Suppression of Microglia-Mediated Neuroinflammation by Small Molecule NF-κB Inhibitors

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ABSTRACT

It has been shown that long-term production of proinflammatory cytokines by activated microglia plays a role in neurodegenerative disorders like Parkinson's disease and multiple sclerosis. Here we investigated if inhibition of the NF-κB signaling pathway, an important factor in the inflammatory response, could alter the chemokine and cytokine profile of the BV-2 mouse microglia cell line. Using the Toll-like Receptor 4 (TLR4) agonist lipopolysaccharide (LPS), we stimulated BV-2 cells and subsequently treated them with two different small molecule inhibitors, MLN4924 (a NEDD8 E1 Activating Enzyme inhibitor) and Celastrol (a NF-κB inhibitor). Cell supernatants and lysates were measured for 26 mouse cytokines/chemokines/growth factors using a R&D Systems™ Mouse Magnetic Luminex® Assay. LPS stimulation increased multiple proinflammatory cytokines, including IL-1α/IL-1F1, IL-1β/IL-1F2, IL-6, IL-27, and TNF-α, and the chemokines CCL4/MIP-1β, CCL5/RANTES, CXCL1/KC and CXCL10/IP-10/CRG-2. Treatment with either MLN4924 or Celastrol resulted in significant decreases in the levels of the proinflammatory cytokines and chemokines. To determine the effects of the inhibitors on the NF-κB pathways, cell lysates of activated cells were analyzed for inhibition of the canonical (IκBα degradation) pathway. The data suggest that MLN4924 and Celastrol inhibits the degradation of IκBα. These results indicate that the small molecule inhibitors, MLN4924 and Celastrol, may be used to suppress microglia, NF-κB-mediated neuroinflammation.

INTRODUCTION

In neurodegenerative diseases, such as Parkinson's disease, inflammation has been shown to contribute to disease pathology. Microglia are central nervous system (CNS) resident immune cells that have a macrophage-like phenotype and, when activated, produce proinflammatory cytokines. Microglia activation by LPS results in neuronal and axonal loss both in vitro and in vivo.1 LPS is an endotoxin from gram-negative bacteria that stimulates the immune system via the innate immune receptor TLR4. The TLR4 signaling pathway activates the transcription factor NF-κB, which is part of a family of transcription factors that regulate several genes involved with inflammation including cytokines and chemokines. The NF-κB proteins are normally sequestered in the cytoplasm via two distinct mechanisms: by an IκB inhibitory protein family member (canonical) and by the C-terminal portion of NF-κB p100 (noncanonical). The canonical activation of NF-κB requires the inducible degradation of its inhibitory IκB protein via phosphorylation and subsequent Ubiquitin-mediated degradation. This allows the release and nuclear translocation of its respective NF-κB dimer.2 Research has shown that processing of the noncanonical NF-κB inhibitor, p100, also requires the Ubiquitin-proteasome system. Interestingly, both degradation of IκBα, the most studied IκB protein, and p100 requires the activity of a SKP1-CUL1-F-box protein (SCF) E3 Ubiquitin ligase. Furthermore, SCF-mediated ubiquitination of IκBα and p100 is dependent on the neddylation of its CUL-1 component by NAE (NEDD8-activating enzyme inhibitor).3 In this study, we examined how MLN4924 (Pevonedistat), a protein neddylation inhibitor, and Celastrol, a known NF-κB inhibitor, could inhibit NF-κB-mediated production of cytokines and chemokines by mouse microglia BV-2 cells.

MATERIALS & METHODS

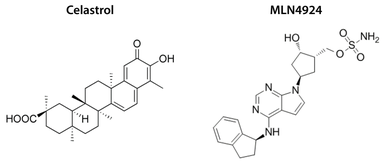
Cell Culture and Treatments
Mouse microglia BV-2 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml), and maintained in a 5% CO2 incubator at 37 °C. Cells were left untreated or treated with 100 ng/mL LPS (Sigma, Catalog # L4516), 300 nM Celastrol (Tocris, Catalog # 3203), 5 µM MLN4924 (R&D Systems, Catalog # I-502), or DMSO for 24 hours. Cells were also pretreated with Celastrol (100 nM, 200 nM, 300 nM) or MLN4924 (1 µM, 5 µM, 10 µM) for 1 hour, washed with PBS, and then treated with 100 ng/mL LPS for 15 minutes or 24 hours. After each incubation period, conditioned media and cells were separated by centrifugation. Conditioned media was aliquoted and stored at -20 °C until analysis. Cells were washed with PBS and then solubilized with Lysis Buffer 17 (R&D Systems, Catalog # 895943) for Luminex® analysis, or Lysis Buffer 6 (R&D Systems, Catalog # 895561) for Western blot. Lysis buffers were supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Catalog # 78440) prior to lysis. Total protein of whole cell lysates was determined via bicinchoninic acid assay (BCA).

Cell Viability
Cell viability was investigated for all experimental setups and aided in determining optimal conditions. Viability was determined via trypan blue staining and assessed by the Countess™ II Automated Cell Counter system.

Luminex® Analysis
Cell culture samples were analyzed for 26 different analytes using the Mouse Magnetic Luminex® Assay (R&D Systems, Catalog # LXSAMSM). Cell culture supernatants were diluted to 1:2 and 1:20 with the assay diluent, and cell culture lysates were run at 5 mg/mL per sample (125 µg/well). The assay was run according to kit instructions and tested on a Luminex® MAGPIX®.

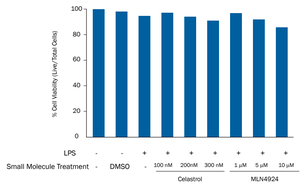
Western blot Analysis
Whole cell lysates were loaded at 10 µg per lane and resolved under reducing SDS-PAGE conditions using 5-20% gradient gels. Proteins were transferred from resolved gels to PVDF membranes using a Semi-Dry Transfer Unit. PVDF membranes were then blocked for 1 – 2 hours in Blotting Buffer (25 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. PVDF membranes were probed using the following primary and secondary antibody pairs: Rabbit Anti-Human Phospho-IκBα (S32/S36) Antigen Affinity-Purified Polyclonal Antibody (R&D Systems, Catalog # AF4809) followed by a HRP-Conjugated Goat Anti-Rabbit IgG Secondary Antibody (R&D Systems, Catalog # HAF008); Sheep Anti-Human/Mouse IκB α Antigen Affinity-Purified Polyclonal Antibody (R&D Systems, Catalog # AF4299) followed by a HRP-Conjugated Donkey Anti-Sheep IgG Secondary Antibody (R&D Systems, Catalog # HAF016); Mouse Anti-Human/Mouse/Rat GAPDH Monoclonal Antibody (R&D Systems, Catalog # MAB5718) followed by a HRP-Conjugated Donkey Anti-Mouse IgG Secondary Antibody (R&D Systems, Catalog # HAF018). Western blots were visualized using the VisULite ECL Western Blotting Substrate (R&D Systems, Catalog # VL001-200) and captured onto X-Ray film.

Figure 1. Small Molecule Inhibitors Celastrol and MLN4924 (Pevonedistat) Were Used to Reduce LPS-Induced Inflammation in Microglia



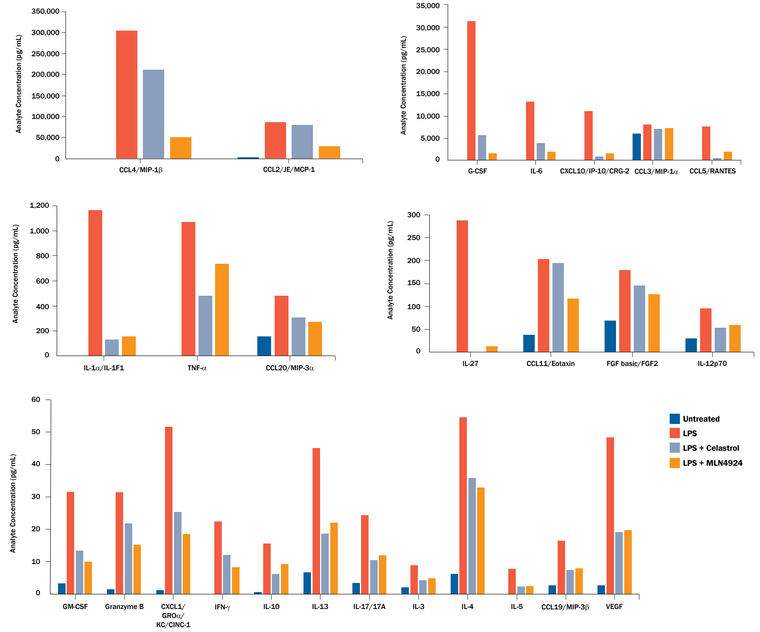
Celastrol has been shown to inhibit TNF-α-induced NF-κB activation. MLN4924 is a NEDD8-activating enzyme inhibitor that can control the activity of Ubiquitin ligases.

Figure 2. Celastrol and MLN4924 are Minimally Cytotoxic



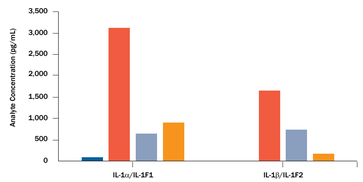
In vitro LPS-stimulated mouse microglia BV-2 cells had limited cell cytotoxicity, as assessed by trypan blue staining, when treated with multiple concentrations of Celastrol or MLN4924 for 1 hour, followed by LPS for 24 hours.

Figure 3. Celastrol and MLN4924 Attenuate Cytokine and Chemokine Secretion by LPS-Activated BV-2 Cells



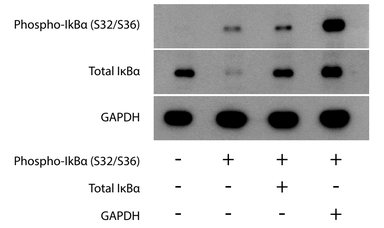
Mouse microglia BV-2 cells were left untreated or pretreated with Celastrol (300 nM) or MLN4924 (10 µM) for 1 hour followed by stimulation with LPS (100 ng/mL) for 24 hours. Cell supernatants were collected and diluted with assay buffer and then analyzed for secretion of 26 different analytes using a Mouse Luminex® Magnetic Assay. Histogram profiles shown are representative of three separate experiments.

Figure 4. Celastrol and MLN4924 Attenuate Cytokine Expression in Lysates from LPS-Activated BV-2 Cells



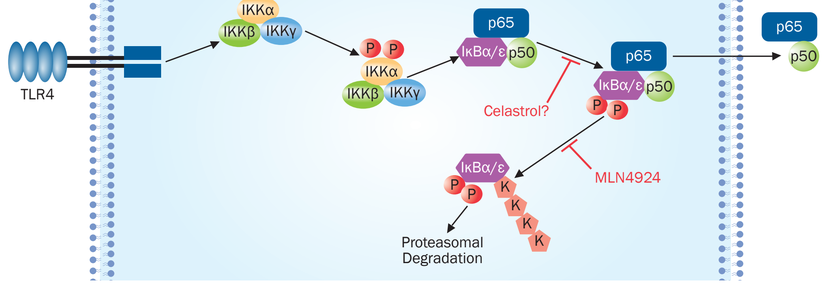
Mouse microglia BV-2 cells were left untreated or pretreated with Celastrol (300 nM) or MLN4924 (10 µM) for 1 hour followed by stimulation with LPS (100 ng/mL) for 24 hours. Cells were washed with PBS, solubilized with Lysis Buffer 17, and analyzed using a Mouse Luminex® Magnetic Assay. Histogram profiles shown are representative of three separate experiments.

Figure 5. Celastrol and MLN4924 Inhibit the Canonical NF-κB Pathway



Mouse microglia BV-2 cells were left untreated or pretreated with Celastrol (300 nM) or MLN4924 (10 µM) for 1 hour followed by treatment with LPS (100 ng/mL) for 15 minutes. Cell lysates were extracted and the relative levels of Phospho-IκBα (S32/S36), total IκBα and GAPDH were determined by Western blot. Celastrol blocks LPS-induced-phosphorylation and degradation of IκBα, while MLN4924 blocks LPS-induced degradation of phospho-IκBα.

Figure 6. Proposed Mechanism of Action for Celastrol and MLN4924



CONCLUSION

The small molecule inhibitors Celastrol and MLN4924 inhibited LPS-induced secretion of several cytokines and chemokines by mouse microglia BV-2 cells including IL-1α/IL-1F1, IL-1β/IL-1F2, IL-6, TNF-α, CXCL10/IP-10/CRG-2, CCL4/MIP-1β, and CCL5/RANTES. This decrease in expression was not due to a significant change to total cell numbers (data not shown) or a decrease in cell viability, but by Celastrol and MLN4924 targeting the NF-κB pathway. Western blot analysis of phospho-IκBα (S32/S36) and total IκBα levels in cell lysates suggest that Celastrol inhibited the LPS-induced degradation of IκBα by blocking IκBα phosphorylation while MLN4924 also inhibited LPS-induced IκBα degradation but did not prevent IκBα phosphorylation. These data suggest that MLN4924 blocks ubiquitination of the phosphorylated IκBα thereby preventing proteasomal degradation (Figure 6). This hypothesis is supported by experiments done in other cell types[4].

REFERENCES

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