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The critical role of JNK and p38 MAPKs for TLR4 induced microglia-mediated neurotoxicity

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ABSTRACT

Identifying signal transduction pathways and understanding their role in microglia-mediated neuroinflammation and neurotoxicity may provide clinical benefits in neurodegenerative diseases. Microglia activation and inflammation is the first line of defense mechanism by the host to remove pathogen/injurious stimuli and initiate the tissue healing process. Inflammation should be tightly regulated. However, dysregulation leads to 'bystander' tissue damage in most neurodegenerative diseases. Evidence suggests that activated microglia excessively secretes chronic neurotoxic factors, including TNF-α, IL1-β, NO and reactive oxygen species (ROS), driving progressive neurotoxicity. The present study provides the molecular mechanisms involved in TLR4 mediated excessive inflammatory responses by microglia. We used LPS stimulated BV2 murine microglia cells, as an in vitro model system to examine TLR4 induced microglia-mediated chronic neuroinflammation. Here we demonstrate that β-amyloid and LPS enhanced TLR4 expression on microglia. LPS stimulation significantly enhanced inflammatory cytokines by microglia in time and dose dependent manner at mRNA and protein levels. Subsequently, phosphorylation of PI3 kinase was observed but it is partially involved in inflammation regulation. In addition, TLR4 activation markedly induces phosphorylation of JNK 1/2, p38 and ERK1/2 MAPKs. Our study revealed that JNK 1/2 and p38 MAPK are crucial players during TLR4 mediated inflammation and neurotoxicity. In addition, combined inhibition of p38 and JNK synergistically increases neuroprotection against Aβ induced neurotoxicity. In conclusion, our findings suggest that TLR4 activation by LPS and β-amyloid significantly increases inflammation while p38 MAPK and JNK 1/2 inhibition provides neuroprotection through negative regulation of NF-κB. Thus p38 MAPK and JNK 1/2 are good therapeutic targets in inflammatory neurodegenerative diseases like Alzheimer's disease.

Key words: Microglia, neuroinflammation, TLR4, Alzheimer's disease, JNK 1/2, p38 MAPKs.

INTRODUCTION

A tight control of innate immunity is essential because morbidity from brain infection / injury can be caused directly by the acute chronic neuroinflammation, as well as by a disproportionate immune response. Recent studies described a direct role of microglial toll-like receptor 4 (TLR4) not only in pathogen/injury clearance but also in exacerbating neurodegeneration [1-2]. However, detailed molecular mechanisms of regulation of excessive inflammatory responses and neurotoxicity by microglia are poorly understood. Microglia, the resident macrophage-like cells, functions as the basic immune defense system of the brain [3]. Evidence suggests that activation of innate immunity in the CNS triggers neurodegeneration through a toll-like receptor 4-dependent pathway [4]. TLR4 induced microglia activation is a characteristic feature of most neurodegenerative diseases including Alzheimer's disease, Multiple sclerosis, Parkinson's disease, stroke, ischemia and amyotrophic lateral sclerosis as well as posttraumatic

brain injury [4-7]. Neurotoxicity induced by β-amyloid or LPS in CNS depends on the presence and activation of microglial TLR4 [1-2, 8-10].

Toll-like receptors (TLRs) recognize pathogen/danger-associated molecular patterns (PAMPs/DAMPs) and induce innate immune responses that are essential for host defense against infection or injury [11]. Among the TLR family, TLR4 functions as a receptor for the endotoxin lipopolysaccharide (LPS) [12], which binds LPS-binding protein (LBP) and the CD14. This complex in turn binds to TLR4 and initiates an intracellular signaling pathway including activation of p38, JNK [13] and ERK MAPKs that regulates gene expression through NF-κB activation [1]. TLR4 functions through an accessory protein (MD-2) [14]. TLR4 activation results in increased secretion of cytokines which dictates the fate of brain pathology.

MAPKs are serine/threonine protein kinases, plays important role in a variety of cellular functions like cell proliferation, differentiation and apoptosis [15-16]. There are three main members of the MAPK family, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPKs exert different biological functions. Activation of ERK1/2 is involved in proliferation and survival while JNK and p38 MAPK are associated with apoptosis [15]. Several studies have shown that LPS induces neuroinflammation via a mechanism mediated by the JNK pathway and induces apoptosis in brain [15, 17].

Microglia are rapidly activated upon tissue damage, can efficiently clear apoptotic cells [18], and can promote neuro-repair through the production of growth factors [3]. The spectrum of activated microglia phenotypes is diverse and generally beneficial. However, when microglia activation becomes exaggerated or dysregulated, the response becomes neurotoxic. Therefore, it is of critical importance to elucidate the mechanisms that are specifically involved in the dysregulated response of microglia which contribute to neuronal damage.

In the current study, we explored whether there is a causative link between microglial MAPK signaling and microglia-dependent neuronal damage. Our study demonstrates that TLR4 activation by LPS and β-amyloid significantly increases inflammation whereas p38 MAPK and JNK 1/2 inhibition provides neuroprotection. Collectively, p38 MAPK and JNK 1/2 are good therapeutic targets in neurodegenerative diseases.

MATERIALS AND METHODS

2.1 Cell culture and treatment

BV2 microglial cells are suitable *in vitro* model system for examining inflammation in primary microglia or animal brain [19]. BV2 microglia cells (kind gift from Prof. Anirban Basu) were maintained at 37°C under an atmosphere of 5% CO2 in DEMEM supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin.

For experiments, BV2 cells were pretreated with ultrapure LPS-Rs-TLR4 inhibitor (Invivogen), PD184352-ERK1/2 inhibitor, SP600125-JNK1/2 inhibitor, SB202190-p38 inhibitor and LY294002-PI3K inhibitor (All 10µM concentration) for 1 hr followed by stimulation with lipopolysaccharide ((LPS1µg/ml) from *Escherichia coli*, serotype 055:B5) or fibrillated β amyloid (5 µM) for 24 hrs. Preparation of fibrillated amyloid β (Aβ): briefly, acetyl-Aβ (Sigma-Aldrich) was dissolved in sterile PBS (100 µM) and stored at −20°C until use. Aβ peptides were aggregated by incubation at 37°C for 4 days and then 5 µM fibrillated Aβ was used to treat BV2 cells. All Inhibitors and LPS are from Sigma Aldrich and cell culture related reagents were purchased from Invitrogen unless otherwise mentioned.

2.2 Semiquantitative RT-PCR

Relative mRNA levels of TLR4, IL-1β, IL-6, TNF-α, iNOS and COX2 in microglia were determined by RT-PCR as described earlier [2].

2.3 Western blotting

BV2 microglial cells were plated in 6 well plates at a density of 1×10^6 cells/well and differentially treated as indicated. Cell lysates were prepared in sodium dodecyl sulphate (SDS)-containing sample buffer and 30µg total protein from each sample were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to PVDF membranes and blocking, then blots were probed with the primary antibodies: rabbit anti-TLR4 (1:500); rabbit anti-phospho p38 (1:1000); rabbit anti-phospho JNK 1/2 (1:500): rabbit anti-phospho ERK 1/2 (1:1000) and mouse anti-β-Actin (1:1000) all from Invitrogen.

2.4 Nitric oxide (NO) measurement

The nitrite (a stable oxidative end product of NO) production in the culture medium was measured using the Griess reagent (Sigma Aldrich) as per manufacturer's instructions. Briefly, culture supernatants (phenol red-free) was mixed with Griess reagents (1:1 ratio) in a 96 well plate for 15 min. Absorbance was measured at 540 nm with microplate reader (BioRad). Sodium nitrite was used as a standard and each experiment was performed in triplicate.

2.5 ROS measurement

Microglial cells were treated as indicated for 24 hrs. Then, cells were washed with PBS and incubated with 5 µM Cell ROX deep red (Invitrogen) in dark for 30 min at 37°C. After washing, intracellular ROS levels were measured by mean fluorescence intensity with a multimode plate reader (Molecular Devises) at 644/655 nm (excitation / emission).

2.6 Neuronal cell survival assay

'Differentiated' neuro2a cells with neuronal morphology used as alternative source of neurons. The neuronal cells were cultured with differentially treated microglia conditioned medium for 48 hrs to examine microglia mediated neurotoxicity. The neuronal cell death was determined with the MTT assay (Sigma) as per manufacturer's instructions.

2.7 ELISA

The production TNF-α in the culture supernatants was measured with sandwich ELISA kits (eBiosciences) according to the manufacturer's instructions.

2.8 Statistical analysis

The data expressed as ± SEM and statistical significance was analysed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A p value less than 0.05 ($p<0.05$) was considered to be statistically significant.

RESULTS

3.1 β amyloid and LPS induced TLR4 mediated neuroinflammation

Microglia are the resident innate immune cells of the brain, provides first line of defense through inflammatory response and removes infectious materials/debris/or plaques by phagocytosis to provide protection during CNS pathologies. TLR4 is reported to be upregulated in brains of patients with AD [1, 20-21]. We investigated effect of β amyloid (Aβ) on TLR4 expression in BV2 microglia cell. We observed that BV2 microglia stimulated with fibrillar Aβ significantly enhanced TLR4 mRNA levels (Figure 1A) accompanied with increased production of inflammatory mediators, NO and TNF-α (Figure 1B-1C) and the effect was TLR4 dependent as evidenced by inhibition of TLR4 (LPS-Rs, a potent TLR4 antagonist) significantly decreased Aβ-induced production of NO and TNF-α (Figure 1B-1C). The observations suggest that Aβ induced TLR4 mediated inflammation in BV2 microglia. For further studies we used LPS, a potent TLR4 ligand, to study TLR4 induced microglia mediate immune responses. Resting microglial cells have highly ramified processes which are very motile. This motility allows microglia to screen the neuronal parenchyma for danger signals, pathogens, metabolic products and deteriorated tissues. Conversion of ramified to amoeboid morphology, increased size of microglia as well as increased expression of several microglia activation markers, are essential features of the inflammatory response of the brain to various insults [22]. Therefore, we initially confirmed that, in our experimental paradigm, LPS treatment resulted in an intense microglia activation and inflammation as evidenced by increased amoeboid morphology (Figure 1D), increased expression of microglia activation markers like TLR4, IL-1β, IL-6, TNF-α, iNOS, and COX-2 (Figure 1E) accompanied with increased production of NO and TNF-α (Figure 1F-1G). Collectively, the data suggests that β amyloid and LPS induced microglia activation and neuroinflammation through TLR4 signalling.

3.2 TLR4 stimulation enhanced neuroinflammation in dose dependent manner

As mentioned above, Aβ induced TLR4 mediated microglia activation and neuroinflammation. We investigated the effect of TLR4 ligand concentration (LPS 10-1000 ng/ml) on microglia mediated inflammation. We observed that LPS dose dependently enhanced expression of TLR4 mRNA and protein levels (Figure 2A-B) accompanied with subsequent increase in pro-inflammatory cytokines IL-1β, IL-6 and TNF-α mRNA levels (Figure 2C). In addition, LPS significantly enhanced production of NO and ROS in a dose dependent manner (Figure 2D-E). The data indicates that TLR4 activation and subsequent inflammatory response depends on concentration of ligand (LPS) and the inflammatory response is directly proportional to dose of the TLR4 ligand. Thus the possible reason for exacerbated neuroinflammation in AD patient's brain could be due to increased TLR4 activation resulting from higher accumulation of Aβ.

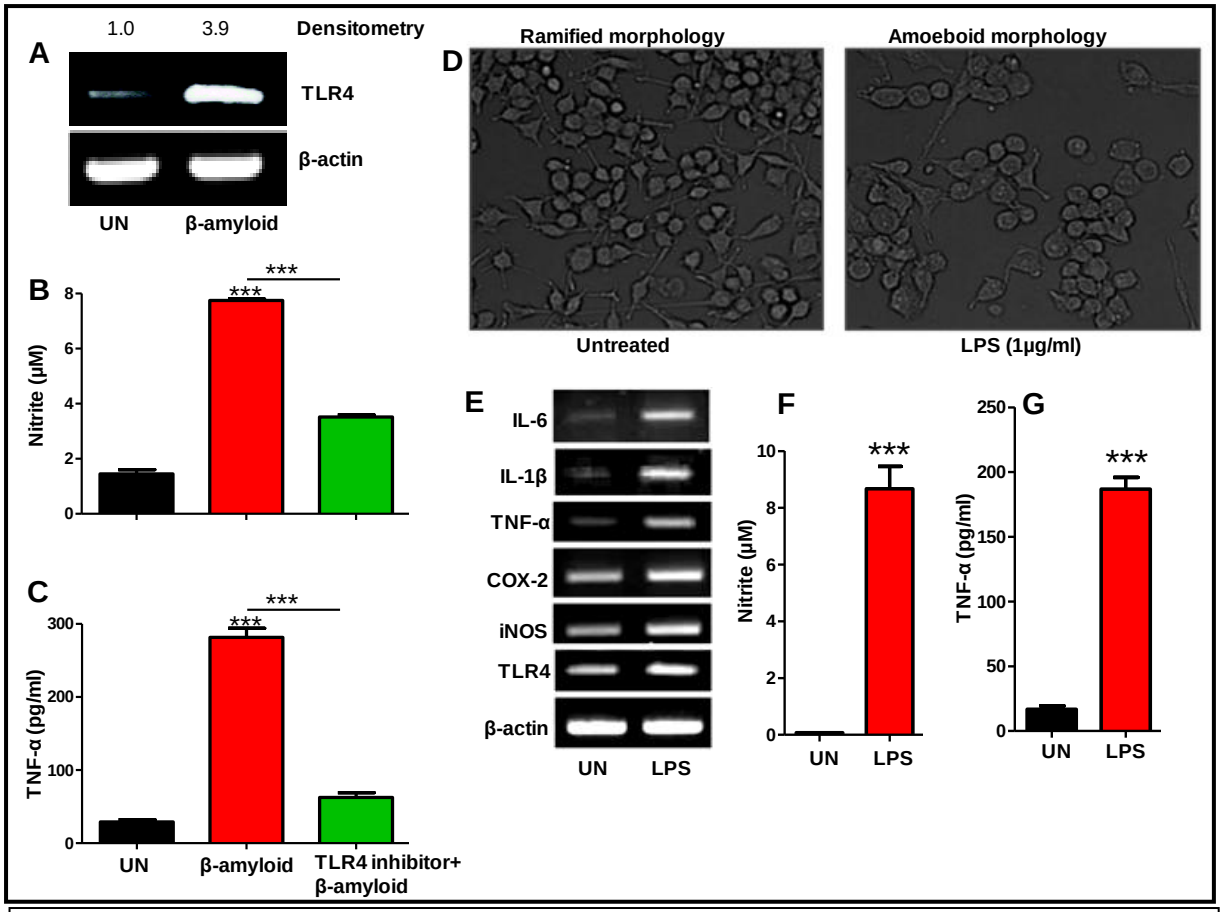

Figure 1. β amyloid and LPS induced TLR4 mediated neuroinflammation. BV2 microglial cells were stimulated with 5μM fibrillar Aβ and TLR4 mRNA expression was assayed by RT-PCR (after 6 hrs) (A), secreted NO and TNF-α in culture supernatant were analysed by Griess reaction and ELISA (after 24 hrs), respectively (B and C). BV2 microglial cells were stimulated with 1μg/ml LPS and ramified or amoeboid morphology was observed under inverted phase-contrast microscope (Olympus) at 400x magnification (D), mRNA levels TLR4, proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and inflammatory mediators iNOS and COX2 was assayed by RT-PCR (after 6 hrs) (E), secreted NO and TNF-α in culture supernatant were analysed by Griess reaction and ELISA (after 24 hrs), respectively (F and G).(Asterisk * indicates p<0.001)**

3.3 PI3 kinase partially regulates TLR4 induced inflammation in BV2 microglia

The phosphatidylinositol 3-kinase (PI3K) signaling plays a key role in regulation of inflammation [23]. We examined the TLR4 induced activation of PI3K in microglia cells. We observed that LPS stimulation significantly increases activation of PI3K (Figure 3A-3B). Activation of PI3K was peaked at 30 min and decreased after 60 min (Figure 3A-3B). To examine the role of PI3K in TLR4 induced inflammation by microglia, we used LY294002, a selective PI3K inhibitor. Microglial cells were pretreated with PI3K inhibitor for 1 hr followed by LPS stimulation for 6 hrs and mRNA levels of proinflammatory were determined. PI3K inhibition does not affect expression of IL-6, TNF-α, iNOS and Cox2 (Figure 3C), whereas inhibition of TLR4 confirms the inhibition of LPS induced inflammation (Figure 3C). Similarly, TNF-α production was partially affected by PI3K inhibition (Figure 3D). The results suggest that inhibition of PI3K partially inhibits TLR4 induced inflammation in BV2 microglia.

3.4 TLR4 stimulation enhanced inflammation and ROS production through activation of p38 and JNK 1/2 MAPKs

Activation of MAPK signaling plays an important role in mediating microglia activation [24]. In addition, JNK and p38 MAPKs are also involved in neuronal death [25]. TLR4 upregulation is also associated with MAPKs activation and neuronal cell apoptosis [26-27]. We therefore investigated whether MAPKs activation are important for TLR4 induced microglia-mediated inflammation and neuronal cell death.

We first investigated the activation of MAPK pathways in our experimental paradigm. BV2 microglia cells were treated with or without LPS for 5-60 minutes and MAPKs were detected by Western blotting using specific antibodies for the phosphorylated (activated) forms of P-JNK, P-p38 and P-ERK1/2. LPS stimulation significantly enhanced phosphorylation of p38, JNK, and ERK1/2 as compared to untreated BV2 microglia (Figure 4 A-C). Activation of p38, JNK, and ERK1/2, was peaked at 30 min, which declined after 60 min (Figure 4A-4C). It should

be noted that LPS did not activated MAPKs in neuronal cells (data not shown). In contrast, neuronal MAPKs (p38, JNK and ERK 1/2) were reported to be induced by soluble, diffusible factors released from activated microglia [25]. These observations suggest that MAPKs activation in neurons is induced by microglia secreted inflammatory mediators. The TNF-α, ROS and iNOS-derived NO has been suggested to play an important role in inducing neuronal cell death, and their blocking may provide neuroprotection. To address the relationship between MAPK signaling and inflammation, we used selective inhibitors for the MAPK pathways: ERK1/2 inhibitor (PD184352), JNK1/2 inhibitor (SP600125), and p38 inhibitor (SB20219. Microglial cells were pretreated with MAPK inhibitors for 1 hr followed by LPS stimulation for 24 hrs and inflammatory mediators were determined. Interestingly, JNK and p38 inhibition significantly decreased production of TNF-α, NO and ROS (Figure 4 D-4F), while the ERK1/2 and PI3K inhibition did not show inhibitory effect on inflammation (Figure 4D-4F). The results suggest that JNK and p38 activation play indispensable role for TLR4 induced inflammation and oxidative stress which may enhance neurotoxicity during pathophysiology.

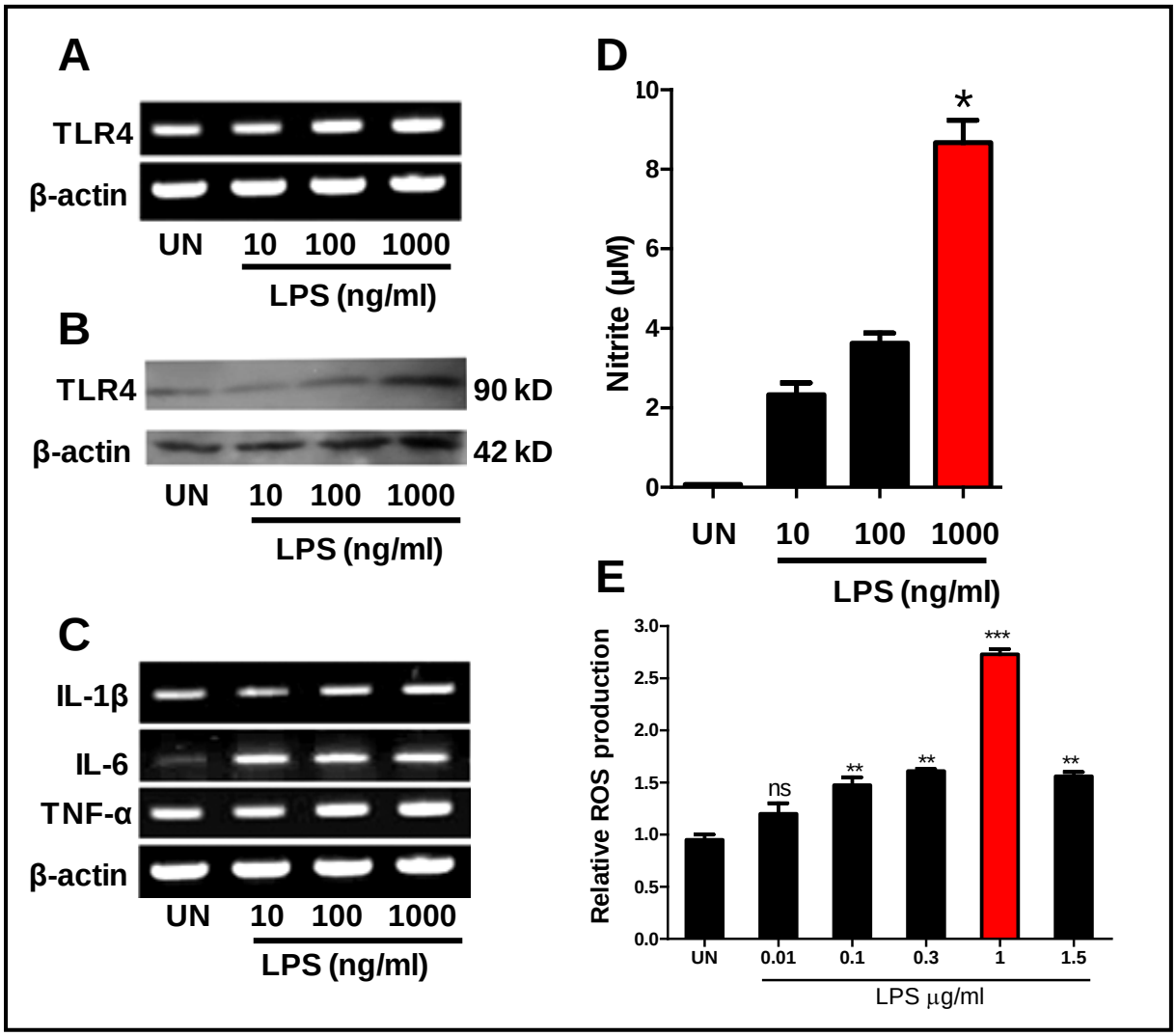

Figure 2. TLR4 stimulation enhanced neuroinflammation in dose dependent manner. BV2 microglial cells were stimulated different LPS concentrations (10-1000 ng/ml); TLR4 mRNA expression was assayed by RT-PCR (after 6 hrs) (A) and TLR4 protein levels were analysed by Western blot (B). Expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) mRNA levels were assayed by RT-PCR (after 6hrs) (C), production of NO and ROS were measured by Griess reagent and Cell-Rox deep red reagent, respectively (D and F). Asterisks * indicates p<0.0001, **indicates p<0.001; *indicates p<0.05**

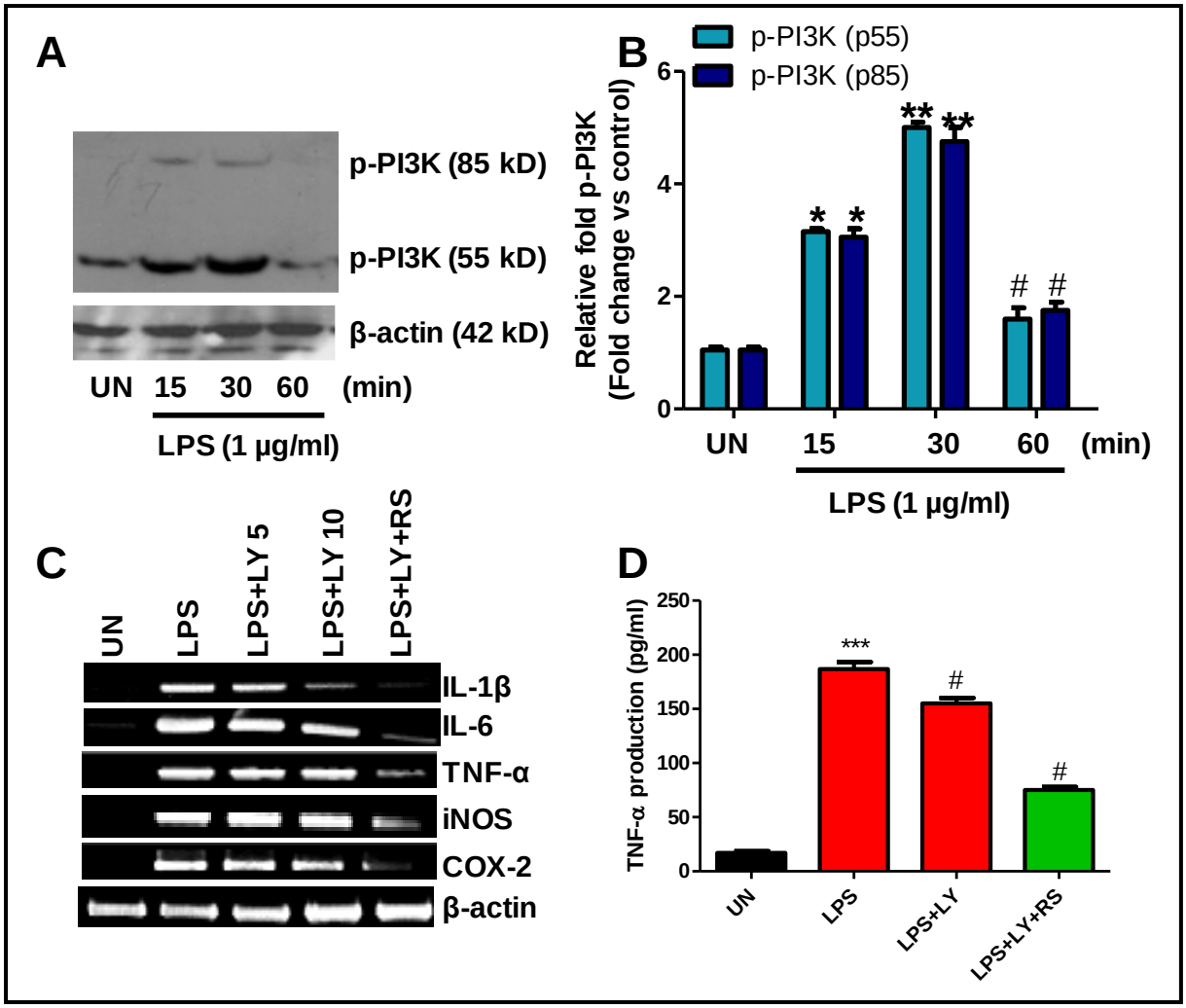

Figure 3. PI3 kinase partially regulates TLR4 induced inflammation in BV2 microglia. BV2 microglial cells were stimulated with LPS for different time point (15-60 min) and phosphorylation of PI3K was assayed by Western blot (A) and relative fold changes were done by densitometry analysis using Image J (B). BV2 microglial cells were pretreated with PI3K inhibitor (LY) for 1 hrs followed by LPS stimulation, mRNA levels of inflammatory mediators was analysed by RT-PCR (after 6 hrs) (C) and secretion of TNF-α was measured by ELISA (after 24 hrs) (D). Asterisks * indicates p<0.0001, **indicates p<0.001; *indicates p<0.05 and # indicate p<0.05 vs LPS treated group/ ****

3.5 Inhibition of p38 and JNK ameliorates Aβ induced Microglia-mediated Neurotoxicity through negative regulation of NF-κB

Consequent to above observations, we examined the possible role of the MAPK signaling in TLR4 induced microglia-mediated neurotoxicity. To examine the functional importance of TLR4 on neuronal cell survival during neuron-microglia coculture. We used the LPS-Rs, a potent TLR4 signaling inhibitor, to investigate the role of TLR4 in Aβ-mediated neurotoxicity in neuron-microglia culture. LPS activated microglia are well known to trigger neuronal cell death through secretion of neurotoxic products and therefore used as a positive control for detection of microglia-mediated neuronal toxicity. Exposure of differentiated neuro2a cells to supernatant from Aβ activated microglia showed significantly increased neuronal cell death however Aβ induced neuronal cell death significantly reversed by TLR4 inhibition as examined by MTT assay (Figure 5A). These results indicate that TLR4 signaling is major contributor in Aβ induced neuronal insults. Microglial cells were pretreated with MAPK inhibitors for 1 hr followed by Aβ stimulation for 24 hrs and the conditioned media (CM) were collected. Neuronal cells were treated with or without CM for 48 hrs and neuronal cell survival were assayed. The inhibition of JNK and p38 significantly ameliorated neurons from TLR4 induced microglia-mediated neuronal death, while inhibition of ERK1/2 did not show a neuroprotection (Figure 5B). In addition, combined inhibition of JNK and p38 synergistically enhanced neuroprotection (Figure 5B). NF-κB is well described for its important role in CNS pathologies, so we investigated the effect of JNK and p38 inhibition of NF-κB. We observed that Aβ enhanced NF-κB activation through TLR4 (Figure 5C and 5D) as evidenced by decreased NF-κB by TLR4 inhibition. Interestingly, JNK and p38 inhibition also results in decreased NF-κB while ERK inhibition did not limited NF-κB (Figure 5C and 5D). Collectively, the

data suggests that inhibition of JNK and p38 provides neuroprotection through negative regulation of NF-κB activation and combined inhibition of JNK/p38 MAPK synergistically potentiates the neuroprotection against microglia-mediated neurotoxicity.

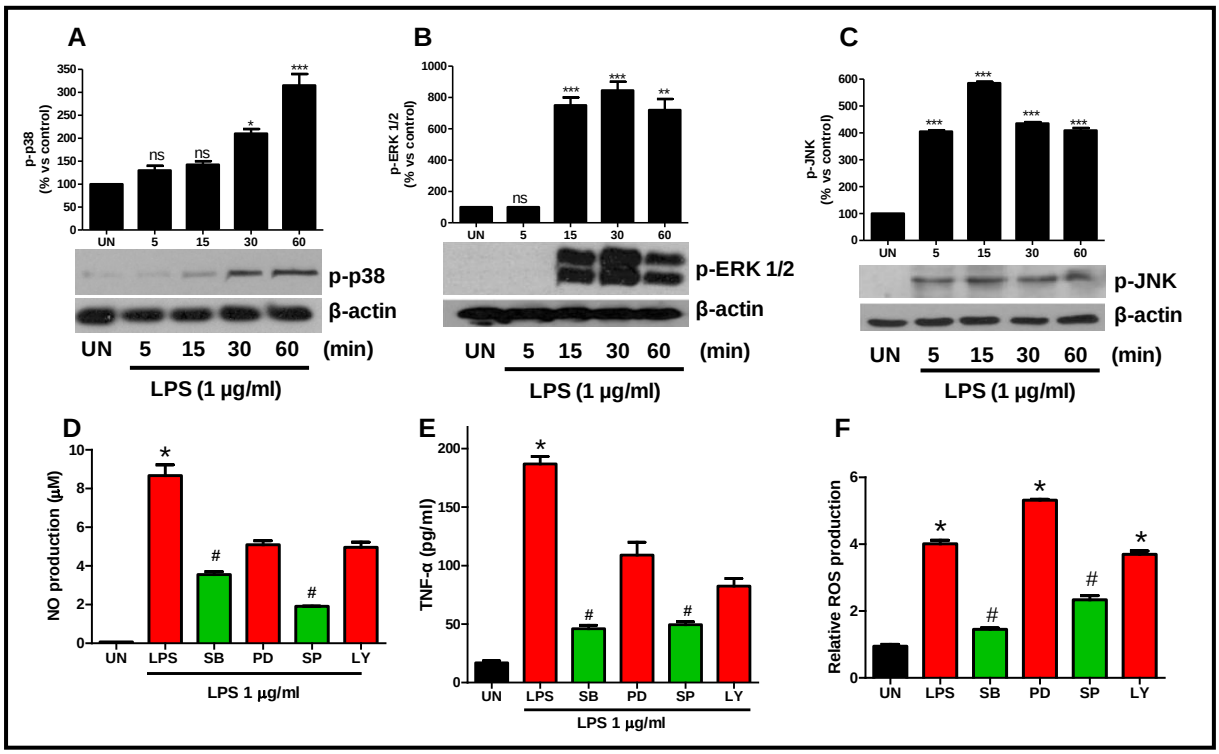

Figure 4. TLR4 stimulation enhanced inflammation and ROS production through activation of p38 and JNK 1/2 MAPKs. BV2 microglial cells were stimulated with LPS for different time point (15-60 min) and phosphorylation of p38, ERK1/2 and JNK was assayed by Western blot (A, B, and C). BV2 microglial cells were pretreated with MAPKs inhibitors for 1 hrs followed by LPS stimulation for 24 hrs and production of NO, TNF-α and ROS were measured (C, D and E). Asterisk *indicates p<0.0001 vs untreated control, # indicates p<0.001 vs LPS treated group

DISCUSSION

In the present study, we demonstrated (i) Aβ and LPS induced TLR4 mediated microglia activation and neuroinflammation, as evidenced by the amoeboid morphology and up-regulation of TLR4, IL-1β, IL-6, TNF-α, iNOS, and COX-2, (ii) PI3K and MAPK are markedly activated in microglia after TLR4 stimulation with LPS and (iii) inhibition of p38 and JNK provide neuroprotection against TLR4 induced microglia-mediated neuronal cell death. We for the first time report that combined inhibition of JNK and p38 MAPK synergistically enhanced neuroprotection during TLR4 induced microglia mediated neuroinflammation and neurotoxicity.

Several evidences suggest that MAPK are rapidly activated in microglia /macrophages with various activating stimuli. In microglia cultures, LPS stimulation induces activation of p38, JNK, and ERK 1/2 [28-30]. Consistent with the studies, we also report activation of p38, JNK, and ERK1/2 in LPS activated BV2 microglia. Importantly, Xie and coworkers shown that MAPK signaling pathways were activated in neurons that had been in co-culture with microglia stimulated with LPS and IFN-γ, but not in neurons treated with LPS and IFN-γ [25]. These evidences suggest that microglia secreted factors like TNF-α, NO and ROS activates p38, JNK1/2 and ERK 1/2 and may be potentiates neuronal loss in aberrant neuroinflammatory conditions.

Several studies have reported that MAPKs (p38, JNK and ERK 1/2) are involved in neuronal cell death. LPS stimulated microglia shows activation of MAPKs, play key roles in increased inflammatory mediators like iNOS, IL-1β, or TNF-α [31-33]; thus, there might be link between activation of MAPKs and increased neuroinflammation. The molecular mechanisms by which MAPK pathways contribute to microglia-mediated neuroinflammation was elucidated using selective inhibitors. Inflammatory mediators like TNF-α and NO produced from β-amyloid or LPS activated microglia enhanced neuronal cell death [34]. However, our findings suggest inhibition of JNK and p38 significantly reduced NO, TNF-α and ROS levels (Figure 4D-F), in contrast inhibition of ERK 1/2 and PI3K did not blocked TLR4 induced microglia-mediated inflammation. These results suggest that the JNK and p38 pathways are important regulators of TLR4 induced microglia mediated inflammation. Furthermore, we investigated the role of

the MAPKs in microglia mediated neuronal cell death. Our observation that inhibition of p38 and JNK provides neuroprotection against LPS activated microglia-mediated neurotoxicity (Figure 5B) suggests the critical role of JNK and p38 MAPKs signaling during the neurotoxicity. In addition, inhibition ERK1/2 did not protects neuronal cell death suggests that activation of ERK1/2 is not a problem in TLR4 induced microglia-mediated neuroinflammation and subsequent neurotoxicity.

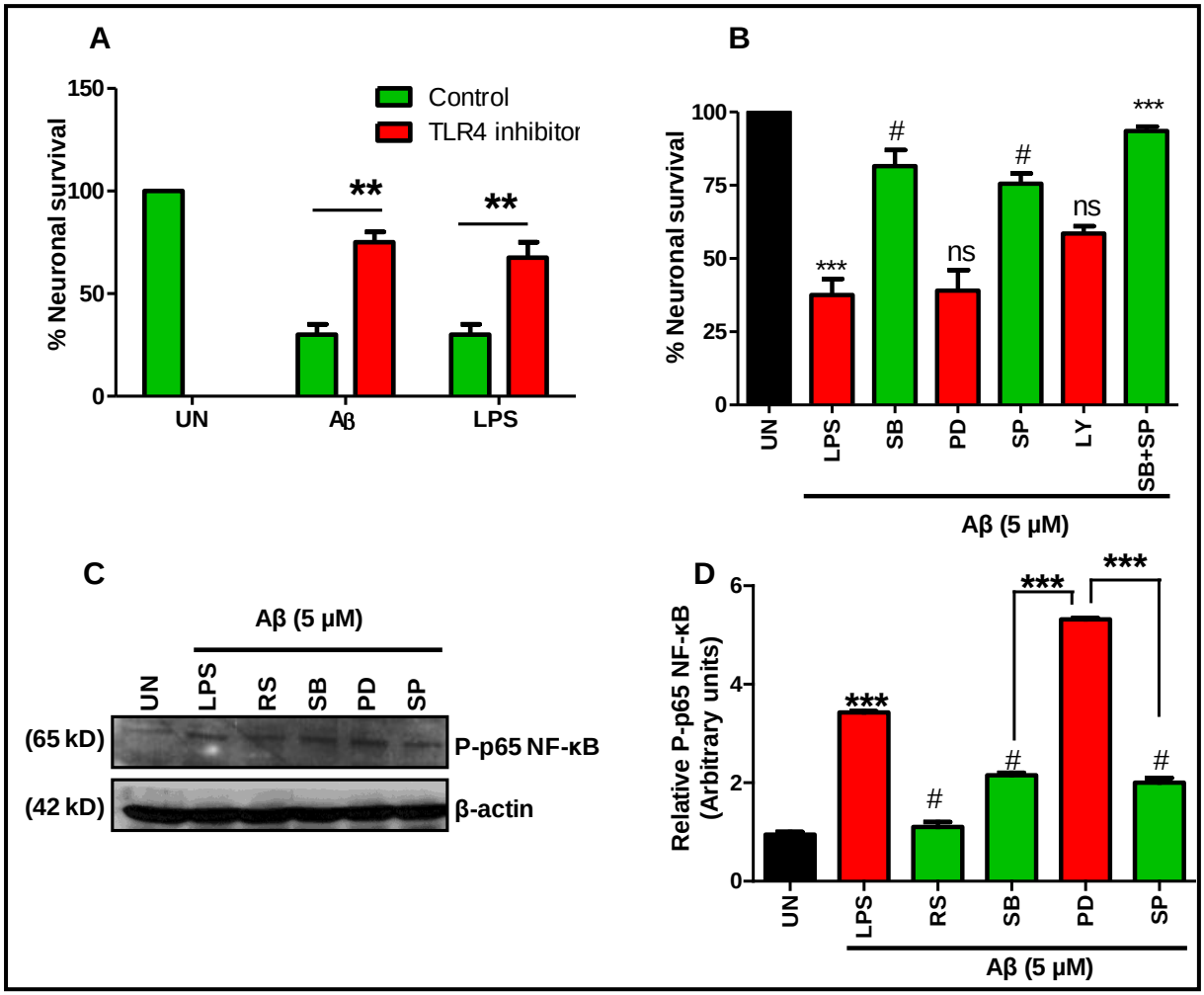

Figure 5. Inhibition of p38 and JNK ameliorates Aβ induced microglia-mediated neurotoxicity. Differentiated neuro2a cells were exposed to supernatants from Aβ or LPS activated microglia either untreated or treated with TLR4 inhibitor. Neuronal cell viability was examined by MTT assay (A). BV2 microglial cells were pretreated with or without MAPKs inhibitors for 1 hr followed by activation with Aβ for 24 hrs and the conditioned media were collected. Differentiated neuro2a cells were exposed to conditioned media for 48 hrs and neuronal cell survival was analysed by MTT assay (B). Differentiated neuro2a cells were exposed to supernatants from Aβ or LPS activated microglia either untreated or treated with TLR4 inhibitor. Differentiated neuro2a cells were exposed to supernatants from differentially treated microglia for 1 hr and NF-κB activation was assayed by Western blot (C), relative P-NF-κB (p65) was assayed by densitometric analysed using ImageJ (=p<0.001, ***=p<0.0001, #=p<0.001 vs Aβ treated group and ns=non significant).**

CONCLUSION

Our findings suggest that combined inhibition of JNK1/2 and p38 MAPK signaling could be a potential therapeutic strategy against brain pathologies like Alzheimer's disease where TLR4 induced microglia-mediated neuroinflammation is implicated in disease progression.

Acknowledgements
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