THE EFFECTS OF ANTIOXIDANT THERAPY ON THE OXIDATIVE STRESS PATHWAY IN MICROGLIAL CELLS

by

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ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disease associated with old age and marked by deficits in memory and cognitive function. AD pathology is characterized by amyloid-beta (Aβ) accumulation, which leads to Aβ plaque formation and ultimately neuronal death. Additionally, Aβ activates microglial cells, which function as immune cells in the brain. Microglial cells secrete proteins that induce inflammation, known as pro-inflammatory cytokines. The chronic activation of microglia engenders oxidative stress in the brain, which further exacerbates AD pathologies. Dr. Kayla Green's lab in the TCU Chemistry Department has successfully created potent small molecules, such as L2 and L4, that act as potent antioxidants. We collaborated with Dr. Green's lab to research the possible, therapeutic effects of L2 and L4 treatment against inflammation in immortalized, BV2 microglial cells. Moreover, the main purpose of the current experiment was to further study the effects of these molecules against key AD pathologies, and to understand L2 and L4's therapeutic potential against inflammation *in vitro*. The overall goal of this research was to demonstrate the capacity of L2 and L4 to minimize the immunological mechanisms that drive AD pathologies. AD is the sixth leading cause of death in America, but the availability of therapies is limited. Our research will contribute to the understanding of the link between the immune system and central nervous system in AD development.

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ACKNOWLEDGEMENTS

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INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that is the most common form of dementia. It affects around 44 million people worldwide, including 5.7 million Americans. Without a viable treatment or cure, AD will likely remain a major healthcare issue as the world's life expectancy increases. AD symptoms include, but are not limited to, cognitive decline, memory loss, loss of language skills, loss of motor function, and personality changes (Weller & Budson, 2018). The two common biological markers of AD are the deposition of extracellular amyloid β (Aβ) plaques and intracellular hyperphosphorylation of tau proteins (Liu et al., 2019). The Aβ plaques are formed from proteolytic cleavage of the amyloid precursor protein, in which one of the fragments is Aβ (Finder & Glockshuber, 2007). These fragments of Aβ aggregate forming the plaques that are believed to be a cause of AD, rather than a consequence. The Aβ plaques are believed to block cell communication at synapses and initiate inflammation, further contributing to AD pathologies (Lahkan 2019).

Two indicators of AD are inflammation and oxidative stress. Inflammation is a natural response within the immune system. It serves to eliminate the cause of cellular damage, as well as to remove dead cells and initiating repair mechanisms (Ahmed et al., 2017). There are two forms of inflammation: acute and chronic. Acute inflammation can be beneficial to the host, whereas chronic inflammation, especially when uncontrolled, can be harmful to cells and lead to cellular damage (Ahmed et al., 2017). Prolonged chronic inflammation and the presence of damaged tissue leads to the activation of microglia and astrocytes, which generate a neuroinflammatory response (Fischer & Maier, 2015). Astrocytes and microglia are the two main cellular mediators involved with the inflammatory response (Wyss-Coray & Rogers, 2012).

Microglial cells are brain immune cells that modulate inflammation in the central nervous system (CNS) in response to injury and infection (Block & Hong, 2005). Microglia account for 10-15% of CNS cells that, along with immune response responsibilities, aids in trophic support and maintaining homeostasis (Zheng et al., 2018). When activated, microglia undergo morphologic alterations changing from resting microglia to activated microglia (Block & Hong, 2005). Activation of microglial cells occurs through inflammation in response to Aβ plaque accumulation (Johnston et al., 2019). Microglia release a variety of proinflammatory soluble factors through the TLR4 pathway,

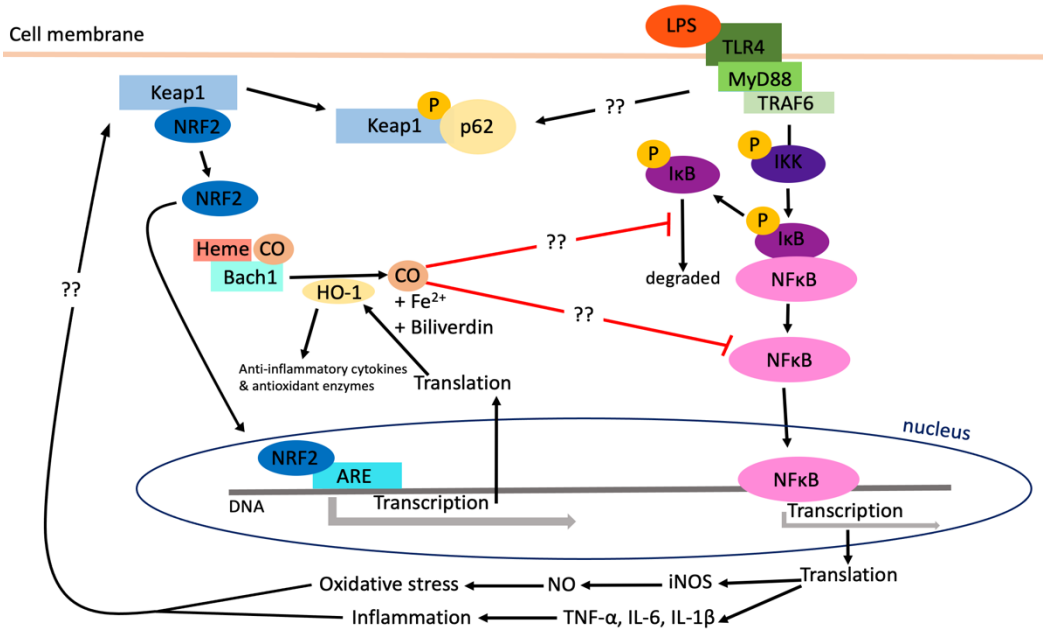

Figure 1. Signaling pathways involved in inflammation and oxidative stress, simplified for the purpose of this study. This figure demonstrates the interaction between theTLR4 pathway and the Nrf2 pathway. The TLR4 pathway is activated by LPS and through the signaling cascade, NFκB is activated and translocates into the nucleus where it acts as a transcription factor for proinflammatory mediators and cytokines. Oxidative stress and inflammation activate the Nrf2 pathway. Nrf2, once activated, translocates into the nucleus and acts as a transcription factor that leads to anti-inflammatory cytokines and antioxidant enzymes. (Figure based on information from the following papers: Ahmed et al., 2017; Cai et al., 2018; Dai et al., 2014; Gong et al., 2015; Ko et al., 2016, Wang et al., 2019. Layout based on pathway from Caitlyn Vilas, TCU Departmental Honors

Figure 1, such as prostaglandin E2, nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin-(IL-6), IL-1β, and tumor necrosis factor (TNF-α). These mediators generate an inflammatory response and lead to oxidative stress, which contributes to the progression and pathogenesis of AD (Lee & Kang, 2014). In response to the damage caused by inflammatory factors, cells activate their intrinsic antioxidant pathway to protect against oxidative stress. Nuclear factor (erythroid-derived-2)-like 2 (Nrf2) is an anti-inflammatory factor that plays a critical role in the regulation of this antioxidant response (Lee & Kang, 2014; Block & Hong, 2005). When activated, Nrf2 will translocate into the nucleus, where it binds to the antioxidant response element (ARE) and serve as a transcription factor that initiates expression of antioxidant enzymes (Johnston et al., 2019). Nrf2 signaling protects against oxidative stress due to inflammation or injury by inhibiting the proinflammatory cytokines, downregulating the proinflammatory pathways, or by expressing antioxidant proteins such as HO-1 (Johnston et al., 2019).

Oxidative stress, which is linked to inflammation, is another important contributor to the pathogenesis of AD. Aβ plaque deposition results in inflammation. The inflammatory response activates NADPH oxidase that causes an increased production of ROS (Fischer & Maier, 2015). Oxidative stress can occur due to the activation of microglial cells and the dysfunction of the antioxidant system leading to expression of proinflammatory cytokines and ROS (Agostinho et al., 2010). Inflammation and ROS are highly controlled processes, and both can lead to the cleavage of APP, and therefore, the production of Aβ. The production and presence of Aβ plaques will lead to further inflammation and oxidative stress. This is a self-perpetuating process in AD that plays a role in the progression and pathogenesis of the disease. Therefore, agents that

lead to a reduction in NO, iNOS, and proinflammatory cytokines and mediators are a target for potential drug therapies (Lee & Kang, 2014).

Metal ion dysregulation has also been linked to neurodegenerative diseases. Abnormal concentrations of certain metals may contribute to pathogenesis of diseases like AD (Liu et al., 2019). Metal ions are important for essential life functions because they bind to metalloproteins which allows for maintenance of cell structure, mediation of cell signaling, regulation of gene expression, and catalyzation of enzymatic activity (Kim et al., 2018). The accumulation of these metal ions, such as zinc and copper, is believed to be a contributor to Aβ plaque in AD pathogenesis (Fischer & Maier, 2015). The use of metal chelators is proving to be a promising way to reduce redox stress and therefore use as a potential therapeutic for neurodegenerative disorders (Liu et al., 2019).

Dr. Kayla Green's lab in the TCU Chemistry Department has successfully created a family of compounds that act as powerful antioxidants that have the capability of radical scavenging and metal ion capture. L2 ($^{OH}PyN_3$) and L4 (1,4,11,13-Tetraazabis(2,6-pyridinophane)-8,17-diol), shown in Figure 2, are N-heterocyclic amines that can chelate metal ions and act as antioxidants (Johnston et al., 2019). In a previous study, Dr. Green's lab showed that L2 and L4 pretreatment could protect HT-22 cells from oxidative stress induced cellular damage in a dose dependent manner (Johnston et al., 2019). L2 and L4 can also activate the Nrf2 pathway that promotes antioxidant properties. Nrf2 signaling protects against oxidative stress as a result of inflammation or injury by inhibiting the proinflammatory cytokines, downregulating the proinflammatory pathways, or by expressing antioxidant proteins (Johnston et al., 2019).

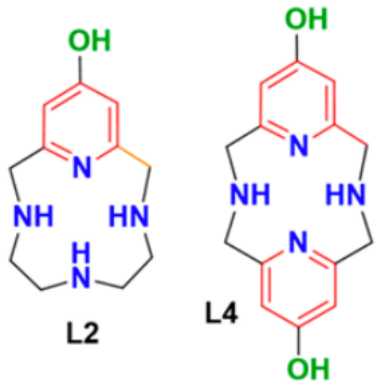

Figure 2. L2 and L4 Compounds (figure taken from Johnston et al., 2019)

This research focused on the ability of L2 and L4 in reducing oxidative stress and specifically, inflammation. BV2 microglial cells, a common model for neurodegenerative diseases, are immortalized murine neonatal cells. These cells are able to induce neuroinflammation via inflammatory responses during endogenous chemical stimulation (Stansley et al., 2012). Microglial cells can be stimulated with lipopolysaccharide (LPS) which results in the production of proinflammatory cytokines (Subedi et al., 2019). LPS is an endotoxin from the cell wall of a gram-negative bacteria which can activate the immune system and induce an inflammatory state (Chen et al., 2012). In this study, BV2 microglia were treated with LPS to induce an inflammatory response to determine the capabilities of L2 and L4 to block the release of proinflammatory cytokines. BV2 cells were seeded in 6-well plates and treated with 6 different concentrations of LPS at three different time intervals. To quantify the amount of TNF-α produced, an Enzyme Linked Immunosorbent Assay (ELISA) was run. Western blotting was done to see the expression of Nrf2 in the LPS treatment. Another experiment was carried out to pretreat the cells with L2 and L4 at several concentrations for 4 hours prior to LPS treatment. A TNF-α ELISA was run again to measure the levels of TNF-α in the pretreatment study. This study focused on TNF-α, since this study aimed to examine the potential therapeutic properties

of the antioxidant compounds L2 and L4 against the inflammatory response of BV2 microglial cells, following stimulation by LPS treatment.

MATERIALS AND METHODS

BV2 Cell Maintenance

The BV2 microglial cells used were maintained in a cell incubator at 37 degrees Celsius and 5% CO_2. The cells were grown in 10cm tissue culture dishes, in complete medium containing 10mL of Dulbecco's Modified Eagle Medium (DMEM – Caisson Laboratories, Smithfield, UT), 5% Penicillin-Streptomycin, 5% L-glutamine, and 5% Fetal Bovine Serum. The cells were monitored and sub-cloned when they reached 80-90% confluency.

To sub-clone the cells, the old medium was aspirated before the wells were washed with 10mL of phosphate-buffered saline (Dulbecco's PBS – Caisson Laboratories, Smithfield, UT). After washing the cells, 5mL of complete medium was added and a cell scraper was used to lift the cells from the bottom of the plate. Cells were pipetted up and down to break up clumps before being transferred into a new 10cm plate, which contained fresh, complete medium and were then returned to the incubator.

Prior to treatment experiments, cells were seeded in 3.5cm 6-well plates. Old medium was aspirated off, cells were washed in 10mL of PBS and 10mL of complete medium was added. A cell scraper was used to loosen cells from the bottom of the plate. Cells were pipetted into a 15mL conical tube and centrifuged for 10 minutes at 1.2 RCF where the cells spun down into a pellet. The supernatant was aspirated off, leaving 2mL solution in the conical tube, where the pellet was broken up. The cells were counted using a hemocytometer. To do this, 10µL of the resuspended cells was added to each side and manually counted using a microscope.

LPS Preparation

The lipopolysaccharide (LPS) used to treat the cells is from the Escherichia coli serotype: 055:B5 and was acquired from SigmaAldrich, St Louis, MO. The LPS was dissolved in PBS to

create a 5µg/mL stock solution before being aliquoted and stored in a -20 degrees Celsius freezer. Before treating the cells with LPS, one aliquot of stock solution was thawed. Concentrations of 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, and 0µg/mL (control) were prepared via serial dilutions of the 5µg/mL stock solution with serum free media.

LPS Treatment of Cells

Cells were first treated with varying concentrations of LPS, and supernatant and lysates were collected at various time points to determine the cytokines produced in the elicited inflammatory response. After being incubated for four hours, one concentration of LPS was added to each well before the plates were returned to the incubator. Cell supernatants and lysates were collected following 4, 8, and 24 hours of treatment. The experimental timeline is shown in Figure 3A. Cell supernatant was aliquoted in microcentrifuge tubes, snap frozen using dry ice, and stored in a -20 degrees Celsius freezer. Cell lysates were stored in lysis buffer containing mammalian protein extraction reagent (MPER; Invitrogen, Waltham, MA), which also contained a protease inhibitor and a phosphatase inhibitor and then snap frozen using dry ice before being stored in the -20 degrees Celsius freezer.

L2 and L4 Pretreatment of Cells

The same protocol for cell seeding was followed. Two 6-well plates were prepared by seeding 200,000 cells per well. After 24 hours of incubation, these cells were pretreated with four different concentrations of L2 or L4 for 4 hours prior to LPS activation. These concentrations were 100µM, 10µM, 1µM, 0.1µM, and a control of 0µM, no L2 or L4. Both plates had an LPS concentration of 0.005µg/mL. Both plates were treated with LPS for 4 hours before cells were collected. The timeline of the experiment is shown in Figure 3B. Collected cells were aliquoted into microcentrifuge tubes and stored in the -20 degrees Celsius freezer.

Supernatant was collected separately from the cell lysates, which were stored in the lysis buffer MPER with the added protease inhibitor and phosphatase inhibitor.

After considering the data, this experiment was repeated with three additional concentrations of L2 or L4 at 50µM, 250µM, and 500µM.

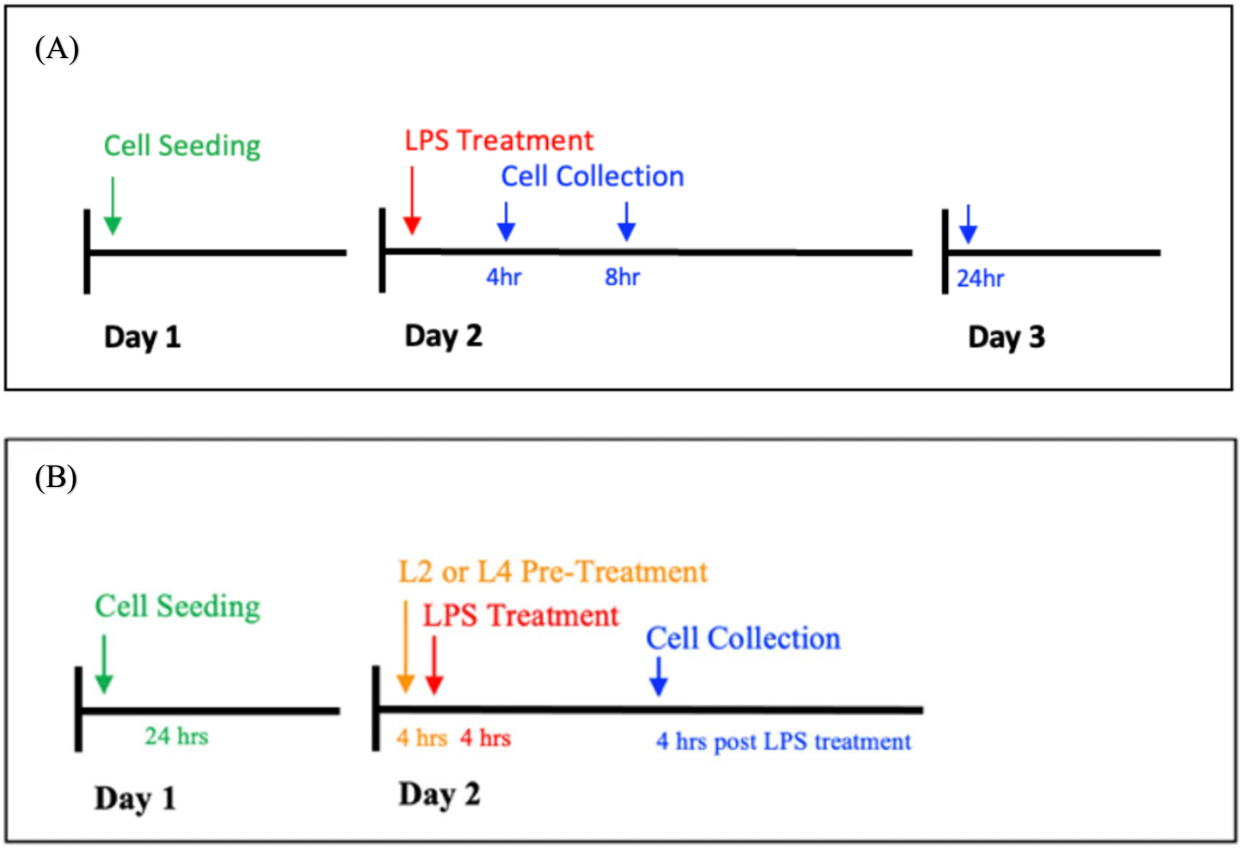

Figure 3. Timelines of experimental procedure. (A) 24 hours after seeding, BV2 microglial cells were treated with six different concentrations (5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, and 0µg/mL) for three different treatment times (4hr, 8hr, and 24hr). Following each treatment time, cell lysates and supernatant was collected. (B) BV2 Microglia were seeded and 24 hours later were pretreated with four concentrations (100µM, 10µM, 1µM, and 0.1µM) for 4 hours before LPS activation for 4 hours, where an LPS concentration of 0.005µg/mL was used.

Enzyme Linked Immunosorbent Assays (ELISA)

Enzyme Linked Immunosorbent Assays (ELISAs) were performed using the collected cell supernatants in order to determine the levels of TNF-α pro-inflammatory cytokines released

by BV2 cells. The BioLegend Mouse cytokine ELISAs – ELISA MAX Deluxe Set or ELISA MAX Standard Set (BioLegend, San Diego, CA) were used to perform this assay. For the LPS treatment samples, one plate was needed to run the samples neat. To prepare for the assay, one 96-well plate was used and coated overnight with 100µL of diluted capture antibody. Then, each plate was washed four times using a wash buffer of PBS and 0.05% Tween-20. The wells were then blocked using diluted Assay Diluent A and incubated for an hour at room temperature on a plate shaker (500 rpm with a 0.3cm circular orbit). The plates were washed four times again, followed by loading 100µL of sample or standard to the appropriate wells after which the plates were sealed and incubated for two hours with shaking. The plates were washed four times and then 100µL of detection antibody was added to each well before they were sealed and incubated for an hour with shaking. Next, the plates were treated with 100µL of Avidin HRP (secondary antibody) per well, the plates were sealed and incubated at room temperature for 30 minutes with shaking. The plates were washed five times, letting the washing buffer soak for one minute every wash. 100µL of TMB substrate solution was added to each well and the plates were incubated for 15 minutes in the dark. Finally, 100µL of stop solution (2N H_2SO_4) was added to each well. The plates were then read on the plate reader, using the protocol for BioLegend TNF-α and BMG plate at an absorbance of 450.

The same protocol was followed for the TNF-α ELISA run for the L2 and L4 pretreatment experiments. The cells were also run neat.

Bradford Assay & Western Blotting

A Bradford Assay was performed to measure the protein levels in each sample of cell lysates. Cell lysates were run neat for the Bradford assay. 96-well plates were used. 5µL of sample or standard were added to the appropriate well followed by 250µL of Bradford reagent

(Bio-Rad Laboratories; Hercules, CA). The plates were incubated in the dark for five minutes before being read on the plate reader. The Bradford Assay protocol was used which is 595nm absorbance with shaking for 15 seconds. Upon analysis, the protein concentrations were used to calculate the appropriate sample to buffer ratio for running Western Blots.

Western blotting was used to analyze Nrf2 protein levels in the samples. β-actin was used as a loading control. Samples were mixed with sample buffer and boiled for five minutes in the 100-degree Celsius dry bath before adding 20µL of sample or 20µL of Precision Plus Protein Dual Color ladder (Bio-Rad Laboratories; Hercules, CA) to the any kD gel and run in the SDS Page apparatus at 200 volts (held constant), 3 amps, 300 watts, for 40 minutes. Once the run was complete, the gel was prepared for transfer by soaking in Towbin for 15 minutes. During this time, the PVDF membrane (Bio-Rad Laboratories; Hercules, CA) was cut and washed with methanol, distilled water, and then washed in TBST. After setting up the appropriate filter paper, gel and membrane orientation, the transfer machine was run at 25 volts, 0.3 amps, and 300 watts for 1:05 minutes. After transfer was complete, the membrane was washed in TBST before ponceau staining and then cutting the membrane. The membrane was then put in a 5% BSA in TBST blocking buffer for two hours on the shaker. The primary antibody for Nrf2 was a rabbit polyclonal (Santa Cruz Biotechnology; Dallas, TX), 1:250 concentration and the primary for β-actin was a mouse monoclonal (Santa Cruz Biotechnology; Dallas, TX), 1:500 concentration. Both primaries were placed on the membrane overnight and placed in the fridge on a rocker. On day two, the primary antibody was removed and an hour's worth of TBST washes was completed before adding the secondary antibody. A goat anti-rabbit Nrf2 (Jackson Immuno Research Labs; West Grove, PA) at 1:25,000 concentration and goat anti-mouse β-actin (Jackson Immuno Research Labs; West Grove, PA) at 1:15,000 concentration were added and the

membrane was put on the shaker for two hours. Another hour's worth of TBST washes was done before the membrane was prepped for imaging.

RESULTS

LPS Induces Production of the Pro-inflammatory Cytokine TNF-α

In order to induce an inflammatory response in the BV2 cells, LPS was added to the cells at increasing concentrations for increased treatment durations. TNF-α production was measured using an ELISA and illustrated a dose-dependent relationship between LPS concentration and TNF-α production. A one-way ANOVA demonstrated that there was a significant main effect of LPS (4 hours, μg/ml) on TNF-alpha secretion in BV2 cells, $F(5, 43) = 8.347$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct the data. Also, post hoc tests revealed that all of the LPS concentrations were significantly different from one another, with the exception of the following concentrations: 0.05 & 0.5 μg/ml, 0.05 & 5 μg/ml, and 0.5 & 5 μg/ml, (ps ≥ 0.362).

Additionally, there was a significant main effect of LPS (8 hours, μg/ml) on TNF-alpha secretion in BV2 cells, $F(5, 45) = 19.893$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct the data. Post hoc analyses revealed that all of the LPS concentrations were significantly different from one another, with the exception of the following concentrations: 0.005 & 0.05 μg/ml and 0.5 & 5 μg/ml, (ps ≥ 0.750).

Furthermore, there was a significant main effect of LPS (24 hours, μg/ml) on TNF-alpha secretion in BV2 cells, $F(5, 42) = 25.311$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct this. Post hoc analyses revealed that all of the LPS concentrations were significantly different from one another, with the exception of the following concentrations: 0.005 & 0.05 μg/ml, 0.005 & 0.5 μg/ml, 0.05 & 0.5 μg/ml, and 0.05 & 5 μg/ml, ps $\geq$ 0.111. Increasing the concentrations of LPS relatively increased the production of TNF-α. Figure

4 shows the results from the ELISA. The 8-hour LPS treatment period generated a rapid elevation of TNF-α levels.

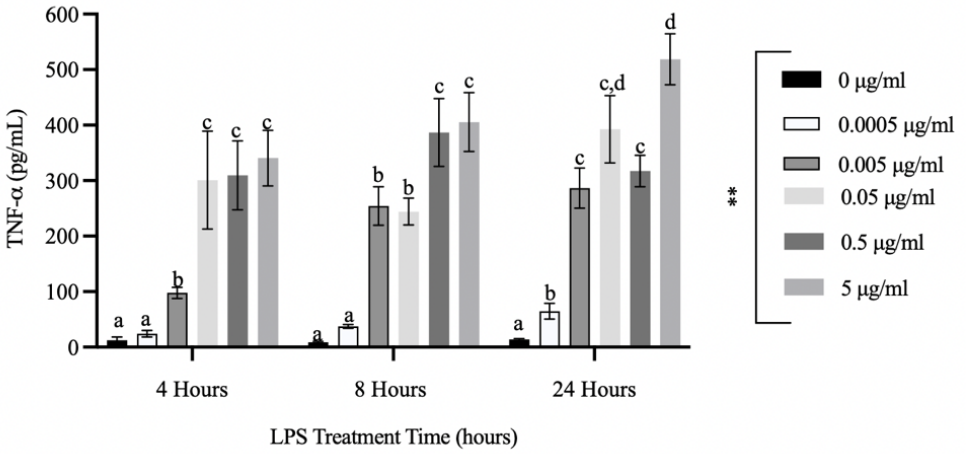

Figure 4. TNF-α ELISA results following LPS treatment. BV2 microglial cells were treated with six different LPS concentrations (5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, and 0µg/mL) at three different timepoints (4hr, 8hr, and 24hr). Results are shown in pg/mL. LPS treatment of 4 hours saw a significant elevation of TNF- α production.

Nrf2 Western Blots from LPS Treatment

Microglia can also release anti-inflammatory mediators like Nrf2. Nrf2 signaling protects against oxidative stress as a result of inflammation or injury by inhibiting the proinflammatory cytokines. Nrf2 Western Blotting was done to see Nrf2 production in the cells following L2 or L4 pre-treatment and 0.005µg/mL of LPS inducing an inflammatory response. The initial piloting stage results from these western blots show that there is the presence of Nrf2 in our samples, Figure 5. β-actin was the loading control, and the bands are present around 40kDa. Nrf2 bands were present around 60-68kDa. Densitometry was not able to be completed for these samples. These results were preliminary, and effective conclusions cannot be drawn from them until the controls and conditions have been investigated.

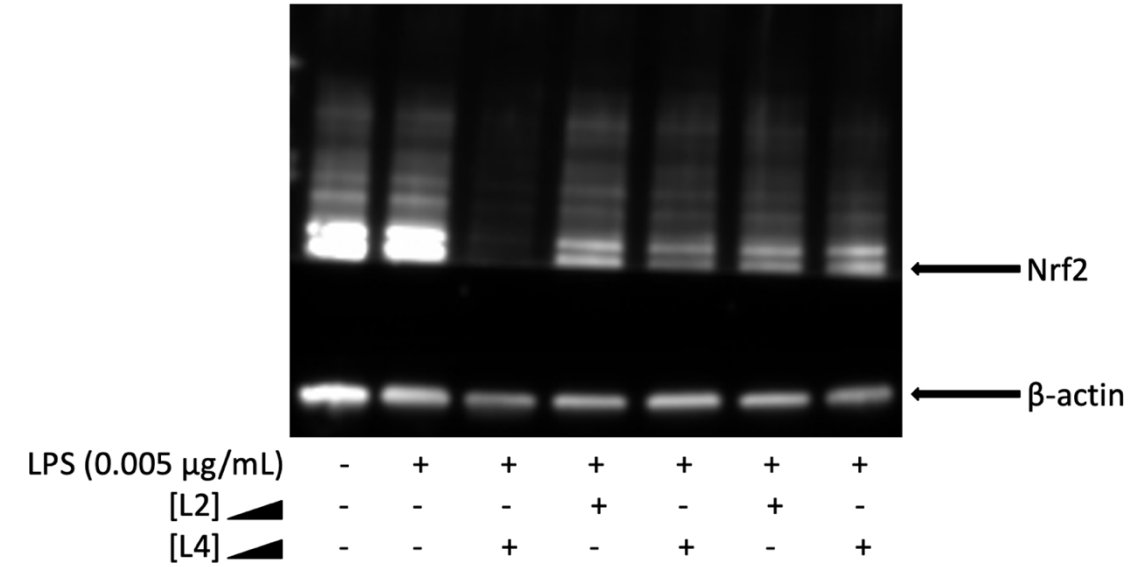

Figure 5. Nrf2 Western Blot. Nrf2 at 60-68kDa. β -actin used as a loading control at 40kDa.

L2 and L4's Capability at Blocking the Inflammatory Response

In order to assess the protective effects of L2 and L4, TNF-α was measured using an ELISA. Cells were pre-treated with L2 or L4 for 4 hours prior to LPS. Also, all wells were treated with 0.005 μg/ml of LPS for 4 hours, with the exception of the first control well. ANOVA revealed a significant main effect of drug treatment, such that L2 attenuated TNF-alpha (pg/mL) production more so than L4, $F(1, 27) = 4.656$, $p = 0.045$. Post hoc tests did not reveal any significant differences between drug treatment concentrations for L2 or L4. However, the difference between 0 μM (LPS only) and 100 μM of L2 pre-treatment was approaching significance, ($p = 0.105$). See Figure 6. Based on these findings, we treated cells with higher concentrations of L2 and L4 to explore greater anti-inflammatory potential in future experiments.

In the next experiment, we measured the anti-inflammatory potential of L2 with higher drug treatment concentrations. ANOVA revealed that the main effect of L2 pre-treatment was

approaching significance, $F(7, 21) = 2.784, p = 0.053$. Post hoc tests revealed significant differences in TNF-alpha (pg/mL) production between BV2 cells treated with 0 µM and 500 µM of L2 ($p = 0.026$), 0.1 µM and 500 µM of L2 ($p = 0.005$), and 1 µM and 500 µM of L2 ($p = 0.002$). See Figure 7.

Additionally, we examined the anti-inflammatory potential of L4 with higher drug concentrations, as shown in Figure 8. ANOVA did not reveal a significant main effect of L4 pre-treatment, $F(6,16) = 1.126, p = 0.419$. Post hoc tests did not reveal any significant differences between treatment conditions. However, post hoc tests did reveal that the difference between 1 µM and 500 µM of L4 treatment was approaching significance ($p = 0.08$). Collectively, this data suggests that L2 is more potent and has greater anti-inflammatory potential in comparison to L4. See Figure 8.

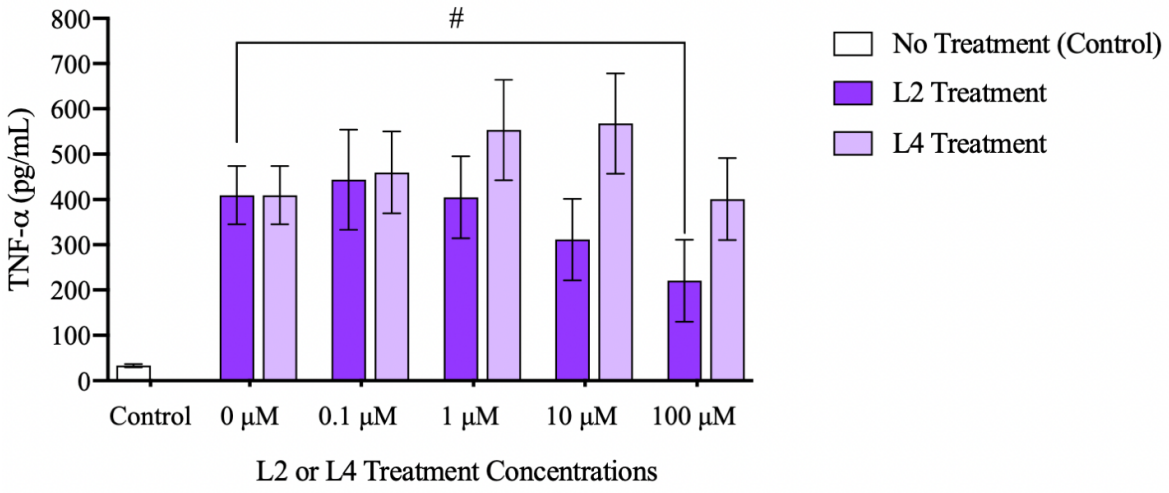

Figure 6. TNF-α ELISA results following L2 or L4 pre-treatment. BV2 microglial cells were pre-treated with five concentrations of L2 or L4 (100µM, 10µM, 1µM, 0.1µM, and 0µM) for 4 hours prior to LPS activation for 4 hours. All wells were treated with 0.005 µg/ml of LPS for 4 hours, with the exception of the first control well. ANOVA revealed a significant main effect of drug treatment, such that L2 attenuated TNF-alpha (pg/mL) production more so than L4, ($p \leq 0.045$). Bars represent mean ± SEM. N's = 2– 6.

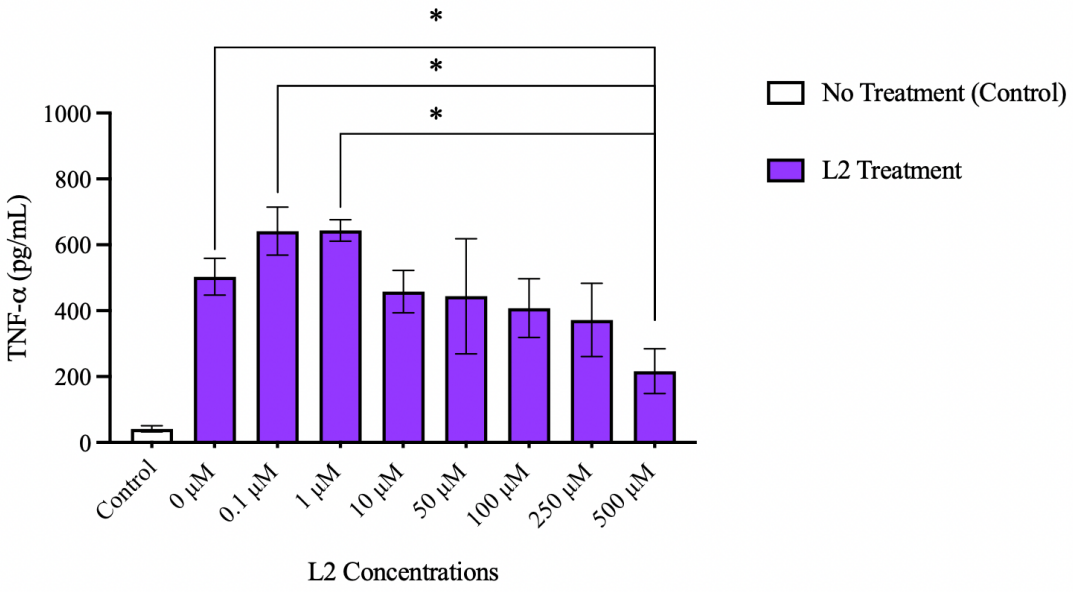

Figure 7. TNF-*α* ELISA results following L2 pre-treatment. All wells were treated with 0.005 µg/ml of LPS for 4 hours, with the exception of the first control well. BV2 microglial cells were pretreated with five concentrations of L2 (100µM, 10µM, 1µM, 0.1µM, and 0µM) for 4 hours before LPS activation for 4 hours.

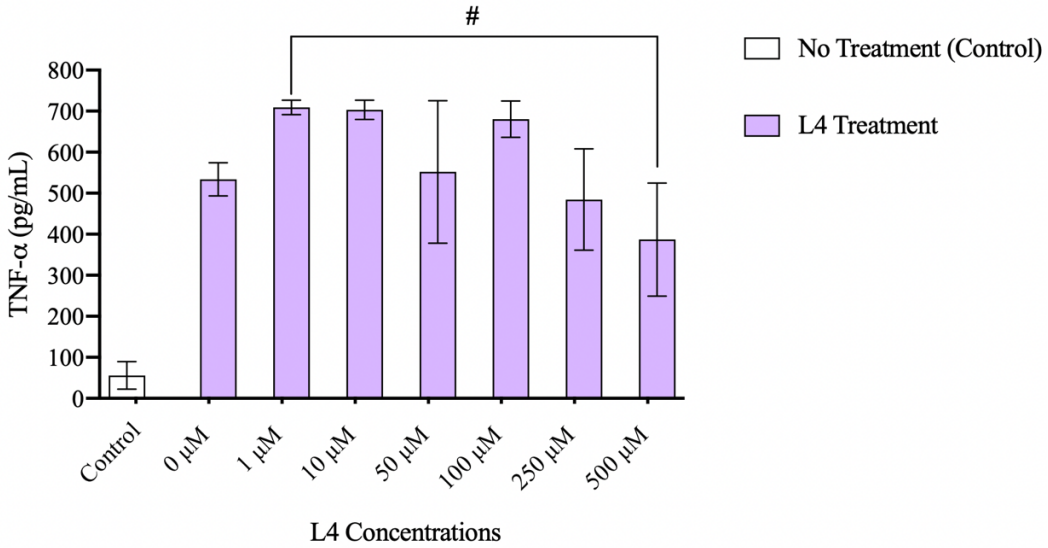

Figure 8. TNF-*α* ELISA results following L4 pre-treatment. All wells were treated with 0.005 µg/ml of LPS for 4 hours, with the exception of the first control well. BV2 microglial cells were pretreated with five concentrations of L4 (100µM, 10µM, 1µM, 0.1µM, and 0µM) for 4 hours before LPS activation for 4 hours. ANOVA did not reveal a significant main effect of L4 treatment. Post hoc tests did not reveal any significant differences between treatment conditions. However, post hoc tests did reveal that the difference between 1 µM and 500 µM of L4 treatment was approaching significance ($p = 0.08$). Bars represent mean ± SEM. Ns = 2 – 3.

DISCUSSION

As previously discussed, two key hallmark pathologies of AD are oxidative stress and inflammation. Oxidative stress induces an inflammatory response by activating microglial cells. Activated microglia produce pro-inflammatory cytokines, pro-inflammatory mediators, NO, and iNOS (Lee & Kang, 2014; Agostinho et al., 2010). Inflammation can also generate oxidative stress by activating NADPH oxidase, which generates ROS (Fischer & Maier, 2015). Furthermore, ROS and inflammation are both implicated in AD pathology, and lead to the amyloidogenic cleavage of APP, which generates Aβ plaques, and in turn increases inflammation and oxidative stress (Finder & Glockshuber, 2007). This is a cyclic process that is detrimental to the brain and leads to a diseased state. As there is no cure for AD, it is imperative that scientists further investigate therapies that could reduce inflammation and oxidative stress in the brain. Therefore, the aim of the current study was to assess the potential, therapeutic capacity of antioxidants against inflammation in microglial cells.

In previous studies, Dr. Green's lab has shown that L2 and L4 molecules have antioxidant properties that are effective in altering the Nrf2 pathway in HT-22 neuronal cells (Johnston et al., 2019). This study sought out to show that L2 and L4 attenuated oxidative stress in neurons, and protected microglia from hydrogen peroxide induced cell death (Johnston et al., 2019). In the current experiment, we first demonstrated that BV2 cells, stimulated with various concentrations of LPS, respond to the inflammatory trigger by producing pro-inflammatory cytokines. ELISAs were used to measure the production of the pro-inflammatory cytokine TNF-α, and the results showed that increasing concentrations of LPS relatively increased expression of TNF-α. This supports other studies that have also demonstrated that LPS induces an inflammatory response in BV2 microglial cells. Next, we pre-treated BV2 microglial cells with

four different concentrations of L2 or L4, and found that while there was a significant main effect of drug treatment in decreasing the production of TNF-α, there were no significant differences between treatment concentrations. Subsequently, we pre-treated microglia with three additional concentrations of L2, and there was a significant decrease in cytokine production, such that an L2 dose of 500μM led to significantly less TNF-α. However, based on collaborator data, this dose is likely killing cells rather than reducing levels of inflammation. Further studies will need to be conducted to measure cell survival following high doses of L2 treatment. Finally, after pre-treating with additional L4 concentrations, there were no significant differences between treatment conditions. Collectively, this data suggests that L2 has greater therapeutic potential against LPS-induced inflammation in comparison to L4.

In terms of future directions for this project, it would be beneficial to explore additional concentrations and treatment times for both L2 and L4. We aim to study the effects of an overnight pre-treatment that could potentially be more efficacious at reducing inflammation in microglia. Additionally, we could first treat cells with LPS, and then treat with L2 or L4 to see if these drugs could potentially rescue cells from LPS-induced inflammation. In these future studies, we aim to look at pro-inflammatory markers such as, TNF-α, IL-1β, and IL-6, as well as anti-inflammatory markers, like IL-10, Nrf2, or HO-1. These experiments and corresponding results will potentially reveal the therapeutic effects of L2 and L4 against LPS-induced inflammation. If proven to be successful, L2 and L4 could potentially be utilized as therapeutic agents for AD and other neurodegenerative diseases that induce oxidative stress and inflammation in the brain. This research is crucial, as AD is currently the sixth leading cause of death in America, and researchers need to find more cost-effective therapies that could hopefully reduce AD prevalence in America.

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