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LPS, A TLR-4 AGONIST AND VIPER A TLR-4 INHIBITOR UPREGULATE PHAGOCYTOSIS OF ZYMOSAN IN BV2 CELLS

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LPS, A TLR-4 AGONIST AND VIPER A TLR-4 INHIBITOR UPREGULATE PHAGOCYTOSIS OF ZYMOSAN IN BV2 CELLS

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Abstract

Microglia cells are the first line of innate immunity defense in the central nervous system (CNS). They play a critical role in maintaining CNS homeostasis by having an active but yet balanced phagocytic activity. However, in various CNS related diseases, microglia cells have been shown to malfunction. In Alzheimer's Disease (AD), hyperactive microglia with impaired phagocytic activity is the main hallmark of this disease, along with the accumulation of amyloid-beta aggregates. Additionally, emerging new studies have suggested a fungal infection etiology to AD, specifically in relation to *Candida albicans (C.albicans)*. Thus, understanding the mechanism of fungal clearance in the CNS is of importance. The main pathogen-associated molecular pattern (PAMP) for *Candida* is its fungal cell wall, which consists of structural carbohydrates β-1,3 glucan and O-mannan, recognized by pattern recognition receptors (PRR) β-glucan receptors found on the surface of microglia. In this study, we showed that the pro-inflammatory PAMP Lipopolysaccharide (LPS) causes an upregulation in zymosan phagocytosis, a fungal cell wall extract from Saccharomyces *cerevisiae* in microglia like BV2 cells. Furthermore, we examined the possible involvement of a β-glucan receptor on zymosan phagocytosis using laminarin, a soluble β-glucan molecule. In addition, we investigated the potential impact of TLR-4' The LPS receptor' on zymosan phagocytosis by treating BV2 cells with a TLR-4 viral inhibitor (VIPER) or a TLR-4 polyclonal antibody showing their antagonistic effects on phagocytosis. Finally, we demonstrated that VIPER has the unique ability to upregulate zymosan phagocytosis while blocking TLR-4 activity in microglia.

Introduction

Alzheimer's disease (AD), the leading neurodegenerative disorder for senile dementia worldwide, is characterized by the accumulation of Aβ plaques, chronic neuroinflammation, and microglial dysfunction (Cornejo & von Bernhardi et al., 2013). Microglial cells are specialized phagocytic cells secluded in the CNS (Kettenmann et al., 2011). Under normal circumstances, microglia promote neuronal homeostasis by surveilling brain tissue, adapting to physiological changes in the CNS, and initiating an innate immune defense, including clearing Aβ aggregates (Hong et al., 2016). However, in AD patients, microglial cells are over-activated, lose homeostatic functions, and exhibit phagocytic impairment of Aβ peptide (Zhang et al., 2014). Upon microglial exposure to LPS or Interferon-ϒ (IFN-ϒ), a neuronal injury response initiates by the secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), and the release of cytotoxic products such as nitric oxide (NO) and reactive oxygen species (ROS) (Henkel J et al., 2009). As a result, these cytotoxic inflammatory markers stimulate Aβ production and neuronal dysfunction (Kitazawa et al., 2005).

Since the accumulation of Aβ aggregates is a hallmark of AD, scientists have geared their research towards finding ways to promote Aβ clearance as a therapeutic approach to AD. However, despite extensive efforts to reduce Aβ to improve AD prognosis, clinical trials have shown that eliminating Aβ causes AD aggravation (Lowe D et al., 2010; Mullane K et al., 2013). These findings have led scientists to rethink the role of Aβ aggregates not as the cause of AD, but in fact, more as an effect of AD (Castellani RJ et al., 2008). Recent research has shown that amyloid depositions play a role in initiating an innate immune response against fungal colonization. For

example, Aβ was shown to inhibit the growth of *C.albicans* and other fungal/bacterial species invading the brain (Kagan BL et al., 2012; Kumar DK et al., 2016). Interestingly, post-mortem brain tissue analysis from AD patients found various types of fungi embedded in these patients' tissues, but not in samples from healthy brains. This compelling correlation between AD and fungal infections further supports the idea that fungi infections may contribute to the etiology of AD (Alonso et al., 2018; Pisa et al., 2015). In fact, 89.6% of AD patients have tested positive for *Candida* antibodies in their serum compared to 8.8% for controls (Alonso et al., 2014). These findings have led researchers to investigate a possible connection between microbial infections and accumulation of Aβ and suggest a possible infectious fungal etiology to AD.

Considering that *C. albicans* is the most prevalent fungal species found in AD patients' brain tissue while being the most abundant commensal fungi in healthy humans, it is crucial to address how *C. albicans* becomes an invasive species causing human pathology. *C. albicans* can change from a commensal fungus to a pathological fungus when the host either has an impaired immune system or an overwhelmed normal immune system (Calderone RA et al., 2001). Once macrophages internalize *C. albican*s in phagosomes, it alters its morphology from yeast to a hyphal state, preventing phagosome maturation and thus evading degradation (Tomalka J et al., 2011). Additionally, the yeast to hyphal transition induces pyroptosis in macrophages, allowing *C. albicans* to escape and thus promoting an infectious phase (Wellington M et al., 2014). Previous work using Intravital microscopy (IVM) has shown that *C. albicans* circulating in the bloodstream can cross the BBB by the binding of Als3 (Fungal invasin) to gp96 heat shock protein receptor, which is uniquely expressed on brain endothelium (Phan QT et al., 2007). Once Als3 binds to gp96, *C. albicans* moves across the brain

endothelium via transcytosis, crossing the BBB (Phan QT et al., 2007). Overall, *C.albicans'* virulence potential, accompanied by its ability to cross the BBB and possible linkage to AD etiology, raises interest in understanding fungal immunity in the CNS.

Microglia cells detect fungal pathogens through PRRs that recognize PAMPs (Lowman et al., 2014). PRRs usually detect PAMPs of the fungal cell wall, including the structural carbohydrates β-1,3 glucan and O-mannan (Davis et al., 2014). There are two main types of PRRs found on innate immune cells, Toll-like (TLR) and C-type lectin (CLR), and both play an essential role in recognizing fungal PAMPs (Brown et al., 2002). In fact, TLRs and CLRs can collaborate to orchestrate an immune response against fungal pathogens such as *C. albicans* (Brown et al., 2002). Specifically, TLR-2, TLR-4, and Dendritic cell-associated C-type lectin receptor-1 (Dectin-1), a β-glucan receptor, were shown to be involved in fungal recognition and immune response in both macrophages and microglia (Brown et al., 2002; Netea et al., 2002; Shah et al., 2008).

Dectin-1 specifically and selectively recognizes β-glucans (Brown & Gordon et al., 2001), including the β-glucans in zymosan, a cell-wall extract from *Saccharomyces cerevisiae* rich in β-glucan, mannan, and chitin (Di Carlo & Fiore et al., 1958). In corneal macrophages, fungal killing is dependent not only on the expression of Dectin-1 but also TLR-4 (Leal et al., 2010), the latter which is widely known for its activation by LPS (Bellocchio et al., 2004; Park & Lee et al., 2013). Additionally, experimental pre-treatment of mice with LPS has correlated with enhanced clearance of fungal pathogens and increased phagocytosis of zymosan by macrophages (Fuentes et al., 2011, Rayhane et al., 2000; Ulevitch & Tobias et al., 1999). Along the same line, pre-treatment of monocytes with LPS was reported to be correlated with an enhanced immune response to

C. albicans due to increased β-glucan receptor expression (Rogers et al., 2013). Despite advances made in identifying the involvement of LPS-activated TLR-4 and β-glucan receptors in fungal clearance in macrophages, the mechanisms behind the interaction of these two receptors in modulating phagocytosis of fungi in LPS-treated microglia remain to be elucidated. Thus, based on previous findings, we hypothesize that stimulating the TLR-4 receptor with LPS in BV2 microglia will enhance phagocytosis of zymosan, which is potentially mediated by a β-glucan receptor.

To test our hypothesis, we first assessed the effect of LPS on the phagocytosis of zymosan by BV2 microglia. We also evaluated the involvement of β-glucan receptor and the TLR-4 receptor on the phagocytosis of zymosan in the absence and presence of LPS. Laminarin, a soluble β-glucan, was used to block the β-glucan receptors before the addition of zymosan, and phagocytosis was then quantified. On the other hand, to study the effect of TLR-4 on phagocytosis of zymosan, an 11 amino acid viral inhibitor peptide derived from Vaccinia protein (VIPER) was used known to block TLR-4's signal transduction pathways. Previously, studies have shown that upon TLR-4 stimulation by LPS, there is an activation of two main pathways, myeloid differentiation primary gene 88 (MyD88)-dependent, and a MyD88-independent pathway (Lu et al., 2008). The MyD88-independent pathway involves the activation of both TIR domain-containing adaptor inducing interferonβ (TRIF) and TRIF-related adaptor molecule (TRAM), ultimately stimulating Type I interferon genes (Kawai et al., 2001). On the other hand, the MyD88-dependent pathway involves the activation of the TIR domain-containing adaptor protein (TIRAP) and the binding of MyD88 adaptor-like (Mal), leading to the production of pro-inflammatory cytokines such as TNF-α (Premkumar et al., 2010). VIPER has been shown to block TLR-4's signal transduction pathways

internally by binding to its intracellular domains TRAM and Mal, inhibiting these pro-inflammatory pathways (Lysakova-Devine, 2019). Thus, VIPER is used in this study to explore the role of the TLR-4 receptor on zymosan phagocytosis.

Materials and methods

BV-2 Cell culture and Reagents

BV-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Corning, Massachusetts), containing 2 mM glutamine and 10% heat-inactivated fetal bovine serum and 100U/mL penicillin, 100µg/mL streptomycin, at 37 °C and 5% CO2. LPS from *Escherichia coli* serotype 055:B5 and Zymosan particles, prepared from *Saccharomyces cerevisiae*, were purchased from Sigma (St.Louis, MO). TLR-4 inhibitor peptide (VIPER) obtained from Novus Biologicals (Centennial, CO). Anti TLR-4 Rabbit polyclonal antibody obtained from Invitrogen (Carlsbad, CA) and Laminarin from Laminaria digitata purchased from Alfa Aesar (Tewksbury, MA).

Phagocytosis Assay

For phagocytosis assays, 2 X 10^4 BV-2 cells/well were seeded onto sterile 8 well chambered Millicell EZ glass slide (Millipore Corporation, Bedford, MA) and allowed to adhere in a humidified 5% CO_2 incubator for 2hr at 37^0C. For studies on the effects of VIPER and TLR-4 on phagocytosis, cells were incubated with/without VIPER (30µM) or TLR-4 Rabbit polyclonal antibody (5µg/mL) for 2 hr, followed by a 16 hr incubation in the presence or absence of LPS (100ng/mL). Zymosan particles were then added at a ratio of 10:1, and cells were allowed to

phagocytize for 1hr at 37^0C in a humidified 5% CO2 incubator. For studies on the effects of laminarin on zymosan phagocytosis, cells were treated with laminarin (200µg/mL) and incubated for 15 min before the zymosan addition. Phagocytosis was terminated by gently washing off non-ingested particles once with DMEM, then twice with warm 1X PBS. Glass slides were air-dried and stained with Wright's stain and examined by light microscopy at X400 and X400 magnification using a Nikon Eclipse 50i photomicroscope. Phagocytosis was determined from photomicrographs at X400 magnification by counting at least 200 BV-2 cells/treatment. Percent phagocytosis was assessed as the number of cells phagocytizing divided by the number of cells non-phagocytizing multiplied by 100%. Photomicrographs of stained cells were captured at X400 magnification using a Nikon Eclipse 50i photomicroscope.

TNF-α and NO Quantification

For TNF and NO quantification, 1X 10^5 BV-2 cells/well were seeded in 200µL of DMEM onto a sterile 12-well chambered Corning Costar Flat Bottom Cell Culture Plate (Corning, NY), and cells allowed to adhere in a humidified 5% CO_2 incubator for 2hr at 37^0C. Cells were then incubated with/without VIPER (30µM) for 2 hr, then stimulated with/without LPS (100ng/mL) for 16 hr. The supernatant was collected and stored at -20^0C for future testing. *TNF-α* was quantified by using Invitrogen TNF alpha Mouse Instant ELISA Kit (Carlsbad, CA) following the manufacturer's instructions. NO levels were measured using the Promega Griess Reagent System (Madison, WI) following the manufacturer's instructions.

Statistical Analysis

Data were obtained from two to three separate experiments in duplicates for each experimental condition. Data presented as mean ± standard deviation, and their statistical significance was analyzed using single-factor ANOVA.

Results

A β -glucan receptor mediates phagocytosis of zymosan in both control and LPS pre-treated BV-2 cells

Previously, we determined that pre-treatment of BV2 cells with LPS significantly increased phagocytosis of zymosan reflected by a significant increase in both the number of cells phagocytizing (percent phagocytosis) as well as the mean number of particles (MNP) phagocytized per cell (SFigure 1, STable 1). Given that the main PAMP in zymosan is β-glucans, we hypothesized that the increased phagocytosis of zymosan we observed after treatment of BV2 cells with LPS was due to the upregulation of a β-glucan receptor in these cells. To test our hypothesis, we used laminarin, a soluble β-glucan, as a competitive inhibitor to interfere with zymosan binding (Brown & Gordon 2001). Control cells were grown in media alone, and LPS-treated cells were incubated with laminarin for 15 min prior to the addition of zymosan; cells were then allowed to phagocytize for 1 hr. The addition of laminarin as a competitive inhibitor significantly decreased percent phagocytosis of zymosan in cells grown in media alone from 29.21% to 9.69% and also significantly abrogated the effects seen in LPS-treated cells, reducing the percent phagocytosis from 79.04% down to 32.32% (Figure 1A & 1B). Furthermore, the MNP phagocytized by LPS-treated cells were significantly decreased by the addition of laminarin (LPS 8.33 ±0.91 MNP; LPS+ laminarin MNP 4.61±0.78).

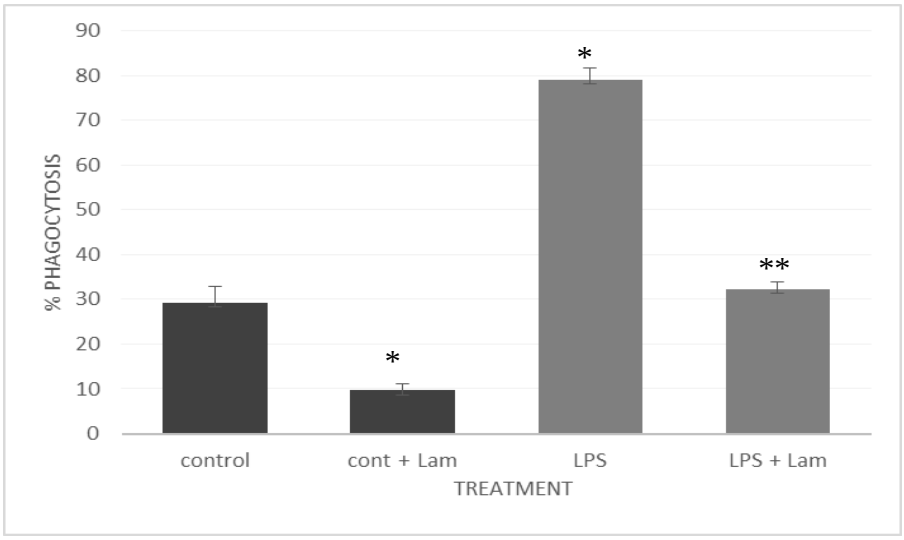


Figure 1A. Laminarin significantly inhibits zymosan phagocytosis in untreated cells grown in medium alone and abrogates LPS-induced upregulation of Zymosan phagocytosis in BV2 cells.

BV-2 cells were either cultured in medium alone or pre-treated with LPS (100ng/mL) and incubated for 16 h; laminarin (200µg/mL) or medium alone were then added for 15 min before the addition of zymosan. Cells were allowed to phagocytose for 1 h, then washed and stained. Phagocytosis was assessed by light microscopy by counting both the number of cells phagocytizing as well as the number of particles phagocytized by each cell. Results are representative of values of means of five independent experiments performed in duplicates. (Compared with control *, P<.0001, compared with LPS **, P<.0001)

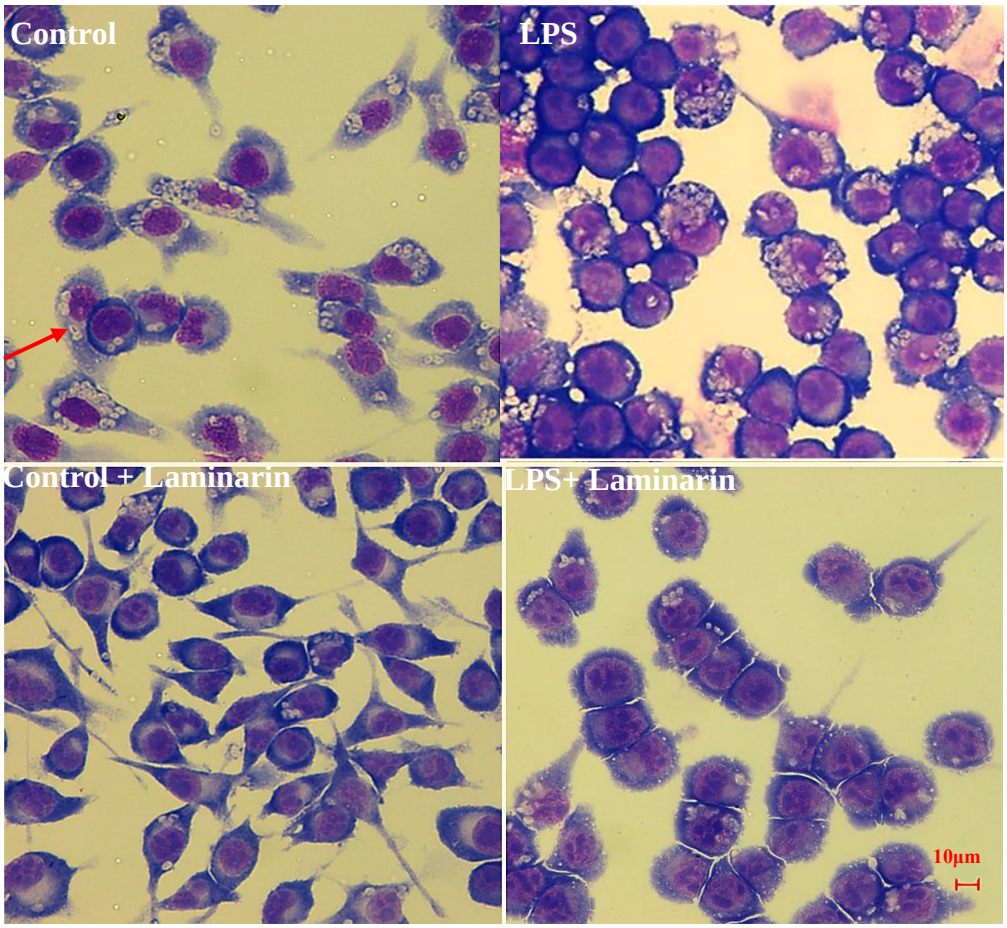


Figure 1B. Comparison of the effects of LPS and laminarin on zymosan phagocytosis. BV-2 cells were pre-treated with LPS (100ng/mL) or medium alone and incubated for 16 h, prior to the addition of laminarin (200µg/mL) for 15 min. Zymosan was then added to all wells at a ratio of 10:1 (zymosan/cell), and cells were allowed to phagocytize for 1 h. Cells were stained with Wright's stain, and photomicrographs were taken at 400X by light microscopy. All the micrographs in this figure were taken at the same magnification (see scale bar in LPS+ laminarin). The arrow in the untreated control shows an intracellular zymosan particle within a phagosome).

TLR-4 Inhibitor peptide (VIPER) significantly increases the uptake of zymosan in BV-2 cells

TLR-4 has been widely reported as the primary receptor recognized and activated by LPS. Therefore, we sought to investigate its possible role in the increase of zymosan phagocytosis observed in BV2 cells pre-treated with LPS. To examine the effect of blocking TLR-4 activity, we used a TLR-4 peptide inhibitor, VIPER, which has been reported to specifically inhibit the Mal and TRAM intracellular intermediates essential for TLR-4 signal transduction activation upon LPS

stimulation (Lysakova-Devine, 2019). BV2 cells were either pre-treated with TLR-4 inhibitor (VIPER) or with media alone and incubated for 2 h; each of these treatments was followed by either the addition of LPS or media alone, cells were then incubated for 16 h. Surprisingly, phagocytosis of zymosan in cells treated with VIPER alone was significantly higher (82.89%) than phagocytosis in control cells (41.18%) and cells treated with LPS (64.11%) (Fig 2). Furthermore, zymosan phagocytosis in BV-2 cells pre-treated with VIPER followed by LPS increased to 82.82% compared to cells pre-treated with LPS and no VIPER (64.11%) (Figure 2), further confirming that VIPER upregulates zymosan phagocytosis in BV2 cells.

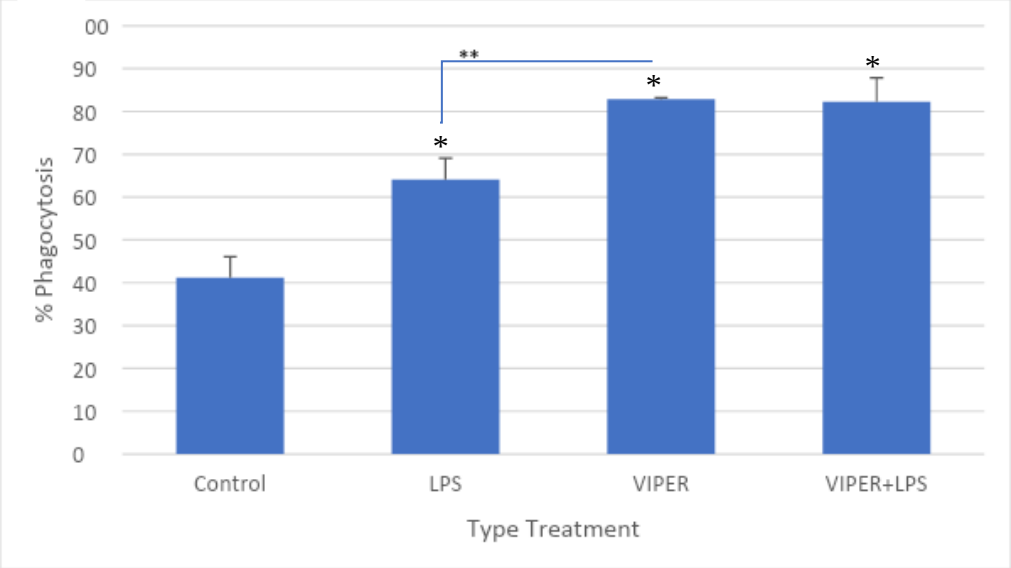


Figure 2. TLR-4 inhibitor (VIPER) enhances phagocytosis of Zymosan in BV-2 cells

BV-2 cells were pre-treated with TLR-4 inhibitor peptide (VIPER at 30μM) or media alone for 2hrs prior to their culture in media or media with LPS (100ng/mL). Zymosan was added at a ratio of 10:1, and cells were left to phagocytize for 1 hr. Results represent the percent of cells phagocytizing zymosan and represent values of means of three separate experiments performed in duplicates. (Compared with control *, $P<0.001$, compared with LPS **, $P<0.001$) N= 3

VIPER inhibits production of TNF-α and NO in BV-2 Cells pre-treated with LPS

Considering the increase in phagocytosis we observed in BV2 cells incubated with VIPER, it was essential to confirm the inhibitory effect of this peptide on intracellular TLR-4 adaptor proteins in BV2 cells. Therefore, we measured TNF-α secretion by BV-2 cells treated with VIPER and compared it to TNF-α levels in cells incubated with LPS alone. We found a significant increase in TNF-α in LPS-treated cells, compared to untreated control and VIPER-alone treated cells (Figure 3A). However, when cells were treated with VIPER for 2hr, followed by LPS treatment for 16hr, the amount of TNFα released by the cells was significantly reduced when compared to cells treated with LPS alone (Figure 3A), confirming that VIPER successfully inhibits TLR-4's activation by LPS in BV2 cells. We also quantified NO production, another inflammatory marker, and found that cells treated with VIPER prior to treatment with LPS had significantly lower NO levels compared to LPS-treated cells (Figure 3B).

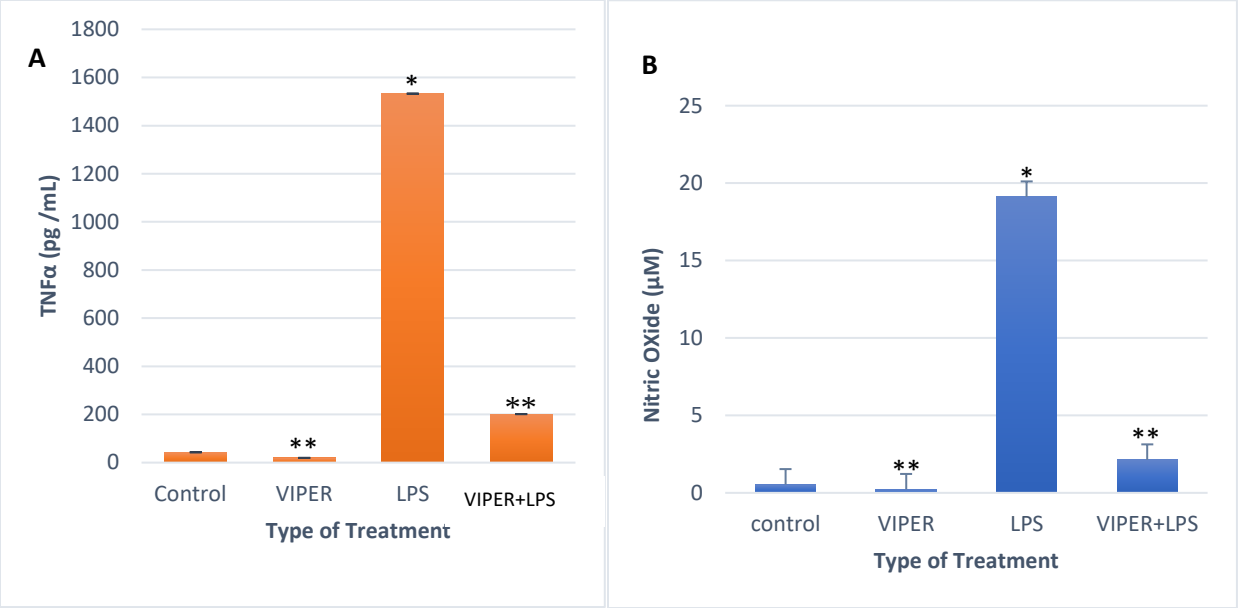


Figure 3. VIPER significantly diminished the production of TNF-α and NO in the BV-2 cell model.

BV-2 cells were pre- treated with/without VIPER (30µM/mL) for 2hr. Cells were then treated with/without LPS (100ng/mL) for 16 h. The supernatants were collected, and the levels of TNF-α and NO were determined using an ELISA Kit and Griess Reagent, respectively. (Compared with control *, $P<0.01$, ** compared with LPS, $P<0.001$) N=3.

TLR-4 polyclonal antibody significantly decreases uptake of zymosan in LPS pre-treated BV-2 cells

Initially, we hypothesized that VIPER, a TLR-4 inhibitor, would downregulate the effect of LPS on zymosan phagocytosis. Instead, VIPER enhanced phagocytosis in LPS-treated cells, and more unexpectedly, treating BV-2 cells with VIPER alone enhanced phagocytosis. These results made VIPER an inadequate tool to determine the role of TLR-4 in the upregulation of phagocytosis by LPS. Thus, we decided to use an anti-TLR4 polyclonal antibody to block TLR-4's extracellular domain, in contrast with the intracellular blocking of TLR-4 described for VIPER, to examine whether this extracellular blocking would abrogate LPS's effect on phagocytosis. Cells were pre-treated with TLR-4 polyclonal antibody or media alone for 2hr before LPS incubation for 16hr. Treatment with TLR-4 antibody alone did not have an impact on the uptake of zymosan compared

to the untreated controls (Figure 4). On the other hand, pre-treatment of cells with TLR-4 antibody followed by LPS significantly decreased the percent phagocytosis of zymosan, to 48.82%, compared to phagocytosis in cells treated with LPS alone 64.11% (Figure 4). Thus, inhibiting the TLR-4 receptor extracellularly with the polyclonal antibody reduces the effect of LPS on phagocytosis but has no effect on phagocytosis of zymosan in untreated cells.

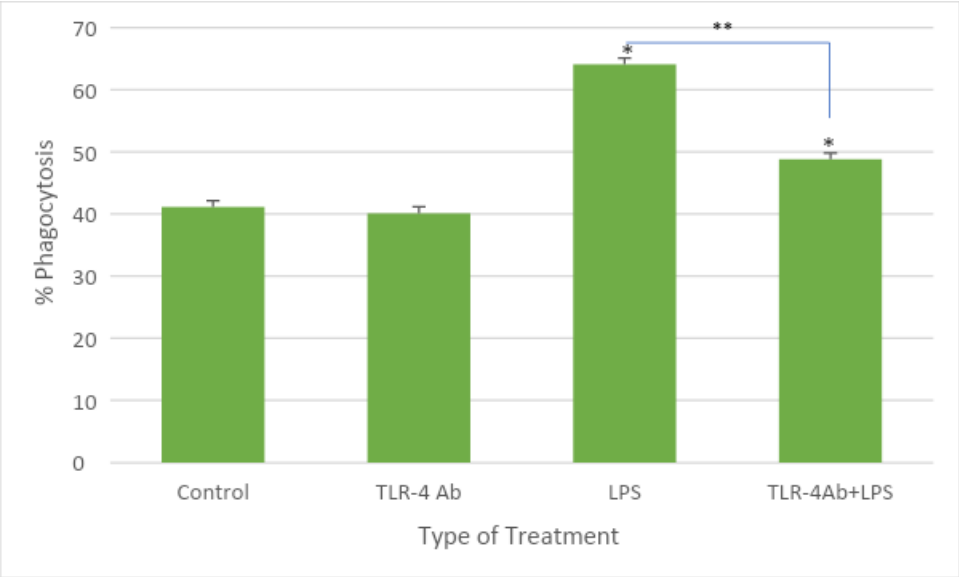


Figure 4. Pre-treatment with TLR-4 polyclonal antibody prior to LPS exposure significantly suppressed LPS-induced increase of phagocytosis of zymosan in BV-2 cells.

BV-2 cells were cultured for two hours in media alone or media with TLR-4 polyclonal antibody(5µg/mL). Cells were then cultured overnight in media alone or with LPS (100ng/mL). Zymosan was added at a ratio of 10:1, and cells were allowed to phagocytize for 1 h. Results represent the percent of cells phagocytizing zymosan and are representative of values of means of three separate experiments performed in duplicates. (Compared with control *, $P<0.001$, ** compared with LPS, $P<0.001$) N=3

VIPER enhancement of zymosan phagocytosis in BV-2 cells is partially dependent on upregulation of the activity of a β -glucan receptor

Our unexpected finding that VIPER alone upregulates phagocytosis of zymosan in BV-2 cells lead us to ask the question of whether this effect is associated with an increase in β-glucan receptor activity, consistent with what we had found earlier with LPS. To investigate this possible correlation between VIPER and the upregulation of β-glucan receptor activity, we used the soluble β-glucan, laminarin, as a competitive inhibitor. The addition of laminarin significantly reduced the upregulation of zymosan phagocytosis by BV-2 cells following VIPER treatment (Figure 5). This result suggests that β-glucan receptor activity is partially (and significantly) responsible for the increased phagocytosis of zymosan stimulated by VIPER (Figure 5), similar to the results reported earlier, where laminarin significantly decreased in the percent phagocytosis of zymosan in LPS-treated BV-2 cells (Figure 1). Laminarin also significantly decreased phagocytosis of zymosan when cells were treated with VIPER, followed by LPS (Figure 5).

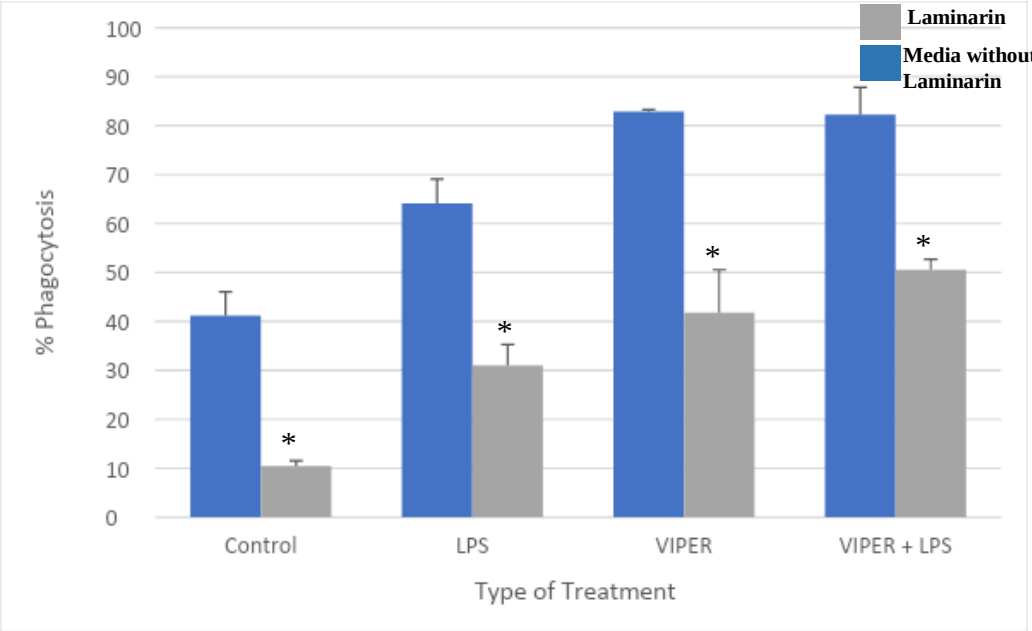


Figure 5. VIPER's amplification on phagocytosis of zymosan is attenuated when the β-glucan receptor is blocked in the BV-2 cell model

BV-2 cells were pre-treated with/without TLR-4 peptide inhibitor (VIPER at 30µM). Cells were then treated with LPS (100ng/mL) or media alone for 16 h, followed by incubation with laminarin (200µg/mL), or media alone, for 15 min. Zymosan was added at a ratio of 10:1, and cells were allowed to phagocytize for 1 h. Results represent the percent of cells phagocytizing zymosan and are representative of values of means of three separate experiments performed in duplicates. (Compared with the same treatment without laminarin **, P<.001)

The effect of LPS and laminarin treatment on phagocytosis

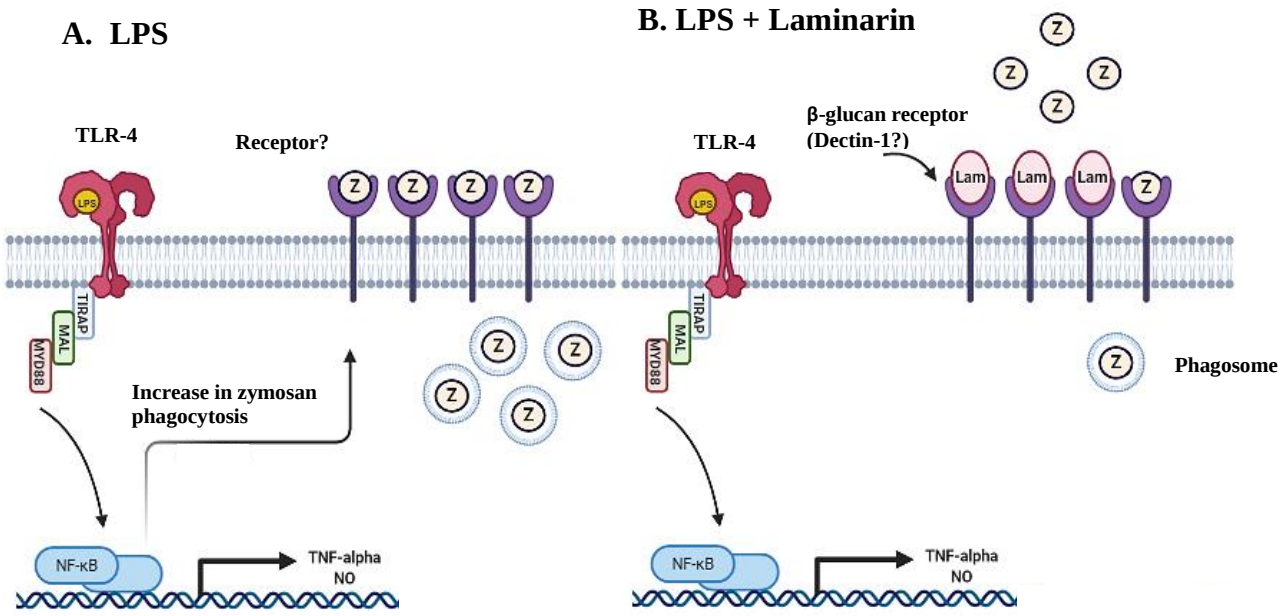


Figure 6. Schematic representation of the effect of LPS and laminarin on phagocytosis. (A) Treating BV2 cells with LPS lead to a significance increase in zymosan (Z) phagocytosis while promoting a pro-inflammatory state via NF-κB activation. (B) LPS treatment followed by the addition of Laminarin (Lam) attenuated zymosan phagocytosis by competitively binding to a β-glucan receptor.

VIPER's potential mechanism on the upregulation of phagocytosis

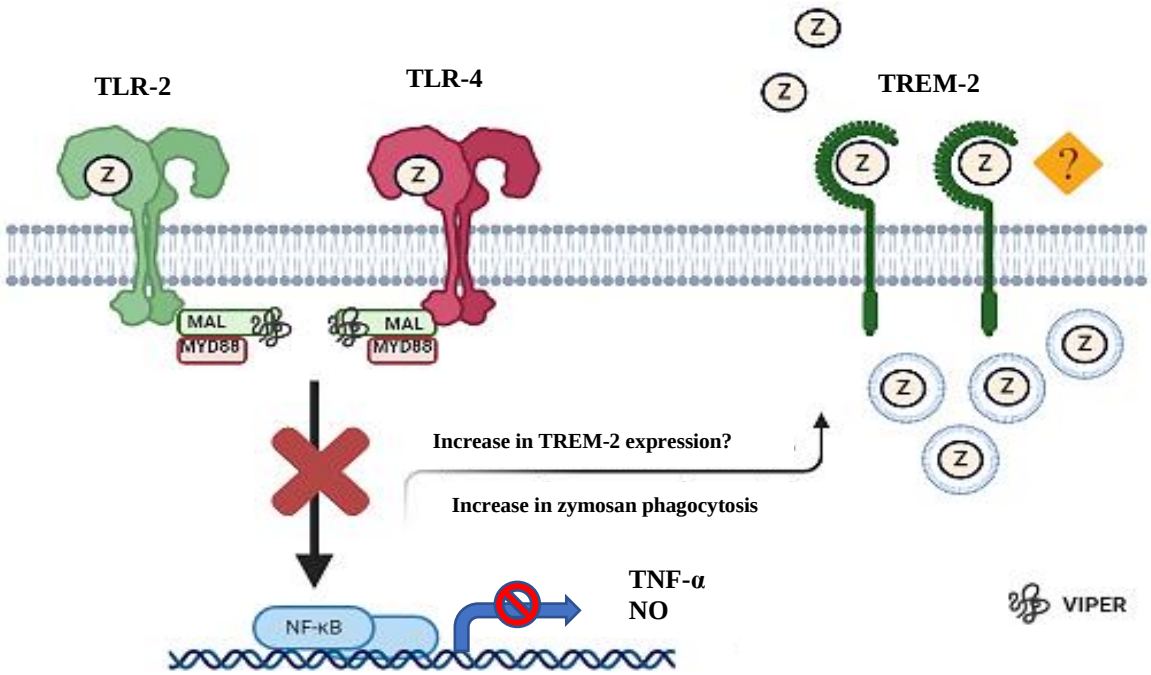


Figure 7. Schematic representation of the effect of VIPER treatment on phagocytosis.

VIPER's inhibition of the Mal adaptor protein, could prevents the activation of NF-κB induced by zymosan's (Z) binding to the TLR-4 and TLR-2 receptors. The upregulation of zymosan phagocytosis could depend on an increase in TREM-2 expression, caused by NF-κB inactivation.

Discussion

In our study, we showed that the addition of laminarin, a soluble beta-glucan that binds to β-glucan receptors, significantly reduces the phagocytosis of zymosan in BV-2 cells. This observation suggests that recognition and engulfment of non-opsonized zymosan in BV2 cells is predominantly mediated by a β-glucan receptor. Interestingly, we found that cells pre-treated with LPS showed a significant increase in phagocytosis of zymosan particles; this effect was partially mediated by a beta-glucan receptor since adding laminarin prior to zymosan significantly (but not completely) abrogated the observed upregulation of phagocytosis (Figure 1 and 6). These results contrast those found for LPS-stimulated RAW-264.7 cells, where laminarin did not abrogate LPS-induced phagocytosis (Fuentes et al., 2011). Instead, the effect was shown to be mediated almost entirely by an increase in Scavenger receptor class A (SR-A1) (Sigola et al., 2016). BV2 microglial cells (unlike RAW-264.7 macrophages) have been shown to express Dectin-1, which is upregulated following exposure to LPS (Shah et al., 2008). Thus we hypothesize that the LPS-induced increase in phagocytosis is partially due to the binding and engulfing of zymosan by Dectin-1 (Figure 6).

A possible way to examine the role of Dectin-1 is to conduct western blot analysis; this will also allow us to quantify Dectin-1 protein levels following the different treatments. In the future, we also plan to block the Dectin-1 receptor with anti-Dectin-1 antibodies following LPS treatment and measure phagocytosis of zymosan to test our hypothesis. However, since laminarin did not completely abrogate the increase in phagocytosis following LPS treatment of BV2 cells (Fig 1A), we posit that other receptors are also involved in this upregulation. Several receptors,

including complement receptor 3 (CR3) and TLR-2 have been previously shown to recognize and bind zymosan in microglia, and could potentially be responsible for the observed upregulation in phagocytosis (Sato et al., 2003; Gitik et al., 2010).

Another aspect we investigated was the role of TLR-4 on the observed upregulation in zymosan engulfment. Since TLR-4 is the primary LPS receptor, we treated cells with VIPER and examined the effects this inhibitor had on LPS modulation of phagocytosis of zymosan. Based on our findings and given VIPER's specified inhibition of the Mal and TRAM adaptor proteins in murine and human-derived cell cultures, we anticipated that treatment with VIPER would significantly reduce phagocytosis of zymosan in LPS activated BV-2 cells (Lysakova et al., 2019). Surprisingly, the inhibitor peptide did not only fail to abrogate the effects of LPS on phagocytosis but instead, it significantly increased phagocytosis when added to BV-2 cells on its own (Figure 2). Furthermore, laminarin partially abrogated the effect of VIPER on zymosan phagocytosis (Figure 5), demonstrating that this upregulation could partially be affected by a β-glucan receptor.

Given the upregulation of phagocytosis by VIPER alone, the results obtained using VIPER and LPS do not provide any light into the involvement of TLR-4 in the LPS-mediated upregulation of zymosan phagocytosis. However, the significant abrogation in zymosan uptake when a polyclonal TLR-4 antibody was added to the cells prior to LPS coincides with our hypothesis that the upregulation of zymosan phagocytosis by LPS in BV2 cells is partially mediated through the binding of LPS to the TLR-4 receptor. Additionally, these results also suggest a novel mechanism underlying the stimulatory effect of VIPER on phagocytosis. We can only speculate on the possible

mechanism(s), given the limited information regarding the impact of VIPER on signal transduction pathways, other than those involving the inhibition of adaptor protein MAL and TRAM. It is important to note that the adaptor protein MAL is also required for TLR-2, TLR-6, and TLR-1 activation (Yang and Ekihiro et al., 2012). Therefore, it is possible that VIPER might be inhibiting these other TLR pathways as well.

The inhibitory effect of VIPER on TLR-4 and possibly TLR-2 is of importance to our work because inactivation of both receptors has been reported to impact the expression of the Triggering Receptor Expressed on Myeloid cells 2 (TREM-2) (Owens et al., 2017). TREM-2 has been associated with enhanced phagocytosis and promoting an anti-inflammatory response in primary microglia (Walter et al., 2016). Furthermore, TREM-2 has been shown to be suppressed by TLR-4's and TLR-2's activation of the transcription factor Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Owens et al., 2017). In our study, we use zymosan to assess phagocytosis; however, zymosan has been shown to activate TLR-2, inducing an inflammatory response via NF- κB stimulation (Sato et al., 2003). In addition, studies have shown that TLR-4 is activated by the mannan PAMP found on *S. cerevisiae*, promoting the release of pro-inflammatory cytokines via NF- κB activation (Tada et al., 2013). Thus, we cannot exclude the fact that TLR-4 might be activated by the mannan component of zymosan in the absence of LPS stimulation (Figure 7). Therefore based on these findings, we hypothesize that the inhibitory effect of VIPER on TLR-4 in conjunction with its postulated inhibitory effect on TLR-2, through the targeting of adaptor protein MAL, could lead to an increase in TREM-2 expression in microglia by suppressing NF-κB activation (Figure 7).

An increase in TREM-2 expression could cause upregulation of zymosan phagocytosis indirectly, by promoting the expression of a β-glucan receptor, or directly by binding zymosan. This latter possibility is based on studies showing that TREM-2 binds various ligands such as Aβ and lipoproteins (Zheng et al., 2018). Therefore, we cannot exclude the possibility that TREM-2 could bind and promote the ingestion of zymosan directly (Figure 7), to test this hypothesis, it is important to measure the levels of TREM-2 transcription and expression prior to and after VIPER addition via RT-PCR and western blot analysis, respectively. Furthermore, using an anti-TREM-2 antibody on microglia before zymosan addition will help assess if TREM-2 impacts phagocytosis of zymosan.

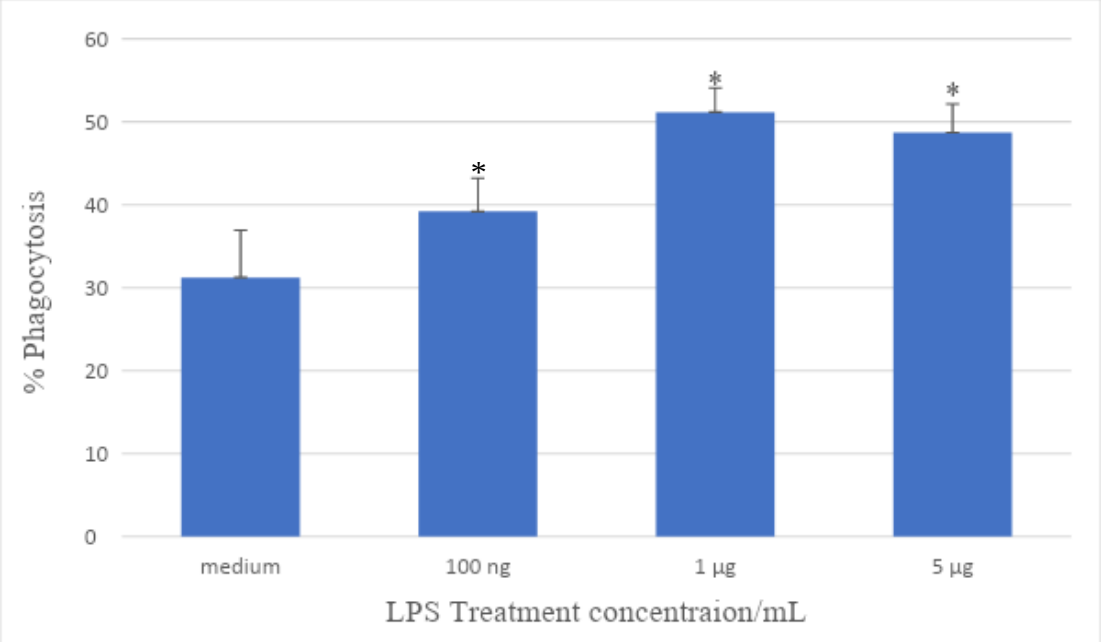
The unexpected effect of VIPER on phagocytosis remains unexplained and could represent an interesting alternative for stimulating the phagocytic activity of microglia while down regulating neuroinflammation, an immune response that is increasingly thought to be the third hallmark of AD (Kinney et al., 2018). Our data shows that VIPER uniquely upregulates zymosan phagocytosis while inhibiting the release of pro-inflammatory markers NO and TNF-α levels in LPS stimulated and in unstimulated control BV2 cells (Figure 2 & 3). These results coincide with previous findings where VIPER downregulated production of pro-inflammatory molecules in LPS pre-treated HEK cells, as well as in *vivo* in hypertensive rats (Lysakova-Devine et al., 2010 & Dang et al., 2014).

Overall, our findings are of importance since AD pathology is postulated to have a fungal etiology, accompanied by hyperactive phagocytically impaired microglia, chronic neuroinflammation, and neurodegeneration (Zhang et al., 2014). Therefore, decreasing the

inflammatory response while enhancing the phagocytic activity of microglia to fungal infections will be beneficial in clearing the fungal burden that is thought to play an important role in AD prognoses. Initially, it will be essential to establish whether the upregulation of phagocytosis is limited to the engulfment of particles with fungal PAMPs, or whether the effects are PAMP-independent; the use of inert beads will solve this question.

Future research should focus on studying the effect of VIPER on phagocytosis and inflammation in primary microglia since they possess remarkable similarities to the in vivo models. Additionally, using AD mouse models that overexpress amyloid-β in an age-dependent manner can be used to evaluate VIPER's therapeutic capabilities. VIPER would be administered to the transgenic mice at different stages of AD; early, middle, and late, intracerebrally, followed by extraction and examination of brain tissue after pre-determined periods of time. This methodology will help assess whether in-*vivo* administration of VIPER is an effective drug for either treatment or prevention of AD. However, one downside of using this transgenic mouse model in this fashion is the lack of information it can provide concerning the role of fungal infection clearance and AD prognoses. Thus, to further examine the role of fungal infection in AD, *C.albicans* could be introduced intracerebrally to transgenic mice at different stages of the disease, followed by an evaluation of the effects of this fungal infection on the progression of AD symptoms. VIPER then could be administered to these fungal infected transgenic mice to examine its therapeutic potential in eliminating the fungal burden and AD treatment. In essence, in *vivo* studies will shed light on VIPER's prospect as a therapeutic drug for AD.

Supplemental Data



SFigure 1. LPS significantly enhances the phagocytosis of zymosan in BV2 cells.

BV-2 cells were either cultured in medium alone or pre-treated with LPS 100ng/mL,1µg, and 5ug respectively and incubated for 16 h; followed by the addition of zymosan. Cells were allowed to phagocytose for 1 h, then washed and stained. Phagocytosis was assessed by light microscopy by counting both the number of cells phagocytizing as well as the number of particles phagocytized by each cell. Results are representative of values of means of five independent experiments performed in duplicates. (Compared with control *, P<.0001)

LPS Treatment	Mean Particle Phagocytized (MPP
0	7.10±1.41
100 ng/mL	10.19[a]± 0.94
1 µg/mL	16.05[a]± 1.98
5 µg/mL	13.99[a] +1.76

[a]P<.0001 compared with control

STable 1. The Effect of LPS on phagocytosis of zymosan.

BV2 cells were cultured in either medium alone or pre-treated for 16h with LPS at 100 ng/mL, 1 µg/mL, and 5 µg/mL, respectively. Cells were then incubated with zymosan and allowed to phagocytize for 1hr. Cells were stained, p assessed by light microscopy, and results are expressed as phagocytic indices ± SEM.

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