

## Original Article

# TAK-242 affects Wallerian degeneration and peripheral nerve regeneration by regulating innate immunity in rats

Le Xiong<sup>1,2\*</sup>, Ruowu Shen<sup>2\*</sup>, Aiyu Ji<sup>3</sup>, Honglin Bian<sup>3</sup>, Guangqiang Sun<sup>2</sup>, Yi Wang<sup>2</sup>, Fengyu Zhang<sup>2</sup>, Jie Liang<sup>4</sup>, Bei Zhang<sup>2</sup>

<sup>1</sup>Department of Clinical Laboratory, Xiangyang NO. 1 People's Hospital, Hubei University of Medicine, Xiangyang 441000, China; <sup>2</sup>School of Basic Medicine, Qingdao University, Qingdao 266071, China; <sup>3</sup>Department of Traumatic Surgery, The Affiliated Hospital of Qingdao University, Qingdao 266003, China; <sup>4</sup>Institute for Immunology, Ruprecht-Karls-University, Heidelberg 69120, Germany. \*Equal contributors.

Received December 14, 2016; Accepted December 22, 2017; Epub March 15, 2018; Published March 30, 2018

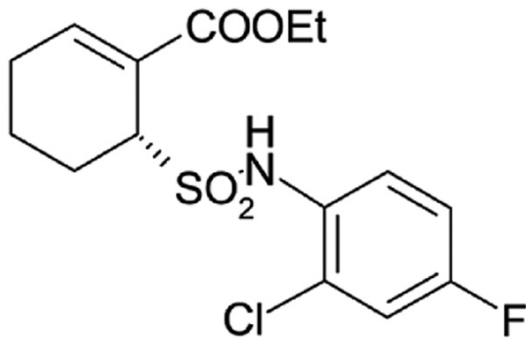
**Abstract:** Objective: The aim of this study is to distinguish whether Toll-like receptor 4 (TLR4) signaling is included in Wallerian degeneration (WD) and nerve regeneration or not after peripheral nerve injury. Methods: The rats were assigned into four groups randomly: sham group (n = 10), control group (n = 10), model group (n = 20), and treatment group (n = 20). The rats were treated with TAK-242 (0.15 mg/kg) or saline. TAK-242, a small molecular that inhibit TLR4 signaling was first given to rats intravenously 1 h before nerve injury and followed by daily injections for 7 consecutive days. Animals were sacrificed 1.5 h, 24 h, 3 d, 4 d, or 7 d after surgery. Real-time quantitative PCR (qRT-PCR) was adopted to test dynamic mRNA expressions of TIR-domain-containing adaptor inducing interferon- $\beta$  (TRIF), interleukin-1 (IL-1 $\beta$ ) and monocyte chemoattractant protein-1 (MCP-1). Immunofluorescence (IF) was used to test the expression of CD68<sup>+</sup> macrophages and iba1<sup>+</sup> Schwann cells in sciatic nerves. Luxol Fast Blue (LFB) staining was applied to test sciatic nerves myelin, and Haematoxylin-eosin (HE) staining was conducted in sciatic nerves tissue to observe the pathological variations. Immunohistochemistry (IHC) was applied to test the form of TRIF and growth associated protein-43 (GAP-43) in sciatic nerve, and sciatic function index (SFI) was applied to assess the recovery of motor function in rats. Results: The expressions of TRIF, IL-1 $\beta$ , MCP-1 and recruitments of Schwann cells and macrophages in sciatic nerve of rats were decreased significantly in treatment group compared with model group after peripheral nerve injury. Myelin clearance and axonal regeneration were delayed in treatment group compared with model group. HE staining also indicated a poor organization of repair site in treatment group in distal stump. Finally, the SFI scores of treatment group at each time point (20, 30, and 40 d post-injury) was lower than that of model group. Conclusions: TLR4 signaling might affect myelin phagocytosis and nerve regeneration during WD in rat after peripheral nerve injury via regulating the innate immune response.

**Keywords:** TAK-242, TLR4, Wallerian degeneration, peripheral nerve injury

## Introduction

Although the apiculate microsurgical reconstructive technique was applied, the patients with nerve lacerations were difficult to recover completely. Wallerian degeneration (WD) is a process that the distal portion of the nerve goes through progressive degeneration after peripheral nerve injury. WD occurs as inflammatory responses of nervous system to axonal injury, which is due to axonal decomposition in the distal nerve stump and increased blood-nerve barrier permeability, as well as receptors

activation of nearby schwann cells and macrophages. The receptors are sensitive to tissue damage [1-3]. Macrophages can swallow degenerative tissue and clear obstacles for nerve regeneration during WD, and the proliferation of Schwann cell is essential for myelin formation and axonal regeneration. In the early stage of injury, shortening the time of WD improves the quality of nerve regeneration. Modulation of inflammatory responses and improvement of axonal regeneration are two main aims at managing peripheral nerve injuries [4].



**Figure 1.** Structure of TAK-242 (resatorvid).

TLR4 is a classic pattern recognition receptor which plays a fundamental role in pathogen recognition and innate immunity activation. It is up-regulated after injury and activate intracellular signaling pathways through MyD88 dependent or independent way, and several others. TLR4 recognizes various microbial structural parts called pathogen-associated molecular patterns (PAMPs), for instance, lipopolysaccharide (LPS) [5]. While in the absence of infection, damage-associated molecular pattern molecules (DAMPs) can also trigger the activation of TLR4 signaling [6]. Normally, there are a lot of necrotic cells in local injured nerve and the HSP60, HSP70 expression at the damaged sites, and ECM parts in damaged tissue [7]. Furthermore, it has been confirmed that TLR4 exist in the nervous system. For example it presented predominantly on glial cells in central nervous system, on microglia in the event of neuropathy [8, 9], and on Schwann cells in peripheral nervous system. It has been reported that injection of LPS can promote myelin debris removal during WD after spinal cord injury [10]. However, there are few studies detected the role of TLR4 in peripheral nervous system injury.

Ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242) (**Figure 1**) is a newly discovered cytokine inhibitor. Study show TAK-242 is optionally combined with Cys747 in the TIR domain of TLR4 and then disordered the connection between TLR4 and TLRAP, TRAM [11, 12]. TAK-242 inhibited TIRAP-mediated nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation and TRAM-mediated NF- $\kappa$ B and interferon sensitive response element (ISRE) activation in HEK293 cells which stably expressing TLR4,

MD-2, and CD14. Meanwhile the TAK-242 treatment also inhibited LPS-induced activation of endogenous interleukin-1 receptor-associated kinase (IRAK-1) in RAW264.7 cells [12]. Moreover, recently study has shown that TAK-242 suppressed the generation of inflammatory cytokines and nitric oxide from LPS stimulated monocytes, macrophages, and PBMCs [13].

It is generally received that macrophages and Schwann cells are involved in myelin debris removal during WD [14, 15]. Macrophages are recruited to the site immediately after peripheral nerve injury, with the amount peaking at 3 d post-injury and Schwann cells reached the peak of proliferation at 3 d post-injury while the peak of demyelination at 7 d post-injury. Inflammatory cytokines/chemokines are expressed at 1 h post-injury at sciatic nerve, with most production occurring at 24 h [16-19]. Axonal restoration can be evaluated by GAP-43 and motor function recovery can be evaluated by sciatic function index (SFI). In the current study, we indicated the influence of TAK-242 on early macrophages/Schwann cells recruitment and myelin debris clearance in rats with sciatic nerve injury, and elucidated the role of TLR4 signaling pathway in the early removal of myelin and axonal restoration during WD after peripheral nerve injury.

## Materials and methods

### *Sciatic nerve injury and animal treatment*

A total of 60 adult male Wistar rats (180~220 g) were randomized into four groups: sham group (n = 10), control group (n = 20), model group (n = 20), and treatment group (n = 20). Anesthetize the rats with 10% chloral hydrate (0.3 ml/kg; intraperitoneal). The model rats were transected right side sciatic nerve with a microsurgical scissor. Repair the nerve by microsuture using 9-0 noninvasive sutures and the skin opening was closed with nylon 4-0 sutures. Model group and treatment group were treated with 0.9% saline or TAK-242 (0.15 mg/kg; Cayman Chemical USA) respectively. The first intravenous injection was given at 1 h before injury, which was followed by daily injections for 7 consecutive days [20]. Rats in sham group were only exposed the sciatic nerve, while rats in control group were exposed the sciatic nerve and then treated with TAK-242.

## Toll-like receptor 4 signaling is involved in Wallerian degeneration

**Table 1.** The primers sequence in the test

Genes	Forward primers	Reverse primers
TRIF	5'-CTGTCATTTCTTGAGCGTGA-3'	5'-CCAGGTTATTGCTTCTGTGG-3'
IL-1 $\beta$	5'-GCCAACAAGTGGTATTCTCCA-3'	5'-CCGCTTTTCATCACACAGGA-3'
MCP-1	5'-AGGACTTCAGCACCTTTGA-3'	5'-TTCTCTGTCATACTGGTCACTTC-3'
$\beta$ -actin	5'-CACCCGCGAGTACAACCTTC-3'	5'-CCCATACCCACCATCACACC-3'

Animals were sacrificed at 1.5 h, 24 h, 3 d, 4 d, and 7 d after surgery.

### *Real-time quantitative RT-PCR (qRT-PCR)*

Extract total RNA from sciatic nerves using trizol according to the manufacturer's protocol (TaKaRa Dalian, China) and RNA purity was estimated by measuring OD260/280 ratio. The RNA was reversely transcribed to cDNA using a Prime-Script reagent Kit (TaKaRa Dalian, China) according to the manufacturer's instructions and RT-PCR was performed using SYBR Green Premix Ex Taq (TaKaRa) on an Eppendorf Realplex<sup>4</sup> real-time PCR System. The PCR program was as below: 95°C 30 s; 40 cycles at 95°C 5 s, 64°C 30 s. All reactions were run in triplicate. Amplification efficiencies were validated and normalized to  $\beta$ -actin and the amount of TRIF, IL-1 $\beta$  and MCP-1 mRNA were calculated according to a standard curve. Relative mRNA levels were quantified using 2<sup>- $\Delta\Delta$ Ct</sup> method [21]. The sequences of all primers are listed in **Table 1**.

### *Immunofluorescence*

The rats were deeply anesthetized after the injection of 10% chloral hydrate. Then sciatic nerve segments were excised and post-fixed in 4% PFA after transcardially perfused and cryo-protected in a 20-30% sucrose gradient until sectioning. Sciatic nerve segments were embedded in liquid nitrogen with optimal cutting temperature (OCT) compound (Sakura Finetek). A 15- $\mu$ m-thick section was cut and mounted onto slides that have permanent positive-charged surfaces (Citoglas Jiangsu, China), and stored at -20°C. Tissue sections were blocked 1 h in 10% normal goat serum (Cwbio Beijing, China) at room temperature, cultured with anti-iba1 or anti-CD68 respectively (Bioss Beijing, China) overnight at 4°C. Following day, sections were cultured by FITC-conjugated secondary antibodies for 2 h, washed with PBS and examined under fluorescence microscope (Olympus IX-73, Tokyo, Japan). Contrast between positive signal and background remained

constant in all images. The histochemical staining part in the sampling area was then gauged using Image-pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA). For the quantification of CD68 and iba1 immunolabeling, the proportional area

of tissue occupied by labeling within a region of interest was measured.

### *Luxol fast blue (LFB) staining*

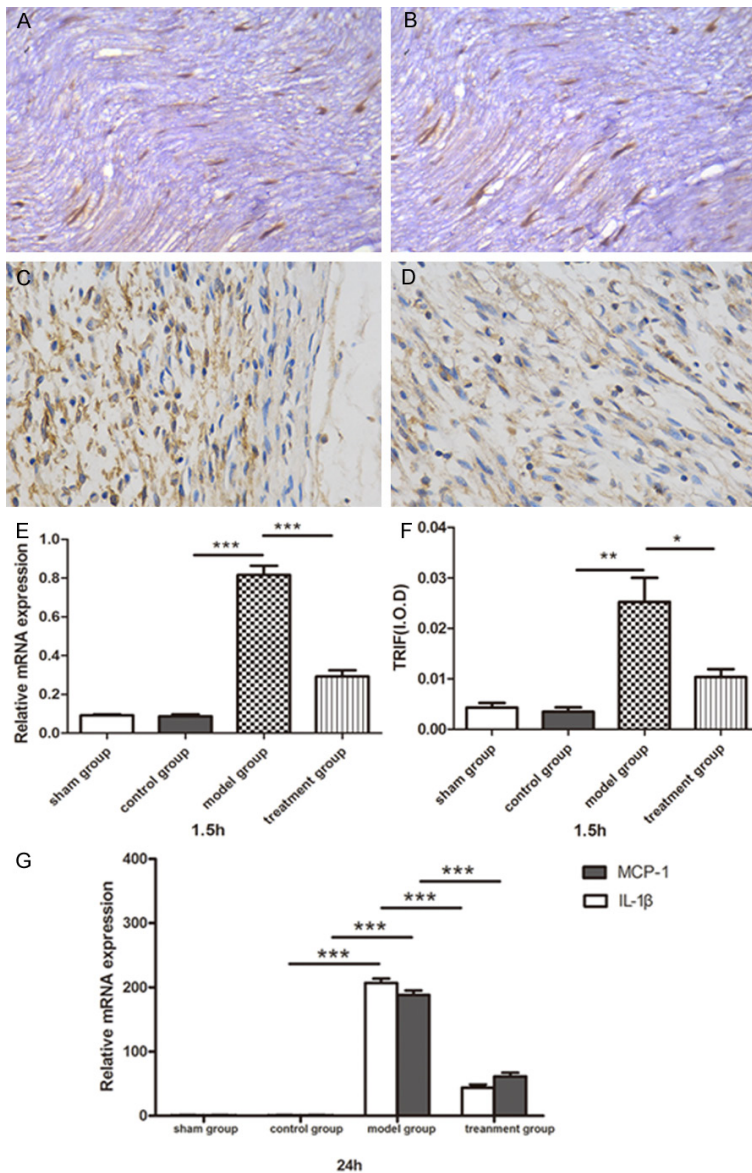
Myelin debris clearance was assessed with LFB staining at 7 d after injury according to standard routine. Dewatered and cultured the sections in 0.1% LFB solution at 37°C overnight. Following day, chill the slides at 4°C, culture in 0.05% lithium carbonate solution, differentiate in 70% ethanol, and covered with glass [10]. Image-Pro plus 6.0 was designed to quantify immune-staining results. Blue staining was seen as positive and positive area of integral optical density (IOD) as a quantitative standard.

### *Immunohistochemistry*

Immunohistochemical SP method was adopted. The tissue sections were cultivated with a specific antibody overnight at 4°C. Next day, the tissue section was cultivated with biotinylated secondary antibody for 20 minutes at 37°C. After washing with PBS, the sections were cultivated with avidin-HRP conjugate for 20 minutes at 37°C and developed with 3,3 diaminobenzidine (DAB). After counterstained with haematoxylin for 5 minutes, the tissue sections were washed and then dewatered in graded ethanol and cleared in xylene (3X). In the end, the sections were covered and examined under microscope. Image-Pro plus 6.0 was designed to quantify immune-staining results. Brown staining was regarded as positive and positive area of integral optical density (IOD) as a quantitative standard [22].

### *Haematoxylin-eosin (HE) staining*

Pathological change in the sciatic nerve distal stump was evaluated at 7 d post-injury. After the rats were sacrificed, vessels were perfused with PBS, followed by 4% paraformaldehyde. Paraformaldehyde-fixed sciatic nerve specimens were dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks.



**Figure 2.** TRIF, IL-1 $\beta$  and MCP-1 expression were reduced in the injured sciatic nerve of rats after treatment with TAK-242. (A-D) TRIF-immunopositive cells (brown staining) from rats in sham group (A), control group (B), model group (C) and treatment group (D) 7 d after surgery. (E) TRIF mRNA level was decreased significantly in rats from treatment group 1.5 h after surgery (n = 3-5 per group). (F) Semi-quantitative analysis: integral optical density of TRIF<sup>+</sup> cell (n = 3-5 per group). (G) IL-1 $\beta$  and MCP-1 mRNA expression were decreased significantly in the sciatic nerve of rats from treatment group 24 h after surgery (n = 3-5 per group). Magnification: 400  $\times$ . The values are the means  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 in comparison with each other.

Longitudinal serial sections of 5  $\mu$ m thicknesses were obtained from a 10 mm length of nerve, including the anastomosis site. Then stain the sections with HE under standard procedures.

#### Walking track analysis

The SFI was adopted to assess the motor function restoration after sciatic nerve lesion. This test was performed for rats at 10, 20, 30, 40, and 50 days after surgery. To assess hind limb performance, each rat was put on a walking track (8.5 cm  $\times$  50 cm) darkened at one end. The suitable size of white office paper was cut to put at the bottom of the track. Immersed the rat's hind limbs in Chinese ink, and permit the rat to walk down the track with its footsteps printing on the paper. The sciatic nerve function index (SFI) was determined according to the Bain calculation formulas [23]. SFI = -100% suggesting complete loss of nerve function, SFI = 0~11% suggesting normal nerve function, SFI = -11%~-100% suggesting part nerve function restored [24].

#### Statistical analyses

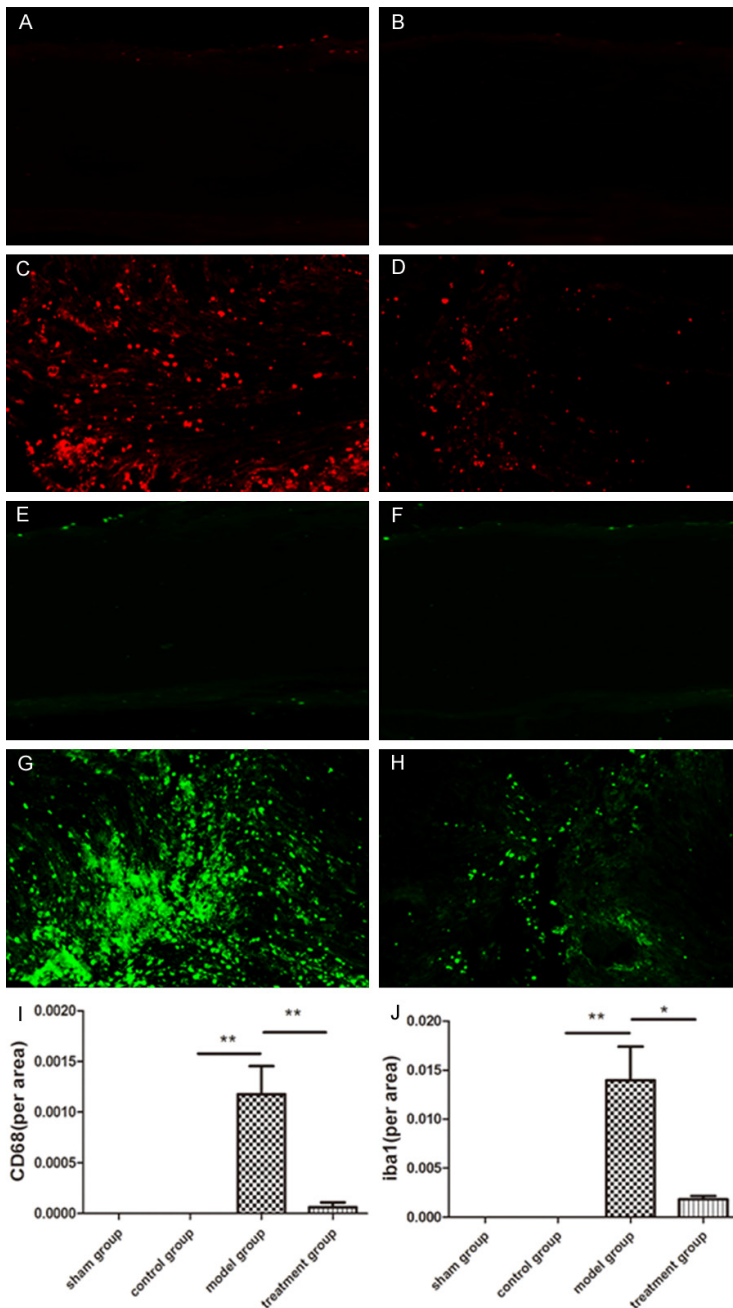
All results were expressed as the mean  $\pm$  SEM. SPSS 17.0 software was used for statistic analysis, and student's t-test was carried out for group comparison. P < 0.05 was considered statistically significant.

#### Results

##### TAK-242 could decrease expression of TRIF, IL-1 $\beta$ and MCP-1

The mRNA level of TRIF from sciatic nerve was measured at 1.5 h post-injury, and the mRNA levels of IL-1 $\beta$  and MCP-1 in sciatic nerve were measured at 24 h post-injury. Results showed that the expression levels of them had little difference between the sham group and the control group. Therefore, TAK-242 won't cause changes in





**Figure 3.** Macrophage and Schwann cell recruitment decreased in rats after treatment with TAK-242. (A-D) Typical fluorescence of CD68-immunopositive macrophages of rats in sham group (A), control group (B), model group (C) and treatment group (D) 3 d after surgery. (E-H) Typical fluorescence of iba1-immunopositive Schwann cells of rats in sham group (E), control group (F), model group (G) and treatment group (H) 3 d after surgery. (I, J) Semi-quantitative analysis of CD68<sup>+</sup> macrophages and iba1<sup>+</sup> Schwann cells in the sciatic nerve distal stump 3 d after surgery (n = 6-8 per group). Magnification: 200 ×. The values are the means ± SEM. \*p < 0.05, \*\*p < 0.01 in comparison with each other.

in model group compared with sham group and control group (Figure 2E and 2G). And the mRNA level of TRIF in treatment group decreased significantly compared with the model group (Figure 2E). Consistent with qRT-PCR result, the protein level of TRIF, which was detected by IHC, also decreased in treatment group (Figure 2F). In addition, IL-1 $\beta$  mRNA increased dramatically in the model group compared with sham and control group, while the TAK-242 treatment decreased IL-1 $\beta$  mRNA to 20%. Similar with IL-1 $\beta$ , MCP-1 mRNA increased significantly in the model group compared with sham and control group and decreased obviously in the treatment group (Figure 2G). The results suggested that TAK-242 could quickly and directly suppress TLR4 signaling and decrease the production of TRIF, IL-1 $\beta$  and MCP-1 in injured sciatic nerve.

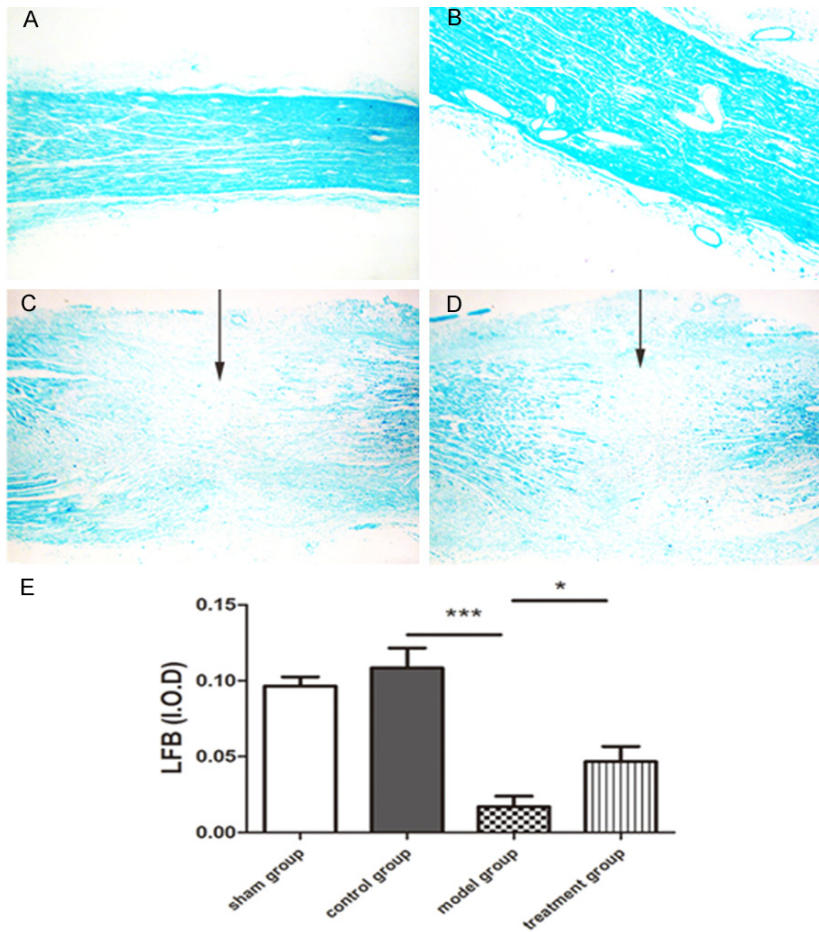
*TAK-242 could inhibit macrophages and Schwann cells recruitment*

Macrophages mediate myelin phagocytosis through receptor-dependent way during WD. To trace the amounts of macrophages/Schwann cells present in lesion site, the expressions of CD68 and iba1 on macrophages/Schwann cells were examined at the injury site of sciatic nerve. A semi-quantitative analysis showed that the quantity of CD68<sup>+</sup> macrophages in model group significantly increased compared with that in sham and control group, and that in treatment group significantly decreased compared with that in model group at 3 d post-surgery (Figure 3I). Similarly, the amounts of iba1<sup>+</sup> Schwann cells were decreased obviously in treatment group at 3 d post-injury

other indicators in rats. While, the mRNA levels of TRIF, IL-1 $\beta$  and MCP-1 increased significantly

amounts of iba1<sup>+</sup> Schwann cells were decreased obviously in treatment group at 3 d post-injury

## Toll-like receptor 4 signaling is involved in Wallerian degeneration



**Figure 4.** Myelin debris clearance was delayed in rats after treatment with TAK-242. (A-D) LFB staining of longitudinal sections of sciatic nerve 7 d after surgery: sham group (A), control group (B), model group (C) and treatment group (D). (E) Semi-quantitative analysis of LFB staining from rats in model group and treatment group 7 d after surgery (n = 3-5 per group). Magnification: 40 ×. The values are presented as means ± SEM. \*p < 0.05, \*\*\*p < 0.001 in comparison with each other. The arrow points to the stoma.

(**Figure 3J**). These results suggested that TAK-242 could decrease early recruitments of macrophages/Schwann cells in the distal stumps of sciatic nerve and TLR4 signaling is important for activating macrophages/Schwann cells during WD.

### *TAK-242 could delay myelin debris clearance*

We made tissue sections from sciatic nerve at 7 d post-injury, and the degree of myelin clearance was assessed by LFB staining. In the sham group and control group, the IODs of LFB staining which used to observe myelin were great, indicating normal myelin structure. While the IOD decreased significantly in model group, indicating demyelization during WD. Sciatic

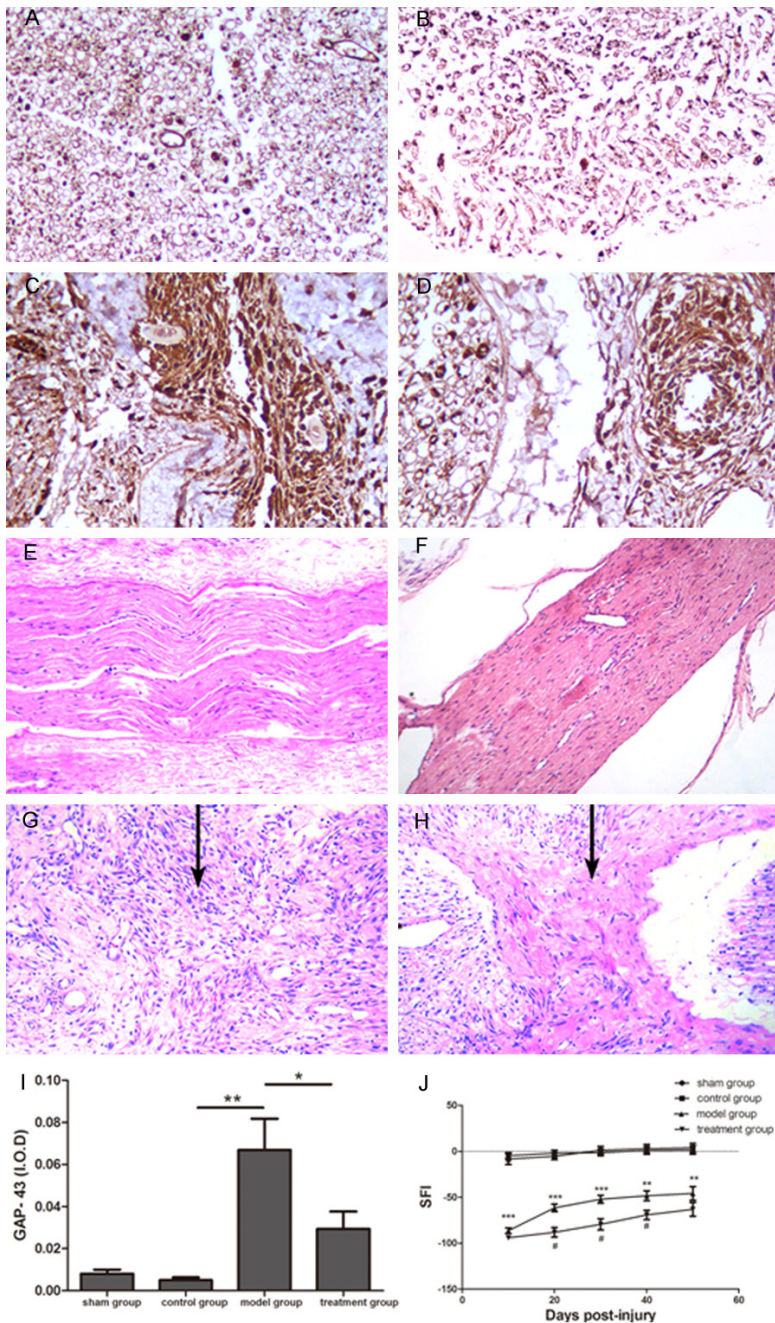
nerve tissues from the treatment group showed a higher IOD compared with the model group, indicating myelin debris clearance delay (**Figure 4E**). These results were consistent with our early finding that recruitments of macrophages/Schwann cells were inhibited by TAK-242 treatment.

### *TAK-242 could delay axonal regeneration and recovery of motor function*

GAP-43-immunopositive profiles were used to evaluate the axonal regeneration (**Figure 5A-D**). The quantification of GAP-43 showed that the IOD increased significantly in model group compared with that in sham and control group, and decreased significantly in treatment group compared with that in model group (**Figure 5I**), which indicating a delay in early axonal regeneration in TAK-242 treated rats.

Histological studies on HE sections of the sciatic nerve distal stump showed the fragmentation of distal axon and myelin, proliferation of Schwann cells, infiltration of macrophage in the distal nerve segment, and phagocytosis of degenerative myelin debris. The sham group and control group exhibited normal axonal organization, and the model group showed a fair organization at repair site during WD. When compared with the model group, the treatment group showed less inflammatory cell infiltration and regenerative axonal continuity from proximal to distal end, indicating a poor organization at repair site (**Figure 5E-H**). This delayed axonal healing in treatment group correlated with the decreased macrophages/Schwann cells recruitment and delayed myelin debris clearance.





**Figure 5.** Motor function recovery and early axonal regeneration were delayed in rats after treatment with TAK-242. (A-D) Transverse sections of GAP-43-immunopositive axon of rats in sham group (A), control group (B), model group (C) and treatment group (D) 4 d after surgery. (E-H) HE staining of longitudinal sections of sciatic nerve 4 d after surgery (n = 3-5 per group): sham group (E), control group (F), model group (G) and treatment group (H). (I) Semi-quantitative analysis of IOD of GAP-43<sup>+</sup> axon 4 d after surgery (n = 3-5 per group). (J) Recovery of motor functions determined by the SFI of rats in model group and treatment group over a circle of 50 d (n = 3-5 per group). Magnification for IHC: 400 ×. Magnification for HE staining: 100 ×. The values are expressed as means ± SEM. \* p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 in comparison with each other. #p < 0.05 in comparison with the model group. The arrow points to the stoma.

Finally, SFI was determined to evaluate the recovery of motor function at 10, 20, 30, 40, and 50 days post-injury. Walking track analysis revealed a spontaneous recovery of neurological function in the model group accompanied with a gradually increased SFI value over 50 days. The scores of sham and control group showed the normal sciatic nerve function. And the SFI values of model group at each time point (10, 20, 30, 40 and 50d post-injury) was lower than that of sham and control group, suggesting that sciatic nerve functions were lost completely. And the SFI values of treatment group at each time point (20, 30, and 40d post-injury) was lower than that of model group, suggesting that treatment with TAK-242 delayed motive function recovery of the severed sciatic nerve (Figure 5J).

## Discussion

Increasing evidence show individuals with peripheral nerve injury are subject to inflammatory response and TLR4 signaling pathway is involved in such inflammatory response. Our study firstly explain that systemic administration of TLR4 signaling inhibitor TAK-242 is able to delay the activation of innate immune responses during WD after peripheral nerve injury in vivo, which was proved by reduced inflammatory cytokines production, decreased macrophages/Schwann cells activation and myelin debris removal, ultimately delayed axonal and motor recovery.

PNS injury is followed by WD, the process that distal portion of the nerve goes through progressive degeneration and inflammatory responses of nervous system to axonal injury due to the production of cytokines and inflammatory mediators. According to different adaptor proteins, there are at least two main distinct pathways, a MyD88-dependent pathway or a MyD88-independent TRIF/TICAM-1 pathway. All TLRs except TLR3 can activate MYD88, leading to the phosphorylation of I $\kappa$ B and the subsequent nuclear localization of NF- $\kappa$ B. Activation of NF- $\kappa$ B triggers production of pro-inflammatory cytokines such as TNF and IL-1 $\beta$ . Conversely, TLR3 and TLR4 can activate IFN regulatory factors (IRF) through the adaptor TRIF, and finally triggers the production of IFN- $\beta$  [25]. TAK-242 can explicitly bind to TLR4 in the midst of 10 TLRs. TLR4 signaling requires the adaptor TIRAP/Mal, which is involved in the MyD88-dependent pathway, or, TRAM/TICAM-2, another adaptor molecule involved in the MyD88-independent pathway. It has been shown that TAK-242 repressed the connection of TIRAP and TRAM with TLR4 [12]. In order to verify whether injection of TAK-242 from tail vein can inhibit the TLR4 signaling, we tested the expression of TRIF from sciatic nerve. The consequence showed that repeated injections of TAK-242 down-regulated the expression of TRIF in the sciatic nerve (**Figure 2E** and **2F**). The two important pro-inflammatory cytokines downstream of TLR4 is IL-1 $\beta$  and MCP-1, IL-1 $\beta$  was secreted by recruited macrophages/Schwann cells and enhanced myelin phagocytosis by turns [18]. While MCP-1 was expressed at 1.5 h post-injury at sciatic nerve with most production at 24 h [16] mainly in Schwann cells. As expected, although the IL-1 $\beta$  and MCP-1 mRNA were up-regulated in sciatic nerve at 24 h after injury, repeated TAK-242 intravenous injections could suppress them (**Figure 2G**). Our results implicated TLR4 signaling play critical role in the process of WD due to the early synthesis of IL-1 $\beta$  and MCP-1.

After nerve injury, it is essential to recruitment macrophages into the nerve membrane to phagocytose myelin debris for its degeneration and regeneration [26]. They accumulate at epineuria initially and begin to move to the endoneurium in quantity 3 days later [27]. In the research, we explained that administration of TAK-242 inhibited the recruitment and activa-

tion of macrophages/Schwann cells in the lesion at 3 days post-injury during WD (**Figure 3D** and **3H**). Moreover, myelin clearance was confirmed at 7 days after injury in degenerating sciatic nerve distal stump following with changes of LFB staining density (**Figure 4A-D**). Quantitative analysis also revealed that TAK-242 treated rats displayed a decline amount of macrophages/Schwann cells and a delay in myelin debris clearance within sciatic nerve distal stump (**Figures 3I, 3J** and **4E**).

Myelin removal can reduce the inhibitory factors of axon growth and provide a more favorable environment for axonal regeneration, which normally starts at 4 day after injury [10, 28]. The level of GAP-43 protein was used for assess axonal regeneration and detected with immunohistochemistry at a distance of 4 mm away from the lesion, similar method has been used by Krekoski et al to evaluate axonal regeneration [29]. Quantification analysis showed an obvious decreased signal in treatment group compared with model group, indicating that the early regeneration was delayed in the rats with inhibited TLR4 signaling (**Figure 5I**). Moreover, HE staining of sciatic nerve at 7 days post-injury showed poor organization at repair site in treatment group, which further supported the results showed above (**Figure 5H**). In a word, the findings showed that TLR4 signaling was critical for raising macrophages/Schwann cells and clearing myelin debris during the process of WD. In addition, we examined the recovery of motor function of rats by SFI and it indicated that treatment with TAK-242 probably delayed the later motive function recovery of the severed sciatic nerve (**Figure 5J**). This advises that TLR4 signaling take part in raising macrophages/Schwann cells and clearing myelin debris in times of WD, and these processes may affect early regeneration of axonal and later recovery of motor function.

In conclusion, TLR4 signaling may affect axonal regeneration and motor function recovery through regulation of early macrophages/Schwann cells recruitment and myelin debris clearance during WD. There is still controversy for the role of different types of macrophages during WD, further study need to be done to clarify. In addition, several factors that cause the activation of TLR4 signaling in peripheral nerve injury also need more in-depth study.



## Acknowledgements

The present study was supported by the Science and technology program of Shandong Province (grant no. J14LK10).

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Bei Zhang, School of Basic Medicine, Qingdao University, Qingdao 266071, China. Tel: +86-532-83780021; E-mail: zhangbei124@aliyun.com

## References

- [1] Gaudet AD, Popovich PG and Ramer MS. Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *J Neuroinflammation* 2011; 8: 110.
- [2] Ha GK, Huang Z, Streit WJ and Petitto JM. Endogenous T lymphocytes and microglial reactivity in the axotomized facial motor nucleus of mice: effect of genetic background and the RAG2 gene. *J Neuroimmunol* 2006; 172: 1-8.
- [3] Temporin K, Tanaka H, Kuroda Y, Okada K, Yachi K, Moritomo H, Murase T and Yoshikawa H. Interleukin-1 beta promotes sensory nerve regeneration after sciatic nerve injury. *Neurosci Lett* 2008; 440: 130-133.
- [4] Girard C, Liu S, Cadepond F, Adams D, Lacroix C, Verleye M, Gillardin JM, Baulieu EE, Schumacher M and Schweizer-Groyer G. Etifoxine improves peripheral nerve regeneration and functional recovery. *Proceedings of the National Academy of Sciences* 2008; 105: 20505-20510.
- [5] Pióciennikowska A, Hromada-Judycka A, Borzęcka K and Kwiatkowska K. Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cellular and Molecular Life Sciences* 2014; 72: 557-581.
- [6] Kato J and Svensson CI. Role of extracellular damage-associated molecular pattern molecules (DAMPs) as mediators of persistent pain. *Prog Mol Biol Transl Sci* 2015; 131: 251-279.
- [7] Willis D, Li KW, Zheng JQ, Chang JH, Smit AB, Kelly T, Merianda TT, Sylvester J, van Minnen J and Twiss JL. Differential transport and local translation of cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons. *J Neurosci* 2005; 25: 778-791.
- [8] Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S, Na HS, Oh SB and Lee SJ. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem* 2007; 282: 14975-14983.
- [9] Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* 2007; 19: 3-10.
- [10] Vallieres N, Berard JL, David S and Lacroix S. Systemic injections of lipopolysaccharide accelerates myelin phagocytosis during Wallerian degeneration in the injured mouse spinal cord. *Glia* 2006; 53: 103-113.
- [11] Kawamoto T, Li M, Kitazaki T, Iizawa Y and Kimura H. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *Eur J Pharmacol* 2008; 584: 40-48.
- [12] Matsunaga N, Tsuchimori N, Matsumoto T and Li M. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Mol Pharmacol* 2011; 79: 34-41.
- [13] Li M, Matsunaga N, Hazeki K, Nakamura K, Takashima K, Seya T, Hazeki O, Kitazaki T and Iizawa Y. A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. *Mol Pharmacol* 2006; 69: 1288-1295.
- [14] Bruck W, Huitinga I and Dijkstra CD. Liposome-mediated monocyte depletion during wallerian degeneration defines the role of hematogenous phagocytes in myelin removal. *J Neurosci Res* 1996; 46: 477-484.
- [15] Stoll G, Griffin JW, Li CY and Trapp BD. Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. *J Neurocytol* 1989; 18: 671-683.
- [16] Carroll SL and Frohnert PW. Expression of JE (monocyte chemoattractant protein-1) is induced by sciatic axotomy in wild type rodents but not in C57BL/Wld(s) mice. *J Neuropathol Exp Neurol* 1998; 57: 915-930.
- [17] Perrin FE, Lacroix S, Aviles-Trigueros M and David S. Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1alpha and interleukin-1beta in Wallerian degeneration. *Brain* 2005; 128: 854-866.
- [18] Shamash S, Reichert F and Rotshenker S. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. *J Neurosci* 2002; 22: 3052-3060.
- [19] Stoll G, Jander S and Myers RR. Degeneration and regeneration of the peripheral nervous system: from Augustus Waller's observations to neuroinflammation. *J Peripher Nerv Syst* 2002; 7: 13-27.

## Toll-like receptor 4 signaling is involved in Wallerian degeneration

- [20] Sha T, Sunamoto M, Kitazaki T, Sato J, Li M and Iizawa Y. Therapeutic effects of TAK-242, a novel selective Toll-like receptor 4 signal transduction inhibitor, in mouse endotoxin shock model. *Eur J Pharmacol* 2007; 571: 231-239.
- [21] Tellinghuisen J and Spiess AN. Comparing real-time quantitative polymerase chain reaction analysis methods for precision, linearity, and accuracy of estimating amplification efficiency. *Anal Biochem* 2014; 449: 76-82.
- [22] Liang J and Zhang B. Preventive effect of halofuginone on concanavalin A-induced liver fibrosis. *PLoS One* 2013.
- [23] Bain JR, Mackinnon SE and Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. *Plast Reconstr Surg* 1989; 83: 129-138.
- [24] de Medinaceli L, Freed WJ and Wyatt RJ. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. *Exp Neurol* 1982; 77: 634-643.
- [25] Stokes JA, Cheung J, Eddinger K, Corr M and Yaksh TL. Toll-like receptor signaling adapter proteins govern spread of neuropathic pain and recovery following nerve injury in male mice. *J Neuroinflammation* 2013; 10: 148.
- [26] Griffin JW, George R and Ho T. Macrophage systems in peripheral nerves. A review. *J Neuropathol Exp Neurol* 1993; 52: 553-560.
- [27] Taskinen HS and Roytta M. The dynamics of macrophage recruitment after nerve transection. *Acta Neuropathol* 1997; 93: 252-259.
- [28] Aguayo AJ, Peyronnard JM and Bray GM. A quantitative ultrastructural study of regeneration from isolated proximal stumps of transected unmyelinated nerves. *J Neuropathol Exp Neurol* 1973; 32: 256-270.
- [29] Krekoski CA, Neubauer D, Graham JB and Muir D. Metalloproteinase-dependent predegeneration in vitro enhances axonal regeneration within acellular peripheral nerve grafts. *J Neurosci* 2002; 22: 10408-10415.