

# An inhibitory alternative splice isoform of Toll-like receptor 3 is induced by type I interferons in human astrocyte cell lines

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**Toll-like receptor 3 (TLR3) recognizes viral double-stranded RNA. It stimulates pro-inflammatory cytokine and interferon production. Here we reported the expression of a novel isoform of TLR3 in human astrocyte cell lines whose message is generated by alternative splicing. The isoform represents the N-terminus of the protein. It lacks many of the leucine-rich repeat domains, the transmembrane domain, and the intracellular Toll/interleukin-1 receptor domain of TLR3. Type I interferons (interferon- $\alpha$  and interferon- $\beta$ ) induced the expression of this isoform. Exogenous overexpression of this isoform inhibited interferon regulatory factor 3, signal transducers and activators of transcription 1, and Inhibitor of kappa B  $\alpha$  signaling following stimulation. This isoform of TLR3 also inhibited the production of chemokine interferon- $\gamma$ -inducible protein 10. Our study clearly demonstrated that the expression of this isoform of TLR3 was a negative regulator of signaling pathways and that it was inducible by type I interferons. We also found that this isoform could modulate inflammation in the brain. [BMB Reports 2015; 48(12): 696-701]**

## INTRODUCTION

Toll-like receptor 3 (TLR3) is a pattern recognition receptor (PRR) that recognizes double-stranded RNA (dsRNA) during viral replication in endolysosomal compartments or on cell surfaces (1). Recognition of viral RNA by TLR3 triggers several intracellular signaling pathways that lead to activation of mitogen-activated protein kinases (MAPKs), nuclear factor  $\kappa$ B (NF- $\kappa$ B), and interferon regulatory factor 3 (IRF3), resulting in the production of pro-inflammatory cytokines and type I interferons (IFNs) (2, 3). TLR3 is expressed in various kinds of cells, including dendritic cells, fibroblasts, intestinal epithelial cells, and astrocytes. Depending on the cell type, TLR3 represents

different cellular response (4-6). Within distinct immunological milieu of the brain, TLR3 has been found to be necessary for the secretion of TNF- $\alpha$ , IL-12p40, and IL-6 in murine microglial cells after being stimulated by polyinosinic-polycytidylic acid (poly(I:C)) (7). In viral infections such as experimental infection with simian immunodeficiency virus and human immunodeficiency virus encephalitis, TLR3 expression is upregulated, leading to the induction of active innate immune responses in the brain (8). Following intracerebroventricular administration of poly(I:C) to mimic viral infection in the central nervous system, TLR3 signaling is activated, resulting in contextual memory and enhanced susceptibility to seizures (9, 10). Similar effects of viral infection on brain function have been reported in encephalitis and associated epileptic activity (11-13). These reports suggest that inflammation as innate immune response is important in neurological disorders and in anti-viral defenses.

Astrocytes perform a variety of physiological functions such as forming the blood-brain barrier, transporting nutrients, and maintaining ion balances for the normal physiology of the brain. In addition, astrocytes play an important role in diseases related to neuro-inflammation (14, 15). Astrocytes express numerous PRRs to allow for the recognition of diverse pathogen-associated molecular patterns (PAMPs) (16-18). We have previously reported that TLR3 is expressed in astrocytes and that its activation induces the expression of IL-6 (19). We have reported that an isoform of TLR3 lacking 192 bp is produced by alternative splicing in primary astrocytes and astrocyte cell lines (20). However, the mechanistic role of this isoform is unclear. In this study, we report a novel isoform of TLR3 that is induced by type I IFNs in astrocyte cell lines. It downregulated poly(I:C)-induced TLR3 signaling. This isoform might have an immune-modulatory function during brain inflammation and other pathological conditions.

## RESULTS

### An isoform of TLR3 is expressed in human astrocyte cell lines

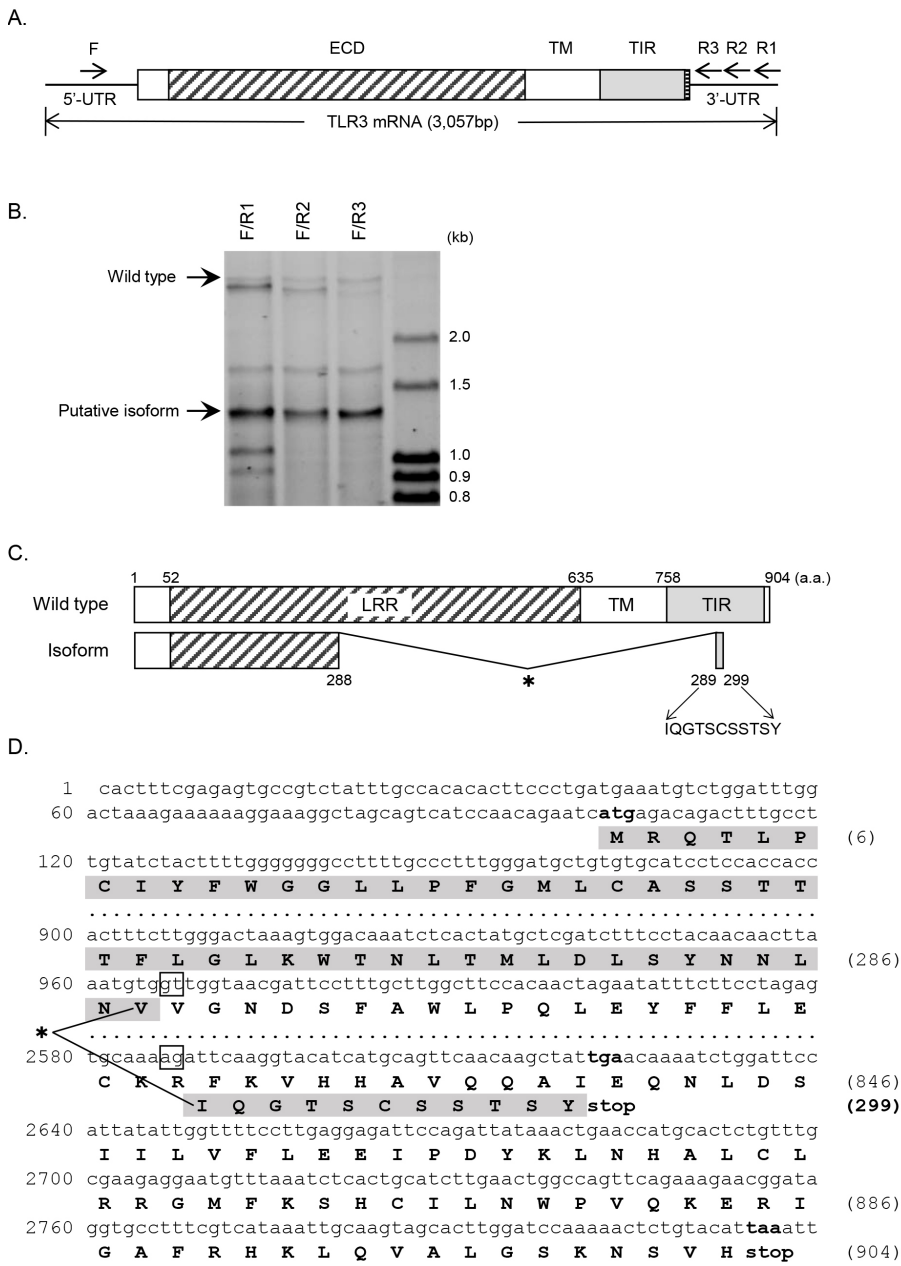
To investigate whether TLR3 has additional splice variants, we examined the expression level of TLR3 transcripts using different sets of primers corresponding to the 5'- or the 3'-UTR. A172 cells expressed a novel isoform of TLR3 that contained

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**Fig. 1.** Identification of a novel splice-isoform of human TLR3 in A172 cells. (A) Schematic diagram of wild type TLR3 gene with depiction of primer sets used for RT-PCR. (B) RT-PCR analysis of the wild type TLR3 and its novel isoform using mRNA from A172 cells. (C) Amino acid sequences of TLR3 and the novel TLR3 isoform. (D) cDNA and protein sequences of wild type TLR3 and its novel isoform. *Numbers* on the left indicate nucleotides, and *numbers* in *parentheses* on the right indicate amino acids. The *asterisks* indicate the portion missing from the isoform. The TLR3 isoform is related to wild type TLR3 by alternative splicing. The splice donor (gt) and acceptor (ag) sequences at positions 966-967 and 2,586-2,587, respectively, are in boxes. The splicing event introduces a frame-shift, resulting in the novel TLR3 isoform with a predicted protein sequence containing 299 amino acids shown in the grey box.

an open reading frame of 900 bp in length, including a stop codon. We obtained similar results using three different sets of primers (Fig. 1A, B). This isoform lacked the region of 966-2,587 bp encoding a considerable number of the leucine rich repeat (LRR) domains, the transmembrane domain, and the intracellular toll/IL-1 receptor (TIR) domain responsible for signal transduction (Fig. 1C). The C-terminal 11 amino acids of this

TLR3 isoform differed from those of the TLR3 wild type as a result of a frame-shift introduced by alternative splicing. The alternative splicing occurred between the donor "GT" (region of 967-968 bp) and acceptor "AG" (region of 2,586-2,587 bp), introducing a frame-shift mutation and a stop codon "TGA" at region of 2,621-2,623 bp, resulting in translation of a 299 amino acid polypeptide (Fig. 1D).

### IFN- $\alpha/\beta$ increases the transcript of the novel isoform of TLR3 in astrocyte cell lines

Upon viral infection, TLR3 will produce type I IFNs *via* activating IRF3. To assess the effect of type I IFNs on the transcription of the novel isoform of TLR3, A172 and NHA cells were treated with IFN- $\alpha$  or IFN- $\beta$ . As a result, the transcription of this novel isoform of TLR3 was increased (Fig. 2A) after treatment with IFN- $\alpha$  or IFN- $\beta$ . The transcript of this novel isoform of TLR3 was detectable starting at 3 h post treatment. Its level was increased until 24 h after IFN- $\beta$  treatment (Fig. 2B).

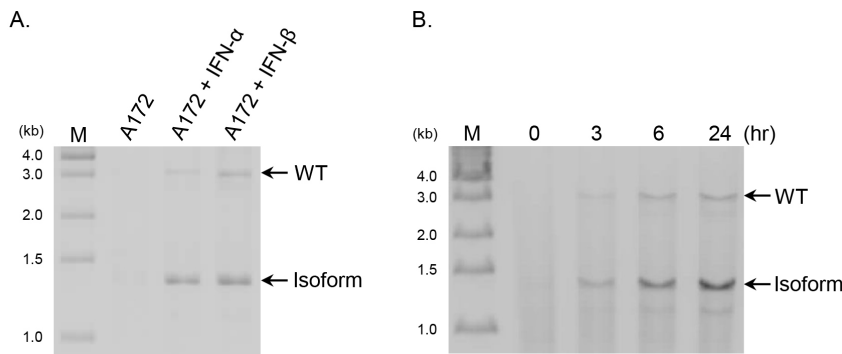
### Overexpression of the novel isoform of TLR3 inhibits TLR3 signaling

Next, we determined whether the expression of the novel

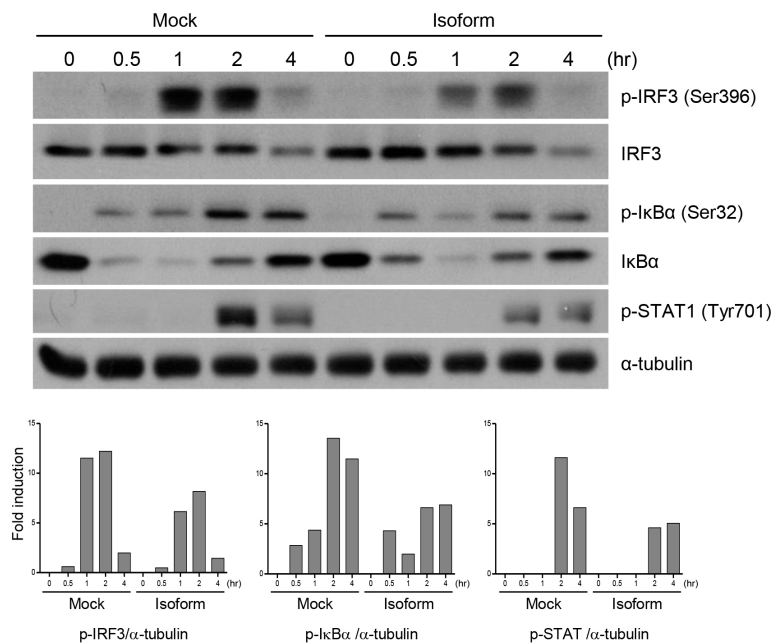
TLR3 isoform would influence TLR3 signaling pathway induced by poly(I:C). Our results revealed that the poly(I:C) treatment induced phosphorylation of interferon regulatory factor 3 (IRF3), Inhibitor of kappa B  $\alpha$  ( $\text{I}\kappa\text{B}\alpha$ ), and signal transducers and activators of transcription 1 (STAT1) *via* TLR3 signaling in the control group (Fig. 3). When the isoform was exogenously overexpressed, phosphorylation levels of IRF3,  $\text{I}\kappa\text{B}\alpha$ , and STAT1 were significantly reduced compared to the mock transfection control (Fig. 3).

### Expression of the novel isoform of TLR3 affects the production of interferon- $\gamma$ -inducible protein 10 (IP-10)

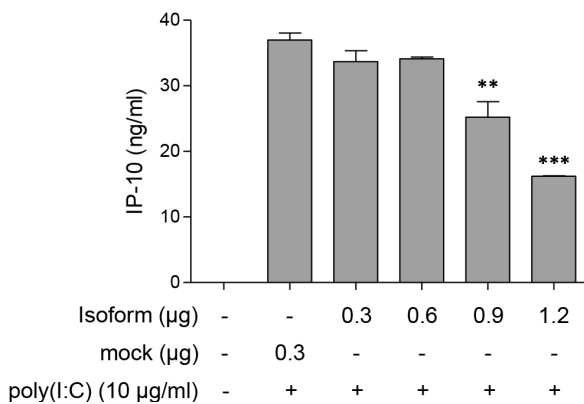
To determine the effect of expression of the novel isoform of TLR3 on the secretion of antiviral chemokines, IP-10 was se-



**Fig. 2.** The TLR3 isoform is a natural transcript in human astrocyte cell lines that is upregulated by type I IFNs. Both A172 (human glioblastoma) and NHA (normal human astrocyte) cells express wild type TLR3 and the novel isoform of TLR3. (A) A172 cells were treated with IFN- $\alpha$  and IFN- $\beta$  for 24 h. (B) NHA cells were treated with IFN- $\beta$  for the indicated time periods. M denotes a 1-kb ladder as nucleotide size marker. The experiments were repeated five times and a representative result is shown.



**Fig. 3.** Overexpression of the TLR3 isoform downregulates poly(I:C)-induced activation of IRF3, STAT1, and  $\text{I}\kappa\text{B}\alpha$  in A172 cell lines. A172 cells were transfected with pCMV-Myc/TLR3 isoform and stimulated with 25  $\mu\text{g}/\text{ml}$  of poly (I:C) for the indicated time periods. Total cell lysates were analyzed by immunoblotting using anti-phospho-IRF3 (Ser396), anti-IRF-3, anti-phospho- $\text{I}\kappa\text{B}\alpha$  (Ser32), anti- $\text{I}\kappa\text{B}\alpha$ , anti-phospho-STAT1 (Tyr701), or anti-tubulin antibody. The experiments were repeated twice and a representative result is shown.



**Fig. 4.** Poly(I:C)-induced secretion of IP-10 was decreased by exogenous expression of the novel isoform of TLR3 in a dose-dependent manner. A172 cells were transiently transfected with pCMV-Myc/TLR3 isoform (0.3-1.2 µg), incubated for 24 h, and stimulated with 10 µg/ml of poly(I:C) for 12 h. Mock vector was transfected at 0.3 µg. Culture supernatants were harvested to measure the production of IP-10 by ELISA. Data represent mean ± S.E. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

lected as the major cytokine induced by TLR3 signaling. Upon ligation with poly(I:C), TLR3 stimulated IP-10 secretion via JAK/STAT pathway. Consistent with the inhibitory effect of TLR3 isoform expression on the activation of IRF3, IκBα, and STAT1 (Fig. 3), the production of IP-10 was decreased in a dose-dependent manner in cells exogenously overexpressing the TLR3 isoform (Fig. 4).

## DISCUSSION

Endosomal TLRs such as TLR3, TLR7, and TLR9 can recognize nucleic acids derived from viruses and bacteria. They play important roles in innate immunity as well as pathological conditions (21). These TLRs can recognize self-DNA and RNA associated with several autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and psoriasis (22, 23). Normally, self-nucleic acids are not detected by endosomal TLRs because there are several regulatory mechanisms, including receptor localization, association with accessory proteins, and proteolytic cleavage (24). For example, protease-mediated regulation of TLR depends on the type of protease that is active. When TLR9 is cleaved by acidic pH-dependent protease such as cathepsins or asparagine endopeptidase, produced C-terminal cleaved fragments of TLR9 can augment TLR9 activation. On the other hand, when TLR9 is cleaved by neutral pH-dependent proteases, produced N-terminal fragments of TLR9 can inhibit TLR9 signaling (25, 26).

In the brain, TLR3 is abundantly expressed in microglia and astrocytes. TLR3 is active in anti-viral immune responses and some autoimmune diseases such as multiple sclerosis and experimental autoimmune encephalomyelitis (27, 28). Recent

studies have shown that TLR3 cleavage by cysteine proteases will produce C- or N-terminal fragments that are involved in the activation of the receptor (29-31).

In this study, we found a novel isoform of TLR3 generated by alternative splicing in astrocyte cell lines. This novel isoform has only LRRs 1-10. It lacks the entire transmembrane and cytoplasmic TIR domains. The expression of this isoform was induced by the treatment with type I IFNs. Interestingly, when the isoform was exogenously overexpressed, poly(I:C)-induced TLR3 activation was significantly reduced. Moreover, the secretion of IFN-responsive chemokine IP-10 was decreased in cells overexpressing this isoform. These negative regulatory effects of this TLR3 isoform may be caused by competition for ligand binding because this TLR3 isoform has dsRNA binding sites (LRR1 and LRR3), whereas it lacks the cytoplasmic TIR domain required for signal transduction (25, 32).

In summary, our results demonstrated that the expression of a novel isoform of TLR3 was induced by type I IFNs and that its protein product negatively regulated TLR3 activity. Therefore, this novel isoform of TLR3 might have immune-modulatory function in brain inflammation and some pathological conditions.

## MATERIALS AND METHODS

### Reagents

Poly(I:C) was purchased from Invivogen (San Diego, CA, USA). Recombinant human IFN-α and IFN-β were purchased from ProSpec (East Brunswick, NJ, USA). Anti-phospho-IRF3 (Ser396), anti-IRF-3, anti-phospho-IκBα (Ser32), anti-IκBα, and anti-phospho-STAT1 (Tyr701) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-tubulin antibody was purchased from Abcam (Cambridge, MA, USA).

### Cell culture

Human glioblastoma cell line A172 was obtained from ATCC and cultured in DMEM medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively) at 37°C in a humidified 5% CO<sub>2</sub> incubator. Normal human astrocyte (NHA) cells were cultured in DMEM/F-12 medium containing 10% FBS, 10 µg/ml of blasticidin, 0.2 µg/ml of puromycin, and penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively) at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### RT-PCR

A172 cells ( $1 \times 10^6$  cells per well) were treated with or without IFN-α or IFN-β (500 units/ml, respectively) for 24 h. NHA cells ( $5 \times 10^5$  cells per well) were treated with IFN-β (500 units/ml). After incubation, total RNA was isolated from A172 or NHA cells using RNeasy Mini kit (Qiagen, Santa Claris, CA, USA). A total of 2 µg of total RNA was used to synthesize cDNA with random primers and M-MLV Reverse Transcriptase (Invitrogen). RT-PCR was performed using ex Taq polymerase

(Takara, Tokyo, Japan). The following primers were used for RT-PCR: F (5'-GAA AGG CTA GCA GTC ATC CAA-3'), R1 (5'-CAA AAA TAG GCC TGA AAT AAG-3'), R2 (5'-TAT GAA TAA ACA AAT TAT AAC AC-3'), R3 (5'-TTT CTC CTT TGC TAA TTG AAT-3'). The following PCR conditions were used: denaturation at 94°C for 1 min, annealing at 50°C for 30s, and extension at 72°C for 2 min. After 30 cycles, an additional extension at 72°C for 10 min was performed. These primers were designed based on human TLR3 mRNA sequences obtained from GenBank (accession No. NM\_003265.2).

### Cloning of the TLR3 isoform

Total RNA was isolated from A172 cells ( $1 \times 10^6$  cells) using RNeasy Mini kit (Qiagen). A total of 3 µg of total RNA was used for cDNA synthesis with M-MLV Reverse Transcriptase (Invitrogen) using forward (5'-G GCC ATG GAG GCC ATG AGA CAG ACT TTG CCT TGT ATC-3') and reverse (5'-CCG GAA TTC TCA ATA GCT TGT TGA ACT GCA TGA TGT-3') primers. A vector for TLR3 isoform expression was constructed by inserting PCR-amplified cDNA digested with SfiI and EcoRI into pCMV-Myc vector (Clontech, Palo Alto, CA, USA).

### Western blot

A172 cells were cultured in 6-well plates at  $3 \times 10^5$  cells per well and transiently transfected with 1 µg of pCMV-Myc (mock) or pCMV-Myc/TLR3 isoform for 24 h. Cells were stimulated with 25 µg/ml of poly(I:C) for indicated time periods. After stimulation, cells were harvested and lysed with lysis buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris, pH 8.0) containing protease and phosphatase inhibitor cocktails (GenDEPOT, TX, USA). Cell lysates were centrifuged and supernatants were stored at -80°C. Protein concentrations of lysates were measured using Bradford protein assay. Fifty micrograms of each protein sample was separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare, Waukesha, WI, USA). Membranes were incubated with anti-phospho-IRF3 (Ser 398), anti-IRF-3, anti-phospho-IκBα (Ser 32), anti-IκBα, anti-phospho-STAT1 (Tyr 701), or anti-tubulin antibody. Protein bands were detected using a West-save up western blot detection kit (Ab Frontier, Seoul, Korea).

### Enzyme-linked immunosorbent assay (ELISA)

A172 cells were cultured in 6-well plates at  $3 \times 10^5$  cells per well and transiently transfected with 0.3-1.2 µg of pCMV-Myc/TLR3 isoform using Eugene 6 reagent according to instructions of the manufacturer (Roche, Basel, Switzerland). Mock vector (pCMV-Myc) was transfected at 0.3 µg per well. After 24 h, cells were stimulated with 10 µg/ml of poly(I:C) for 12 h. Culture supernatants were harvested and stored at -80°C. ELISA was performed with a human cytokine IP-10 assay kit (BD Bioscience, San Jose, CA, USA). The optical density (O.D.) at 450 nm was determined.

### Statistical analysis

One-way ANOVA was performed for statistical analysis using PRISM version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered statistically significant.

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