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Role of TRIF Small Interference RNA (siRNA) in Chronic Experimental Allergic Encephalomyelitis (EAE)

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Background: Multiple sclerosis (MS) is an autoimmune disease causing multifocal demyelination and axonal injuries in the central nervous system (CNS). Toll-interleukin-1 receptor (TIR)-domain containing adaptor protein-inducing interferon beta (TRIF) is an important adaptor protein for Toll-like receptors (TLRs) and can modulate the immune response via regulating cytokine secretion. This study investigated the potential function of TRIF in MS mice via small interference RNA (siRNA).

Material/Methods: Isolated mouse lymphocytes were processed using TRIF siRNA, followed by RT-PCR assay to quantify TRIF expression level. An experimental allergic encephalomyelitis (EAE) model was prepared in C57BL/6 mice immunized with MOG 35–55. TRIF siRNA or controlled siRNA were intravenously applied to evaluate the neurological function of animals. Serum levels of IFN-γ and IL-2 were observed.

Results: Specific siRNA effectively decreased the TRIF expression in mouse dendritic cells and this siRNA improved the EAE severity and neurological scores. Further assays showed that both IFN-γ and IL-2 levels in the siRNA treatment group were significantly lower than in controls.

Conclusions: The expression of TRIF can be down-regulated by siRNA, thereby alleviating the severity of EAE via its inhibition of interleukin and cytokine release. This may provide new insights for future treatment of MS.

MeSH Keywords: **Encephalomyelitis, Autoimmune, Experimental • Multiple Sclerosis, Chronic Progressive • Trifluridine**

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Background

As an autoimmune disease that attacks the central nervous system (CNS), multiple sclerosis (MS) leads to multifocal neural demyelination and axonal injuries. Due to the lack of effective treatment, most patients survive with various disabilities that severely impair the quality of life [1]. Currently, the most important animal model of MS is experimental allergic encephalomyelitis (EAE), which is induced by immunizing sensitive animals using specific antigens and is manifested with CNS white matter demyelination [2–4]. Specifically, MOG 35–55-immunized C57BL/6 mice had EAE similar to clinical MS, making it an ideal animal model [5–7]. Immune dysfunctions in EAE include the breakdown of myelin sheath by autoantibodies, the activation of auto-reactive T cells, and related cytokine expression modulation [7–9].

Toll-like receptors (TLRs) play a critical role in both innate immunity and acquired immunity. Recent studies show that Toll-interleukin-1 receptor (TIR)-domain containing adaptor protein-inducing interferon beta (TRIF) is an important molecule in TLRs-induced signal transducing pathway. Further studies about the TRIF-related pathway have established its role in immune response and pathogenesis, in addition to novel drug targets against certain allergic disease [10]. The role of TRIF in either human MS or EAE animal models, however, still needs to be further elucidated [11]. This study therefore aimed to investigate the effect of TRIF in EAE using specific small-interference RNA (siRNA) and related neurological methods.

Material and Methods

Culture of lymphocytes

The animal study protocol was pre-approved by the animal experiments ethics committee of the Heilongjiang Hospital. After anesthesia, C57BL/6 mice were sacrificed by decapitation. The peritoneal cavity was opened after skin sterilization. The spleen was removed, ground, filtered, and rinsed by Hank's solution. The collected spleen cell suspension was then centrifuged at 2000 rpm for 3 min. After discarding the supernatant, 30 mL erythrocyte lysate was added for 5-min incubation. After the complete lysis of erythrocytes, the mixture was re-centrifuged at 2000 rpm for 3 min to discard erythrocyte debris. After washing by Hank's solution, lymphocytes were re-suspended in RPMI-1640 medium (Yubo BioTech, China) with 10% fetal bovine serum (FBS). The final concentration of cell suspensions was adjusted to $8 \sim 10 \times 10^6$ per mL. Cells were cultured using DMEM culture medium (Yubo BioTech, Shanghai, China) containing 5 mM D-glucose at 37°C with 5% CO₂. The experimentation with animals was governed by the Regulations of Experimental Animals of Heilongjiang Hospital

Authority and was approved by the Animal Ethics Committee of Heilongjiang Hospital.

siRNA transfection

One day before the transfection, cultured lymphocytes were inoculated into Petri dishes without antibiotics. Cells with 30% confluence were transfected with siRNA of ICOS or non-specific (NS) controlled siRNA (Santa Cruz, USA) using Lipofectamine 2000 (Invitrogen, USA) according to the manual's instructions. In brief, diluted liposome solutions (1:50) were mixed with equal volumes of siRNA (0.2 mM), followed by 20-min incubation at room temperature. The mixture was then added into the cultured dish, which was filled with 5 mL serum-free DMEM culture medium. After 6 h, the medium was changed to DMEM with 10% serum. Lymphocytes were collected after 48 h for RT-PCR assays.

RT-PCR for TRIF expression in lymphocytes

Cells (5×10^6) in all groups were collected and extracted for total RNA using the Trizol kit (Invitrogen, USA) following the manual's instructions. First-strand cDNA was then synthesized using total RNA as the template. TRIF-specific primers were designed by Primer 5.0 software with the following sequences: TRIF-F, 5'- CUC CAC GAC UCA AUA TA-3'; ICOS-R, 5'- TTC ATG UGC UAA CGC AG-3'; β -actin-F, 5'- GGT GTG ATG GTG GGT ATG GGT-3'; β -actin-R, 5'- CTG GGT CAT CTT TTC ACG GT-3'. Quantitative RT-PCR was carried out using SYBR PCR kit (Invitrogen, USA) following the manual's instructions with the following condition: pre-denature for 5 min at 94°C; 30 cycles of amplification, each containing 1-min denature at 94°C, 1-min annealing at 60°C, and 3-min elongation at 72°C; ending with 5-min elongation at 72°C. The products were visualized by 1% agarose gel electrophoresis. Relative expression level of mRNA was calculated using $2^{-\Delta\Delta C_t}$ method based on the standard curve.

The induction of EAE using MOG 35-55

We purchased 20 female C57BL/6 mice (age between 6–8 weeks, body weight=16~20 g) from the animal center of our university. At the first day, 100 μ L MOG 35-55 emulsion (containing 100 μ g MOG35-55 purchased from Sheldon Biotech, Canada; 500 μ L complete Freund's adjuvant and 500 μ g inactivated mycobacteria) was subcutaneously injected into bilateral inguinal regions. On the same day and 48 h after the immunization, 400 ng pertussis toxin (Qifu Biotech, China) was intraperitoneally injected.

EAE disease score plot

We used the following standard of neurology to evaluate the condition of EAE: Grade I, partial drooping of the tail; Grade II, complete drooping of the tail; Grade III, complete drooping of the tail and slow movement; Grade IV, partial paralysis of hind

Figure 1. Relative expression level of TRIF mRNA in cultured lymphocytes. The mRNA level was calculated as the ratio between TRIF and actin level. *, $p < 0.05$ compared to control (No Tx) group.

Table 1. Disease incubation and progressive period of EAE mice.

Group (mice)	Incubation period (d)	Progressive period(d)	EAE disease score
NS siRNA (20)	15.25±1.65	6.12±1.32	4.09±1.21
TRIF siRNA (20)	19.86±1.45*	4.02±1.16*	3.02±0.65*
Control (20)	15.65±1.53	6.21±1.28	4.16±1.12

* $p < 0.05$ compared to control and NS siRNA group.

limbs; Grade V, complete paralysis of hind limbs; Grade VI, agonal status. After the generation of the EAE model, 100 μ L of TRIF-specific siRNA or non-specific (NS) siRNA was injected into the tail vein of animals. The neurological behavior was evaluated 24 h after the injection.

Enzyme-linked immunosorbent assay (ELISA) for IL-2 and IFN- γ expression levels

Blood samples were collected from mouse tail veins. Lymphocytes were extracted as described previously and were cultured in DMEM (with 10% FBS and 5 mM D-glucose) at 37°C with 5% CO₂. After 48-h incubation, supernatants from culture medium was quantified for IL-2 and IFN- γ levels using ELISA kit (BD Corp., USA) following the manual's instructions. Each sample was tested in triplicate and the average value was recorded.

Statistical analysis

SPSS 17.0 software was used to process all collected data, which are presented as mean $\pm$ standard deviation (SD). The comparison between 2 independent groups was done by t test. One-way analysis of variance (ANOVA) was applied for multiple group comparisons. Statistical significance was defined when $p < 0.05$.

Results

TRIF expression can be down-regulated by siRNA

As suggested by RT-PCR assay, siRNA can effectively suppress the expression of TRIF in lymphocytes *in vitro* (Figure 1, $p < 0.05$).

Neurological evaluations of EAE mice after treatment

EAE is mainly manifested with slow movement, mental fatigue, decreased appetite, and body weight loss. In our study, EAE mice began to show limb weakness, unstable movement, and further paralysis at 15 days after the immunization. The application of siRNA alleviated the disease condition when compared to the model or siRNA control group, as the treated mice had more days until disease onset, shorter durations of EAE, and lower scores for neurological dysfunction (Table 1, $p < 0.05$).

Serum IL-2 and INF- γ levels in EAE mice

ELISA showed that after 1 week of disease onset, serum IL-2 and INF- γ levels were significantly decreased in siRNA-treated animals compared to siRNA controls (Figure 2, $p < 0.05$).

Figure 2. Serum cytokine levels of EAE mice. Both IL-2 and IFN- γ were tested for their serum levels in EAE mice treated with TRIF siRNA or controlled NS siRNA. *, $p < 0.05$ compared to controls.

Discussion

Both MS and EAE have immune dysfunctions, including the production of autoantibody and activation of autoreactive T cells, both of which can damage the myelin sheath. Various reactive T cells have been identified from both cerebrospinal fluid (CSF) and peripheral blood in MS patients [12]. The exact pathogenesis mechanism of MS is not clear, but it is very possible that the breakdown of body immune balance and related autoimmune reaction causes this disease [13–15]. TRIF plays a critical role in immune response because the activation of transmembrane protein receptors TLR3 and TLR4 transduce their signals via the TRIF-inducing pathway, making TRIF a crucial target molecule for downstream signal transduction [16–18].

Downstream targets of TRIF include TRIF family-associated NF- κ B activator binding kinase 1 (TBK1) and I- κ B kinase (IKK) [19]. The signal subsequently transfers to the nucleus by phosphorylation [20] to induce the gene expression of type I interferon [21]. Recent studies have revealed the importance of the TRIF-signaling pathway in the immune response against microbial infections [22,23]. Recent *in vitro* and *in vivo* studies have identified the participation of TRIF-inducing signal pathways in the production of multiple inflammatory factors, making it an important research focus. Related studies showed that the LPS-inducing TLR4/TRIF pathway works in a cascade pathway to facilitate the secretion of IL-18, which can up-regulate TNF- γ and cause liver damage.

A previous study on MS using the EAE model reported that polyinosinic-polycytidylic acid, a double-stranded RNA analog, induced endogenous IFN-beta to suppress EAE via TLR3 signaling pathway [24]. Evidence was also presented that R(+)

WIN55,212-2 strongly induced IFN-beta expression in MS patient peripheral blood mononuclear cells and enhanced the therapeutic efficacy of murine MS model EAE [25]. It has been suggested that type I IFN suppresses EAE development via constraining IL-17 and induction of IL-27, indicating a protective role of TRIF against EAE [11]. We designed siRNA to target the TRIF gene and for the first time utilized it in EAE animal models. Interestingly, our results were in contrast to previous data and showed that the decreased mRNA level of TRIF *in vitro* and *in vivo* significantly favors neurological functions in siRNA-treated animals compared to controls. Unlike gene knock-out technique, which alters DNA sequence, RNA silencing reduces the expression of specific genes and in turn mounts a variety of responses for regulation of life activities, the mechanism of which may be distinct from that in the gene knock-out model. Here, we showed that RNAi-mediated TRIF knock-down improved the neurological outcomes of EAE animals and their neurological functions.

As the development of MS is modulated by multiple factors, the present study revealed that the down-regulation of IL-2 and IFN- γ in serum of EAE animals with TRIF siRNA treatment correlated with the alleviation of neurological dysfunctions. It has been reported that the expression of IL-2 in CNS was decreased during the recovery period of EAE, but was elevated during the induction and acute phases of the disease. Other scholars have generated EAE models using IL-2 gene knockout mice and found that only one-quarter of transgenic mice had EAE onset, in contrast to nearly 100% incidence in wild-type littermates [17]. Such results proved the lower sensitivity of IL-2-deficient mice to EAE. Further studies also elucidated the involvement of IL-2 in chronic renal failure after transplantation as other TRIF-inducing inflammatory factors, including IL-6 and IL-10, facilitate the progression of the disease [27, 28]. Clinical signs of EAE were also significantly lower in mice lacking IRF3, with impaired proliferation of Th17 cells [29]. These findings provide new insights for developing prevention and treatment methods. Other studies found that the substances released after tissue injury can work as endogenous ligands to activate relevant TLRs to exert a role in tissue recovery via TLR/TRIF-inducing cytokine release [30]. Our study further demonstrated the potentiation of serum IL-2 and IFN- γ secretion by TRIF using siRNA intervention in EAE animals.

Conclusions

TRIF siRNA can extend the incubation period of EAE, shorten its progressive period, and alleviate the neurological dysfunction, thereby protecting against EAE, possibly via its suppression of cytokine IL-2 and IFN- γ secretion.

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