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RNF11 modulates microglia activation through NF-κB signalling cascade

Nirjari V. Dalal[a,b,*], Elaine L. Pranski[a,b], Malu G. Tansey[a,c], James J. Lah[a,b], Allan I. Levey[a,b], Ranjita S. Betarbet[a,b]

[a] Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA 30322, United States
[b] Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, United States
[c] Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322, United States

HIGHLIGHTS

- ► RNF11 associates with A20 in the glial system.
- ► RNF11 is a negative regulator of NF-κB signalling pathway in microglia.
- ► Overexpression of RNF11 is protective against LPS-induced cytotoxicity.

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ABSTRACT

Microglia are resident macrophages in the central nervous system (CNS) that play a major role in neuroinflammation and pathogenesis of several neurodegenerative diseases. Upon activation, microglia releases a multitude of pro-inflammatory factors that initiate and sustain an inflammatory response by activating various signalling pathways, including the NF-κB pathway in a feed forward cycle. In microglial cells, activation of NF-κB signalling is normally transient, while sustained NF-κB activation is associated with persistent neuroinflammation. RING finger protein 11 (RNF11), in association with A20 ubiquitin-editing complex, is one of the key negative regulators of NF-κB signalling pathway in neurons. In this study, we have demonstrated and confirmed this role of RNF11 in microglia, the immune cells of the CNS. Coimmunoprecipitation experiments showed that RNF11 and A20 interact in a microglial cell line, suggesting the presence of A20 ubiquitin-editing protein complex in microglial cells. Next, using targeted short hairpin RNA (shRNA) knockdown and over-expression of RNF11, we established that RNF11 expression levels are inversely related to NF-κB activation, as evident from altered expression of NF-κB transcribed genes. Moreover our studies, illustrated that RNF11 confers protection against LPS-induced cell cytotoxicity. Thus our investigations clearly demonstrated that microglial RNF11 is a negative regulator of NF-κB signalling pathway and could be a strong potential target for modulating inflammatory responses in neurodegenerative diseases.

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1. Introduction

Microglia are resident macrophages of the central nervous system (CNS) that respond to injury and eliminate infection [32]. They regulate the innate immune response, communicate with other brain cells, and act as scavengers by removing dying cells [24,35]. Any insult to the brain, exogenous or endogenous, that disturbs its homeostasis leads to microglial activation. Pro-inflammatory and cytotoxic factors, released by activated microglia, have been implicated in initiation and sustenance of neuroinflammatory responses that exacerbate the underlying neuropathology of several neurological diseases such as Huntington's disease, Alzheimer's disease (AD), and Parkinson's disease (PD) [17,29]. The microglial NF-κB signalling pathway is one of the key players in neuroinflammation [21,43,11]. In addition, p65 a subunit of NF-κB, was observed to be localized to the nuclei of activated microglia in

Abbreviations: AD, Alzheimer's disease; CNS, central nervous system; co-IP, coimmunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκBs, inhibitors of NF-κB; IP, immunoprecipitation; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; PD, Parkinson's disease; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; RNF11, RING finger protein 11; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Tax1, human T-cell leukemia virus type 1; TAX1BP1, Tax1 binding protein 1; TLR, Toll-like receptors; TNF, tumor necrosis factor; TNFAIP3, tumor necrosis factor alpha-induced protein 3; TNFR, tumor necrosis factor receptor.

* Corresponding author at: 615 Michael St., Suite 500, Atlanta, GA 30322, United States. Tel.: +1 404 727 6136.
E-mail addresses: nvdalal@emory.edu (N.V. Dalal), epransk@emory.edu (E.L. Pranski), malu.tansey@emory.edu (M.G. Tansey), jlah@emory.edu (J.J. Lah), alevey@emory.edu (A.I. Levey), rbetarb@emory.edu (R.S. Betarbet).

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PD brain tissue [11] implicating NF-κB activation in activated microglia.

In the CNS, NF-κB has crucial roles in both neuronal development and degeneration [31]. Dysregulation of NF-κB signalling can result in defective neuronal development and tumorigenesis, or chronic neuroinflammation and neuronal death [43,11,4,14,26,12] [27]. Thus the duration of NF-κB activation is tightly regulated for normal homeostasis through mechanisms such as phosphorylation, transcriptional modulation and protein–protein interaction [25]. For instance, NF-κB target genes, such as the ubiquitin-editing protein A20 (also known as tumor necrosis factor, alpha-induced protein 3 or TNFAIP3), IκBs (inhibitors of NF-κB), and cylindromatosis, act as inhibitors of NF-κB signalling [13]. Recent studies have demonstrated the critical role A20 plays in regulating NF-κB signalling cascade, in complex with Tax1 (human T-cell leukemia virus type 1) binding protein 1 (TAX1BP1, also called TXBP151 or T6BP), Itch (also known as AIP4), and RNF11 [19,1,41,40].

RNF11 is a putative E3 ubiquitin ligase that has been associated with various types of cancers, as well as PD [1,42]. Additionally, RNF11 enhances TGF-β and EGFR endosomal signalling by interacting with Smad4 and Smurf2 [22]. Moreover, RNF11 is an essential component of the A20 ubiquitin editing complex and negatively regulates NF-κB signalling, as demonstrated in a monocytic cell line [40] and neurons [33]. In the current study, we investigated the association of RNF11with the A20 ubiquitin-editing complex in microglia, the immune cells and mediators of neuroinflammation in the CNS, and determined its role in the NF-κB signalling pathway, inflammatory response and cell survival.

2. Materials and methods

2.1. Cultured cell lines

BV2 murine microglia cells were a kind gift from Dr. Elisabetta Blasi at University of Modena and Reggio Emilia in Italy [3]. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine (Lonza), inactivated fetal bovine serum (Lonza), and 1% penicillin/streptomycin (Bio Whittaker). Primary microglia cultures were prepared and harvested as described in [33]. All cultures were maintained at 37 °C in 5% CO_2.

2.2. Reagents

Cells were stimulated with 10 ng/ml TNF-α (R&D Systems) and a range of doses for LPS (Sigma) as indicated in figure legends, for 24 h following a 1 h serum starvation period.

2.3. RNA interference

The constructs used for knockdown of RNF11 and overexpression of RNF11 has been previously described [33]. Lentiviruses were produced by the Emory University Viral Vector Core facility.

2.4. Immunoblotting and co-immunoprecipitation

As previously described, cells were grown for 24 h, rinsed with PBS, and harvested as described in [1]. Immunoprecipitations (IPs) were performed as described in [33], using antibody against rabbit RNF11 (polyclonal antibody) described in [1]. Controls IPs were performed with beads alone to demonstrate specificity. Analysis of IPs completed using immunoblotting. Samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Primary antibodies against A20 (Abcam), β-actin (Abcam) and RNF11 were used for immunoblotting. Membranes were scanned using the Odyssey Image Station (LiCor).

2.5. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed, as described previously [33], on a 7500 Fast RT-PCR instrument (Applied Biosystems) using TaqMan PCR master mix (Applied Biosystems) and gene-specific TaqMan probes (Applied Biosystems) against RNF11 (Mm00450014_m1), TNF-α (Mm00443258_m1), A20 (Mm00437121_m1), MCP-1 (Mm00441242_m1), IL1-B (Mm99999061_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Applied Biosystems). RNA samples were run in triplicate. Gene expression was normalized to the house-keeping gene GAPDH and relative expression was calculated for each gene using $2^{-\Delta\Delta Ct}$ method.

2.6. Cytotoxicity assay

Quantification of cell death and cell lysis was performed using the Roche Cytotoxicity Detection Kit. After 24 h of LPS stimulation, the assay was performed and analyzed following manufacturer's instructions. Culture medium was used as control.

2.7. Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 4.03 software (Graph Pad Software, Inc.). One-way analysis of variance (ANOVA) with Tukey's posttest was performed for qRT-PCR analysis of RNF11 levels. Standard two-way ANOVA was used to analyze the mRNA expression of inflammatory markers, and cytotoxicity assay. Bonferroni post-tests were performed for all two-way ANOVAs. Statistical significance was set at $P < 0.05$. All experimental results are presented as mean ± SEM for at least three independent experiments.

3. Results

3.1. RNF11 mRNA expression levels are similar in primary microglia and BV2 microglial cell line

We used BV2 cells, a murine microglia cell line [3] for our investigations since (1) they have been used extensively to study neuroinflammatory responses [17,43], and (2) we could genetically manipulate RNF11 expression levels more efficiently. Furthermore, we established that BV2 cells had similar RNF11 expression levels as primary microglia, by measuring relative mRNA levels of RNF11 using qRT-PCR (Supplemental Fig. 1).

3.2. RNF11 associates with A20 and is a negative regulator of NF-κB pathway in microglial cell line

Previous studies in monocytic and neuronal cells suggested that interaction of RNF11 with the A20 ubiquitin-editing protein complex is crucial for mitigation of the canonical NF-κB signalling pathway [40,33]. To determine if there is an interaction between RNF11 and A20 in microglia, we performed co-immunoprecipitation (co-IP) experiments in BV2 cells. Immunoprecipitates with RNF11 antibody from endogenous cells at steady state and following LPS and TNF-α stimulation were enriched with A20 immunoreactivity while control co-IPs, lacking RNF11 antibody, were absent for A20 immunoreactivity (Fig. 1A). LPS and TNF-α stimulation however did not enhance the association of A20 and RNF11 in BV2 cells with IP western (Fig. 1A). These findings indicate that RNF11 associates with A20 in microglia suggesting that RNF11 could modulate activation of NF-κB signalling pathway in microglia through the A20 ubiquitin-editing protein complex.

Given the strong association of NF-κB activation with activated microglia and neuroinflammation [17,12] we examined the

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