PRO-INFLAMMATORY CYTOKINE PRODUCTION BY BV2 MICROGLIAL CELLS

by

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PRO-INFLAMMATORY CYTOKINE PRODUCTION BY BV2 MICROGLIAL CELLS

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ABSTRACT

Alzheimer's Disease (AD) is a neurodegenerative disease that primarily affects elderly populations. AD engenders memory loss and cognitive decline, and its prevalence is rapidly growing. It is estimated that 14 million Americans will have AD by the year 2050. Therefore, it is imperative for researchers to examine the underlying biological mechanisms responsible for AD. Previous research has demonstrated that chronic inflammation is linked to the hallmark AD pathology, amyloid beta (Aβ). Aβ is a protein that disrupts neuronal communication and increases the production of effector proteins called pro-inflammatory cytokines. Microglia function like immune cells in the brain, and when they are activated by inflammatory triggers, such as Aβ, they secrete pro-inflammatory cytokines. Although cytokine release is initially a healthy response, excess cytokine production is harmful to the brain and exacerbates AD pathologies. Prior research has demonstrated that pro-inflammatory cytokines are upregulated in the serum of AD patients. Therefore, cytokines are a crucial target for AD therapeutics. This project examined the temporal inflammatory response of microglial cells following lipopolysaccharide (LPS) insult. LPS is a component of common bacteria and can induce inflammation in microglial cells. We treated cells with several different concentrations of LPS and assessed cytokine production at several different timepoints. Here we show that microglia treated with LPS produce cytokines in a dose- and time-dependent manner. In addition, the production of different cytokines does not follow the same temporal pattern. These data will help us pinpoint proper testing procedures for therapeutic compounds that are currently under development.

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INTRODUCTION

Alzheimer's Disease (AD) is a neurodegenerative disease that is the primary cause of a progressive decline in cognitive ability, known as dementia. It typically presents as episodic short-term memory loss, and can progress to impaired judgement, loss of language skills, and deficits in motor function. It is the sixth leading cause of death in the United States, with a global prevalence estimated at 24 million, and is predicted to quadruple by 2050 (Kumar et al., 2021). As the population ages, research into AD will become vital to enable more effective treatments to reverse its effects. The hallmark pathologies of AD are extracellular plaques composed of amyloid beta (Aβ), and intracellular aggregates known as tau tangles. The tangles originate from hyperphosphorylated tau protein that misfolds and impairs axoplasmic transport in neurons, while Aβ is generated during improper cleavage of the amyloid precursor protein (Fischer et al., 2015). These aggregates appear to have neurotoxic properties and can interfere in synaptic function, increase the rate of neuronal death and synaptic rarefaction, and promote chronic inflammation (Lakhan et al., 2021).

Evidence indicates that AD pathologies are exacerbated by inflammation and oxidative stress (Ahmed et al., 2017). Inflammation occurs as a natural response to harmful stimuli, such as pathogens or cell damage, and is caused by immune cells and the small molecule mediators they release, such as cytokines (Ahmed et al., 2017). However, constant production of these cytokines leads to chronic inflammation, which is associated with Aβ plaque formation and tau hyperphosphorylation, the two primary pathologies of AD (Wang et al., 2019). Meanwhile, oxidative stress is a critical factor in the development of neuroinflammation. As immune cells are recruited to the site, reactive oxygen species (ROS) are generated (Ahmed et al., 2017). Although essential to the body because they act as intermediates in important cell functions, such

as cellular respiration, excess accumulation is harmful, and labeled as oxidative stress. Increased levels of Aβ are associated with increased ROS in the hippocampus and cortex, two areas that are targeted in AD (Cheignon et al., 2018).

Microglial cells, the resident immune cell in the brain, can be activated by substances such as Aβ or lipopolysaccharide (LPS). LPS, derived from the cell wall of gram-negative bacteria, is shown to be toxic to neurons in the presence of microglia, and triggers inflammation in vivo and in vitro models (Block et al., 2005). LPS can induce the microglia to perform a variety of functions, including the release of proinflammatory cytokines, like interleukin (IL)-1β, IL-6, and TNF (tumor necrosis factor)-α (Wang et al., 2019). While they are important in brain development and maintenance, chronic activation of microglial cells can become harmful (Dai et al., 2014). Indeed, microglia may contribute to the mechanisms behind cognitive dysfunction and neuronal damage in AD (Stansley et al., 2012).

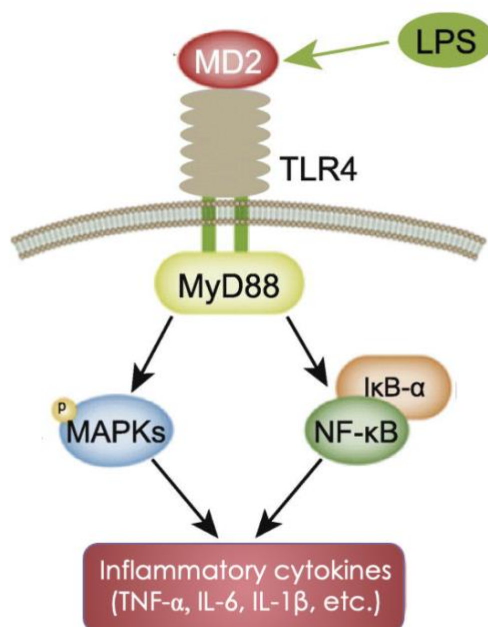

Figure 1. Signaling pathway involved inflammation, simplified for the purpose of this study. This figure represents the stimulation of the TLR4 pathway by LPS. LPS binds to the TLR4 receptor, which causes activation of MyD88. Then, NF-κB is activated by MyD88, then translocates into the nucleus. There, it acts as a transcription factor for proinflammatory cytokines (Figure adapted from Zhang et al., 2020).

In order to study inflammation in microglia cells, BV2 microglia, a murine cell line derived from C57BL/6 mice, were utilized in the current study (Blasi et al., 1989). BV2 cells grown in vitro are optimal for studying neurodegenerative disorders because of their rapid replication rate and ease of access vs. in vivo work (Stansley et al., 2012). Dai et al. also demonstrated that an LPS treatment of BV2 cells results in increased NF-κB, p-IκB, and IL-1β, as well as decreased IκB. The first three molecules are important in transmitting information to the cell to stimulate inflammation, while decreased IκB further indicates this trend. This information together supports the presence of the TLR4 signaling cascade, described in Figure 1, following LPS stimulation in microglial cells (Dai et al., 2014).

The primary purpose of this study is to identify the rate at which pro-inflammatory cytokines, mainly IL-1β, TNF-α, and IL-6, are released in response to LPS. In this study, we seeded BV2 microglial cells in 6-well plates and treated them with varying concentrations of LPS, to stimulate an inflammatory response. The supernatants and cell lysates were collected at five different timepoints following LPS treatment, to understand the temporal regulation of cytokine production. An Enzyme Linked Immunosorbent Assay (ELISA) was performed to quantify IL-1β, TNF-α, and IL-6. These data will provide important information to the Neurobiology of Aging Collaborative when designing potential therapeutics that target inflammation. This research will be beneficial for identifying pro-inflammatory cytokine production in the brain and identifying potential anti-inflammatory therapeutics that may be used to treat AD.

MATERIALS AND METHODS

BV2 CELL MAINTENANCE

BV2 microglial cells were cultured in a cell incubator at 37º C and 5% CO_2. Cells were grown in 10cm tissue culture plates in complete medium, consisting of 10 mL of Dulbecco's Modified Eagle Medium (DMEM), 5% Penicillin-Streptomycin, 5% L-glutamine, and 3% Fetal Bovine Serum. Cells were monitored and passaged upon reaching 80% confluency. The medium was aspirated off the dish, and cells were washed with 5 mL PBS. Then, 5 mL of complete medium was added, and cells were scraped from the bottom of the dish, then transferred to a new 10 cm dish with fresh complete medium and placed back in the incubator.

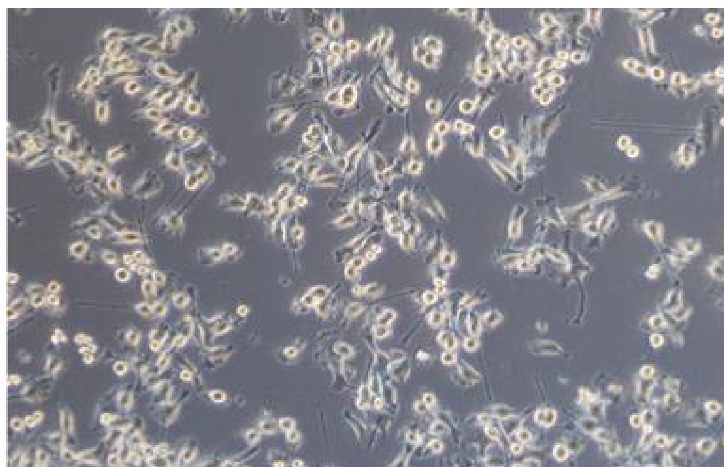

Figure 2. Image of a plate during LPS treatment showing BV2 microglial cells

LPS CELL TREATMENT

To prepare for this experiment, the old medium was aspirated off the cells, then PBS was added to wash the cells. Medium was added again, then cells were scraped and counted using a hemocytometer. Cells were seeded in 6-well plates approximately 12 hours before treatment with LPS. Overnight, the cells adhered to the bottom of the plate. Cells were treated with six different concentrations of LPS, and supernatants were collected at five different time points. LPS concentrations used were 5µg/mL,

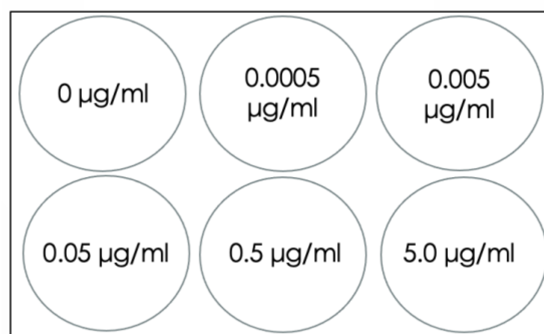

Figure 3. LPS concentrations in a 6-well plate (1 plate used per timepoint)

0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, and a control of 0µg/mL. The supernatant of each LPS concentration was collected at 2, 4, 8, 12, and 24 hours. Cell supernatants were collected and aliquoted into microfuge tubes and stored in a -20º C freezer.

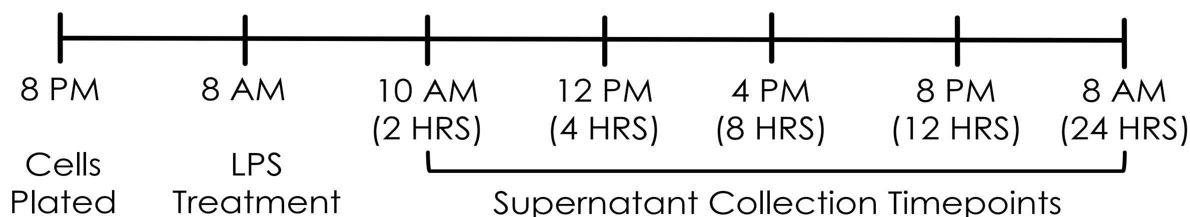

Figure 4. Time sequence of LPS treatment and supernatant collection

Enzyme Linked Immunosorbent Assays (ELISA)

Following cell removal, the cell supernatants were thawed on ice. From this supernatant, the cytokines TNF-α, IL-1β, and IL-6 were measured using an ELISA in accordance with instructions from the kit manufacturer (BioLegend, San Diego, California). The procedure was performed in accordance with the protocol provided by the manufacturer. Briefly, 96-well plates were coated overnight with 100µL of diluted capture antibody. The following morning, each plate was washed four times using a wash buffer of PBS and 0.05% Tween-20. The wells were then blocked with an assay diluent and incubated for one hour at room temperature on a plate shaker set at 500 rpm. Samples were placed into a U-bottom plate in preparation for transfer into the 96-well assay plate. The plates were then washed another four times, and loaded with 100µL of either sample or standard, after which the plates were sealed and incubated on the plate shaker for two hours. The plates were washed four times and treated with 100µL of detection antibody

in each well, then sealed and incubated for another hour on the shaker. After another four washes, 100μL of Avidin-HRP was placed in each well, and the plate was sealed and incubated for thirty minutes. The plate was washed five times, with a minute in between each wash to allow the wash buffer to soak. Following this, 100μL of substrate solution was added to each well, and the plate was placed in the dark, unsealed, for fifteen minutes. Finally, 100μL of stop solution (2N H_2SO_4) was added to each well. The plates were read on a microplate reader at an absorbance of 450 nm.

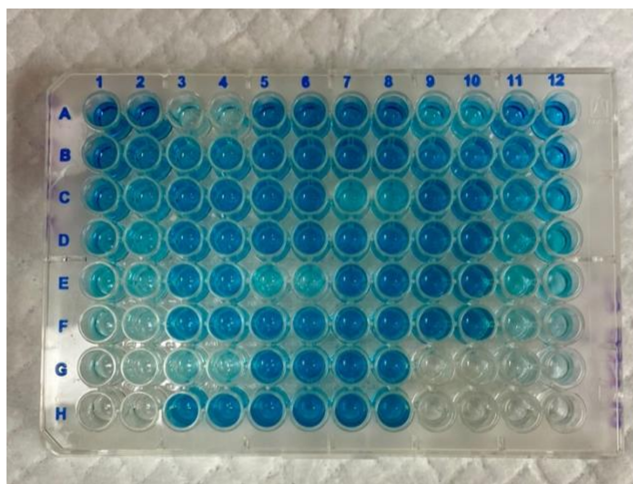

Figure 5. ELISA plate following administration of colorimetric substrate

RESULTS

Temporal and LPS Dose-Dependent TNF-a Production

In order to stimulate an inflammatory response in BV2 microglial cells, LPS was added at increasing concentrations at increasing treatment durations. TNF-α and IL-6 production, measured by ELISAS, both demonstrated a dose-dependent relationship with LPS concentration and treatment duration. Analysis of Variance (ANOVA) revealed a significant main effect of LPS treatment, $F(5,90) = 25.76$, $p \leq 0.001$, and a significant main effect of treatment duration, $F(4,90) = 9.686$, $p \leq 0.001$, such that TNF-alpha production elevated with increasing concentrations of LPS and treatment time. Additionally, this was qualified by a significant interaction, $F(20,90) = 1.838$, $p = 0.036$.

Post hoc analyses did not reveal any significant differences between treatment conditions after 2 hours of LPS treatment. However, post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 µg/mL and 0.005 µg/mL ($p = 0.046$), and 0 µg/mL and 0.5 µg/mL ($p = 0.048$) LPS for 4 hours. Additionally, post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 µg/mL and 0.005 µg/mL ($p \leq 0.001$), 0 µg/mL and 0.05 µg/mL ($p \leq 0.001$), 0 µg/mL and 0.5 µg/mL ($p = 0.008$), 0 µg/mL and 5 µg/mL ($p \leq 0.001$) LPS for 8 hours.

Furthermore, post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 µg/mL and 0.0005 µg/mL ($p = 0.002$), 0 µg/mL and 0.05 µg/mL ($p \leq 0.001$), 0 µg/mL and 0.5 µg/mL ($p \leq 0.001$), 0 µg/mL and 5 µg/mL ($p \leq 0.001$), 0.005 µg/mL and 0.5 µg/mL ($p = 0.015$), of LPS for 12 hours. Finally, post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 µg/mL and 0.0005 µg/mL ($p \leq 0.001$), 0 µg/mL

and 0.05 µg/mL ($p \leq 0.001$), 0 µg/mL and 0.5 µg/mL ($p \leq 0.001$), 0 µg/mL and 5 µg/mL ($p = 0.023$), 0.005 µg/mL and 0.5 µg/mL ($p = 0.015$) of LPS for 24 hours. See Figure 6.

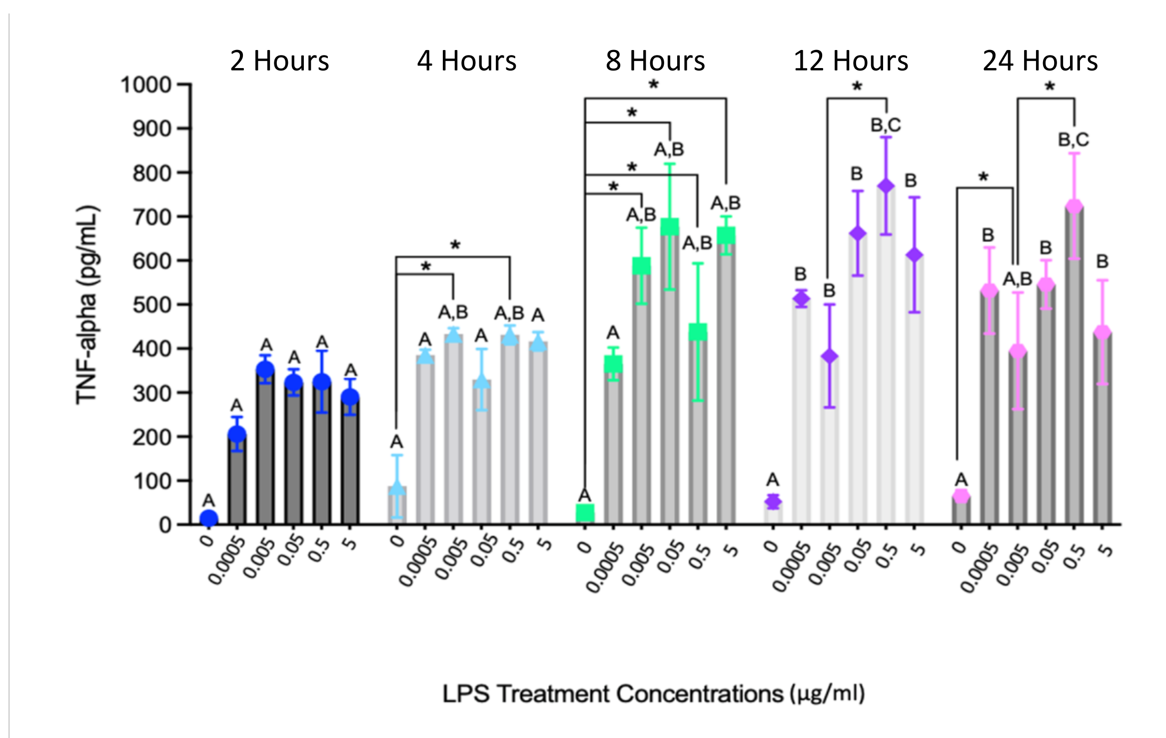

Figure 6. TNF-*α* ELISA results following LPS treatment. BV2 microglial cells were treated with six different LPS concentrations (5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, & 0µg/mL) for five different treatment times (2hr, 4hr, 8hr, 12hr, & 24hr). Different letters (A, B, C) represent significant differences at p≤ .05. Bars represent mean ± SEM. N's =3. Results are shown in pg/mL.

As shown in Figure 6., increasing concentrations of LPS significantly increased the production of TNF-α, starting at 4 hours of treatment time. TNF-α production plateaued around 8 hours possibly indicating that maximal production has already occurred by that time point and increasing the time or LPS concentration does not increase cytokine production.

Temporal and LPS Dose-Dependent IL-6 Production

ANOVA revealed a significant main effect of LPS treatment, $F(5,77) = 5.547$, $p \leq 0.001$, and a significant main effect of treatment duration, $F(4,77) = 4.773$, $p = 0.003$, such that IL-6 production elevated with increasing concentrations of LPS and treatment time. See Figure 7. Post

hoc analyses did not reveal any significant differences between treatment conditions following 2 or 4 hours of LPS treatment. However, post hoc analyses revealed a significant difference in IL-6 production between cells treated with 0.0005 µg/mL and 5 µg/mL ($p = 0.047$) of LPS for 8 hours. Additionally, post hoc analyses revealed a significant difference in IL-6 production between cells treated with 0 µg/mL and 0.5 µg/mL ($p = 0.006$), 0 µg/mL and 5 µg/mL ($p = 0.002$), 0.0005 µg/mL and 0.5 µg/mL ($p = 0.007$), 0.0005 µg/mL and 5 µg/mL ($p = 0.002$), 0.005 µg/mL and 0.5 µg/mL ($p = 0.014$),0.005 µg/mL and 5 µg/mL ($p = 0.004$), 0.05 µg/mL and 0.5 µg/mL ($p = 0.028$), 0.05 µg/mL and 5 µg/mL ($p = 0.008$) of LPS for 12 hours.

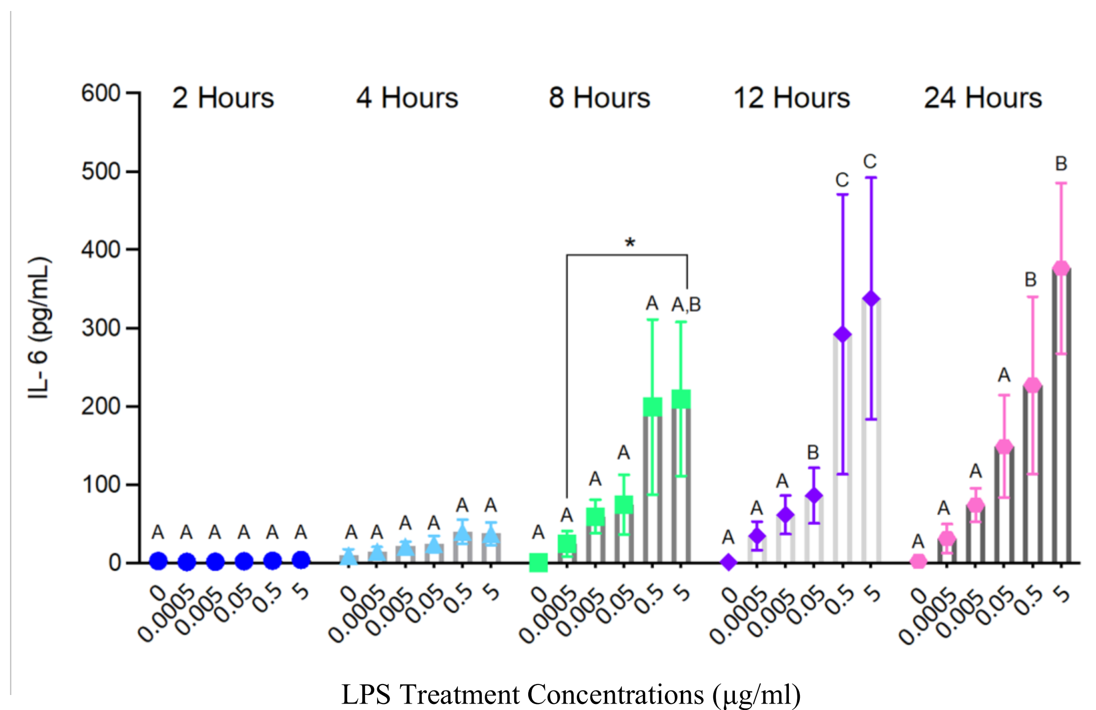

Figure 7. IL-6 ELISA results following LPS treatment. BV2 microglial cells were treated with six different LPS concentrations (5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, & 0µg/mL) for five different treatment times (2hr, 4hr, 8hr, 12hr, & 24hr). Different letters (A, B, C) represent significant differences at $p \leq 0.05$. Bars represent mean ± SEM. N's =3. Results are shown in pg/mL.

Finally, post hoc analyses revealed a significant difference in IL-6 production between cells treated with 0 µg/mL and 0.5 µg/mL ($p = 0.048$), 0 µg/mL and 5 µg/mL ($p \leq 0.001$), 0.0005

μg/mL and 5 μg/mL ($p \leq 0.001$), 0.005 μg/mL and 5 μg/mL ($p = 0.004$), 0.05 μg/mL and 5 μg/mL ($p = 0.029$) of LPS for 24 hours. These data demonstrate that LPS treatment did not have a significant impact on IL-6 secretion until cells were treated with 5 μg/ml of LPS for 8 hours. See Figure 7.

LIMITED LPS-INDUCED IL-1β PRODUCTION IN BV-2 CELLS

As compared to the previous cytokines, there was no significant main effect of LPS treatment on IL-1β. This cytokine, while produced in sufficient amounts in primary microglia, is not readily released in BV2 microglial cells, as seen in previous literature (Stansley et al., 2012).

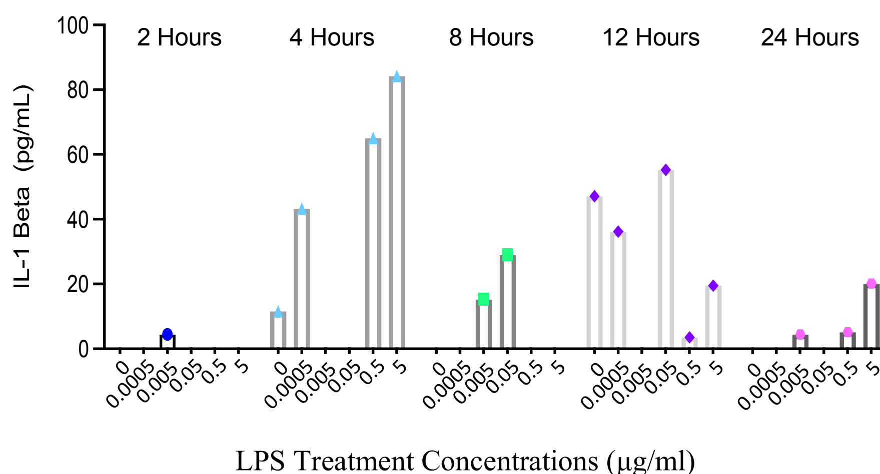

Figure 8. IL-1β ELISA results following LPS treatment. BV2 microglial cells were treated with six different LPS concentrations (5μg/mL, 0.5μg/mL, 0.05μg/mL, 0.005μg/mL, 0.0005μg/mL, & 0μg/mL) for five different treatment times (2hr, 4hr, 8hr, 12hr, & 24hr). Results are shown in pg/mL.

DISCUSSION

As hypothesized, there was a significant main effect of LPS treatment and treatment time on TNF-α and IL-6 production. TNF-α was produced rapidly after treatment, whereas IL-6 production did not start to increase readily until the highest concentration of LPS in the 8-hour time point. This is consistent with previous studies performed on rat microglia, which indicates higher initial levels of TNF-α, and IL-6 taking longer to be produced (Ishijima et al., 2021). Because of this, any future experiments focused on studying TNF-α and IL-6 will likely have different timepoints. Additionally, seeing substantial TNF-α at a 4-hour time point indicates that if we are to study the signal transduction pathway that leads to its upregulation, we would have to look much earlier than the previous collection times. Considering that TNF-α production is approaching significance at 0.05 μg/ml, this indicates that early signaling events, such as activation of NFκB, could occur within minutes. At higher LPS concentrations, TNF- α would likely be detectable after just one hour.

However, there was no significant effect on IL-1β production. As found in previous literature, BV2 microglial cells have nearly undetectable levels of IL-1β (Stansley et al., 2012). . This was consistent with our findings during this experiment. Because our BV2 cells do not express significant amounts of IL-1β following LPS stimulation, there may be a defect in the cell line, specifically in the NLRP3 inflammasome. Activation of this structure is required to cleave pro-IL-1β, the compound made by these cells in response to LPS, into IL-1β, the active cytokine. To further investigate this, we can conduct experiments on NLRP3 activation to determine if this is the issue. If that is the case, then a different microglial cell line that consistently produces IL-1β may be more useful, or a macrophage cell line. Using a macrophage cell line could also

simplify experiments on primary mouse macrophages, as the procedure to remove them from animals is simpler than attempting to remove microglial cells in the brain.

These data will provide guidance for future projects within the Neurobiology of Aging Collaborative as they continue to investigate anti-inflammatory therapeutics. This experiment has provided valuable information that can be used to evaluate when a specific cytokine starts to be produced in response to LPS. Following this, data can be collected on the efforts of a compound at attenuating the inflammatory response in the microglia.

REFERENCES

Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2017). Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1863*(2), 585–597. https://doi.org/10.1016/j.bbadis.2016.11.005

Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., & Bistoni, F. (1989). Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *Journal of Neuroimmunology, 27*(2-3), 229-32. https://doi.org/10.1016/0165-5728(90)90073-v

Block, M. L., & Hong, J.-S. (2005). Microglia and inflammation-mediated neurodegeneration: Multiple triggers with a common mechanism. *Progress in Neurobiology*, *76*(2), 77–98. https://doi.org/10.1016/j.pneurobio.2005.06.004

Cai, B., Seong, K.-J., Bae, S.-W., Chun, C., Kim, W.-J., & Jung, J.-Y. (2018). A synthetic diosgenin primary amine derivative attenuates LPS-stimulated inflammation via inhibition of NF-κB and JNK MAPK signaling in microglial BV2 cells. *International Immunopharmacology*, *61*, 204– 214. doi: 10.1016/j.intimp.2018.05.021

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C., & Collin, F. (2018). Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox biology*, 14, 450–464. https://doi.org/10.1016/j.redox.2017.10.014

Dai, X.-J., Li, N., Yu, L., Chen, Z.-Y., Hua, R., Qin, X., & Zhang, Y.-M. (2014). Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. *Cell Stress and Chaperones*, *20*(2), 321–331. doi: 10.1007/s12192-014-0552-1

Fischer, R., & Maier, O. (2015). Interrelation of Oxidative Stress and Inflammation in Neurodegenerative Disease: Role of TNF. *Oxidative Medicine and Cellular Longevity*, *2015*. https://doi.org/10.1155/2015/610813

Gong, X., Ivanov, V. N., & Hei, T. K. (2015). 2,3,5,6-Tetramethylpyrazine (TMP) down-regulated arsenic-induced heme oxygenase-1 and ARS2 expression by inhibiting Nrf2, NF-κB, AP-1 and MAPK pathways in human proximal tubular cells. *Archives of Toxicology*, *90*(9), 2187–2200. doi: 10.1007/s00204-015-1600-z

Horvath, R.J., Nutile-McMenemy, N., Alkaitis, M.S., & Deleo, J.A. (2008). Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures. *Journal of Neurochemistry, 107*. doi: 10.1111/j.1471-4159.2008.05633.x

Ishijima, T., & Nakajima, K. (2021). Inflammatory cytokines TNFα, IL-1β, and IL-6 are induced in endotoxin- stimulated microglia through different signaling cascades. *Science Progress*. https://doi.org/10.1177/00368504211054985

Ko, S. H., Rho, D. J., Jeon, J. I., Kim, Y.-J., Woo, H. A., Kim, N., & Kim, J. M. (2016). Crude Preparations of Helicobacter pylori Outer Membrane Vesicles Induce Upregulation of Heme Oxygenase-1 via Activating Akt-Nrf2 and mTOR–IκB Kinase–NF-κB Pathways in Dendritic Cells. *Infection and Immunity*, *84*(8), 2162–2174. doi: 10.1128/iai.00190-16

Kumar, A., Sidhu, J., Goyal, A., Tsao, J. (2021). Alzheimer Disease. *Stat Pearls.* https://www.ncbi.nlm.nih.gov/books/NBK499922/

Lakhan, S. E. (2021). *Alzheimer Disease*. Practice Essentials, Background, Anatomy. https://emedicine.medscape.com/article/1134817-overview.

Stansley, B., Post, J., & Hensley, K. (2012). A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *Journal of Neuroinflammation*, *9*(1). https://doi.org/10.1186/1742-2094-9-115

Wang, L.-Y., Huang, C.-S., Chen, Y.-H., Chen, C.-C., Chen, C.-C., & Chuang, C.-H. (2019). Anti- Inflammatory Effect of Erinacine C on NO Production Through Down-Regulation of NF-κB and Activation of Nrf2-Mediated HO-1 in BV2 Microglial Cells Treated with LPS. *Molecules*, *24*(18), 3317. doi: 10.3390/molecules24183317

Wang, W., Tan, M., Yu, J., & Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Annals Of Translational Medicine, 3*(10), 7. doi:10.3978/j.issn.2305-5839.2015.03.49

Zhang, Y., Chen, H., Zhang, W., Cai, Y., Shan, P., Wu, D., Zhang, B., Liu, H., Khan, Z.A., Liang, G. (2020). Arachidonic acid inhibits inflammatory responses by binding to myeloid differentiation factor-2 (MD2) and preventing MD2/toll-like receptor 4 signaling activation. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease, 1866*(5), 165683. doi:https://doi.org/10.1016/j.bbadis.2020.165683