietic origin, other cell types such as epithelial cells and skin cells also perform a vital role in innate immunity. Among these include epithelial cells in the airways, which come into contact with a significant amount of microbial material and protect the body from serious infection. These cells in particular express many TLRs, which is critical for the detection of many types of viruses, bacteria, and other microbes [22].

Of the plethora of disease-causing microbes, of recent importance is Severe Acute

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Respiratory Syndrome Coronavirus 2 (SARS CoV-2). This virus is the cause of the disease COVID-19 and due to factors such as novelty, virulence, latency, lack of preventative measures such as vaccines, and multiple common variants led to a severe pandemic, resulting in the deaths of millions of people worldwide [23]. There are four main structural proteins that are coded for by the virus, the S, M, E, and N proteins. The spike protein (SP), also known as the S protein, binds to the angiotensin converting enzyme 2 receptor in human cells and initiates membrane fusion/cellular intake [24]. As the S protein is a key part of the infection process, it quickly became the target antigen for vaccine production and became the focus of many studies. There are two main subunits that make up the SP, an S1 and an S2 subunit. Cleavage of this site occurs after binding to ACE2 and initiates membrane fusion between the viral envelope and the cell it is bound to, with the S1 subunit containing the receptor-binding domain.

The S1 subunit has been found to cause COVID-19-like disease symptoms by itself in mice and in endothelial cells in humans. Even without infection as the treatment in this study contained no viral genomic material, these mice still exhibited many symptoms commonly seen in disease, such as lung injury and upregulation of pro-inflammatory cytokines in BALF and serum after instillation of the S1 subunit of the SP [25]. Looking at additional cell types in humans, SP overexpression has been shown to upregulate the

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expression of ACE2 after overexpression of the SP in bronchiolar epithelial cells and human embryonic kidney cells through JAK-STAT signaling [26]. Additionally, the SP has been shown to initiate innate immune response in human peripheral blood monocyte macrophages via activation of the NF-κB pathway [27]. SARS CoV-2 infection itself has also been shown to activate signaling pathways such as MAPK and NF-κB in airway epithelial cells [28].

As these studies have demonstrated the pro-inflammatory response caused directly by the SP and highlighted some of the signaling pathways involved in the immune response to both the SP and viral infection itself, it is also important to observe the response of airway epithelial cells and if they play a role in the innate immune response seen after SP exposure. The aim of this study is to characterize the immune response resulting from SP treatment and highlight which signaling pathways are involved in the transduction of this response. To observe this, bronchiolar epithelial (BEAS-2B) cells were used, treated with the S1 subunit of the SARS CoV-2 SP, and response from genes involved in the MAPK, NF-κB pathways was measured via western blot while pro-inflammatory biomarkers were measured in a multiplex assay.

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3.2 Materials and Methods

3.2.1 Cell Culture and Treatment

Bronchiolar epithelial cells (BEAS-2B) passage number 6-7 were plated in 96-well and 6-well plates pretreated with a coating solution composed of 0.01 mg/ml fibronectin, 0.03 mg/ml collagen, and 0.01 mg/ml bovine serum albumin. Cells were cultured in serum-free BEGM media for two days, then media was removed and fresh BEGM was added containing either vehicle treatment, 5 ug/mL LPS for 30 minutes or one hour, or 1 nM or 10 nM of SARS CoV-2 Spike Protein (SP) was added for 30 minutes, one hour, two hours, or 24 hours.

BEAS-2B cells plated in 6-well plate only received the 10nM SP treatment and following

treatment, plate was transferred to ice and media was removed, cells were treated with RIPA buffer containing phosphatase and protease inhibitors, scraped with a cell scraper, and lysate collected in microcentrifuge tubes. These tubes were then sonicated four times at four seconds each, centrifuged for 13 minutes at 12000 rpm, and protein lysate collected in a new microcentrifuge tube. Protein levels were then analyzed by BCA assay.

Following SP treatment, supernatant was collected from BEAS-2B cells plated in the 96-well plate and stored at -20°C for one day, then transferred to -80°C storage until use.

3.2.2 Multiplex Assay of Vascular Inflammation Response

Supernatant was thawed from -80°C and run on the LegendPlex Human Vascular Inflammation Panel 1 per manufacturer's instructions. Samples were then transferred to a

fresh flat-bottom 96-well plate and read on NovoCyte flow cytometer. Bead analysis was done via Qognit.

3.2.3 Protein Assay via Western Blot

15 ug of protein and 10 uL of SeaBlue Standard were loaded into 8-12% BisTris gel and run at 100 volts for 60 minutes and 150 volts until the protein bands reached the bottom of the gel. After pre-treatment in methanol for 30 seconds, gel was transferred to a PVDF membrane and ran at 56 volts for two hours.

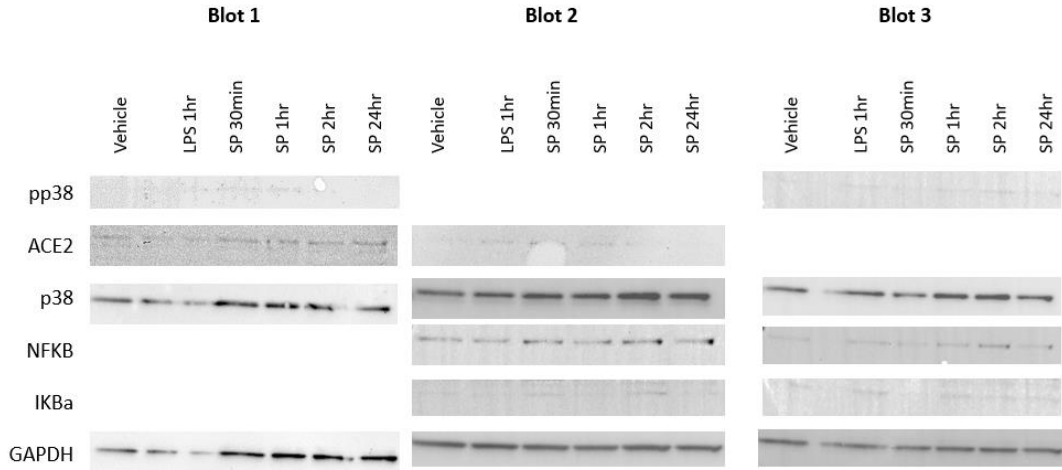
PVDF membranes were washed with TBST and blocked with 5% BSA in TBST for one hour. Membranes were then incubated overnight with primary antibody in 5% BSA, then incubated with secondary antibody conjugated with horseradish peroxidase in 5% BSA the following day. After exposure to West Dura/Pico, membranes were imaged and protein levels were measured through ImageJ gel analysis.

3.3 Results

3.3.1 Western Blot Analysis

SP treatment did not significantly affect levels of p38 or phospho-p38 at any time-point in comparison to vehicle treatment, although LPS treatment had no significant increase in expression of both phospho-p38 and p38 (Figure 3.1a). While not significantly increased, NF-κB p65 showed higher expression as a result of SP treatment, with longer exposure resulting in greater levels of protein expression (Figure 3.1b). SP treatment also resulted in lower levels of protein expression for ACE2.

a)



b)

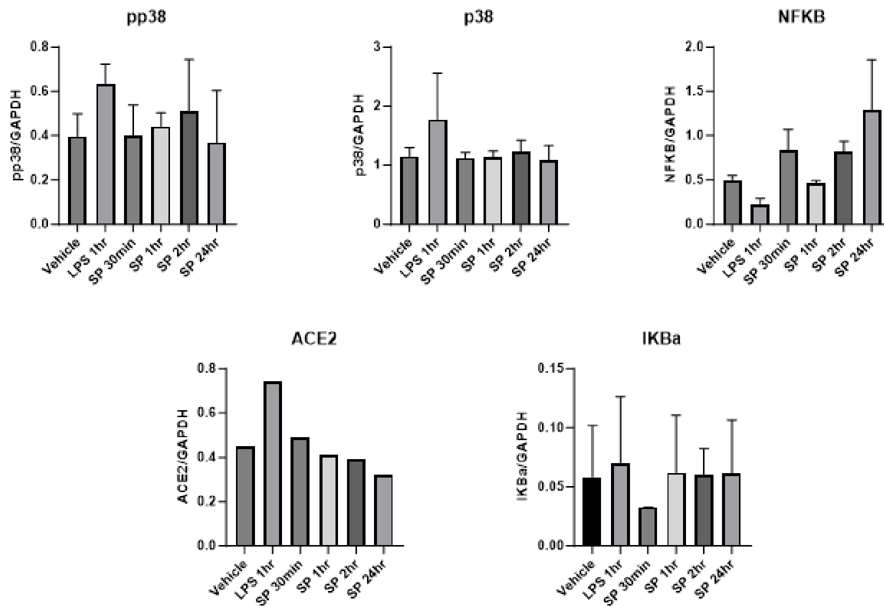


Figure 3.1 a) Western blot imaging of protein collected from BEAS-2B cell line depicting vehicle, LPS and SP treatments. Primary antibodies were derived from rabbit or mouse and diluted to manufacturer instructions in 5% BSA. Secondary antibodies were derived from goat and conjugated with horseradish peroxidase for imaging and diluted to manufacturer instruction in 5% BSA. b) Quantification of Western Blot imaging with signal normalized to GAPDH expression

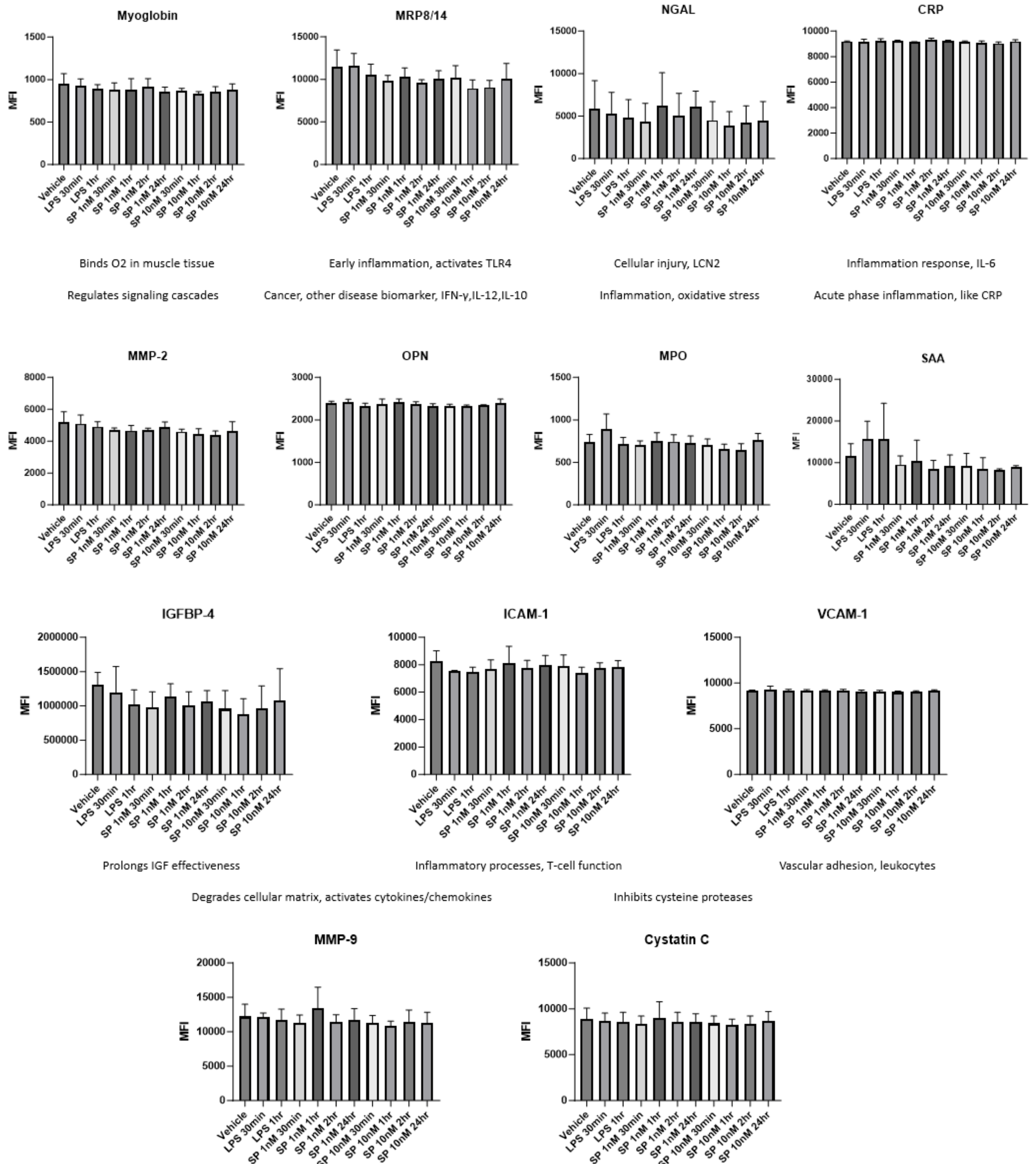


Figure 3.2 Analyte levels measured via LegendPlex Human Vascular Inflammation Panel 1 multiplex assay for BEAS-2B cells treated with vehicle, LPS, or SP.

3.3.2 Multiplex Assay

While no significant change was observed in any of the 13 analytes studied in the multiplex assay, several analytes did see a reduction in levels after prolonged SP treatment. Analytes such as MRP-8/14, NGAL, and MMP-2, each involved in early inflammation, cellular injury response, and regulation of signaling cascades respectively, were part of this trend showing lower expression after exposure to SP at higher concentration and for longer time-points (Figure 3.2).

3.4 Discussion

In contrast to the overexpression experiment performed on BEAS-2B cells transfected with the S protein, this study saw no increase in ACE2 expression after SP treatment, showing reduced protein levels after treatment by the S1 subunit. One underlying reason for this could be that the intracellular response in the transfection experiment caused the upregulation of ACE2, while this study only incubated cells with the SP extracellularly. This could also explain the downregulation in ACE2 expression seen with longer exposure to the SP in this study, as further exposure extracellularly can reduce open receptor-binding domains, limiting the binding of antibodies.

Another observation made by measuring protein levels is that NF-κB levels were upregulated after longer incubation period with the SP. This supports experimental data from other studies, indicating that the S1 subunit does activate pro-inflammatory signaling via the NF-κB signaling pathway.

While the p38 MAPK signaling pathway was shown to be activated by SARS CoV-2 infection, this study showed that the S1 subunit itself does not contribute to this response in BEAS-2B cells.

The multiplex assay results differed in that they did not show any significant upregulation as a result of SP treatment, instead showing downregulation that furthered with longer incubation times. This was more prevalent in analytes like MRP8/14, NGAL, MMP-2, IGFBP-4, and SAA. Many of these analytes that showed downregulation are involved in signaling pathways and are typically part of the early immune response. One potential explanation for this could be that the pathways that release these analytes are triggered early on after SP incubation and levels drop over increased exposure time.

3.5 Conclusion

Following treatment of BEAS-2B cells by the S1 subunit of the SARS CoV-2 spike protein, ACE2 protein levels were downregulated, the p38 MAPK pathway experienced no significant changes, and the NF-κB signaling pathway showed activation from longer exposure to the SP.

All analytes in the multiplex assay experienced no significant changes after treatment with the SP.