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말초신경 손상 후 슈반세포의 활성화와 Wallerian degneration 에서 톨유사수용체 3 의 역할 규명

Toll-like Receptor 3 contributes to inflammatory Schwann cell activation and Wallerian degeneration after peripheral nerve injury

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ABSTRACT

Toll-like receptor 3 contributes to inflammatory Schwann cell activation and Wallerian degeneration after peripheral nerve injury

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It is well known that Schwann cells play an important role in Wallerian degeneration after peripheral nerve injury. Recently, it was reported that toll-like receptor (TLR) signaling contributes to Wallerian degeneration. Moreover, previous studies had found that TLR ligands-induced inflammatory Schwann cell is activated via TLR2 and 3. However, the role of TLR3 in Wallerian degeneration after peripheral nerve injury is still poorly understood. Hence, the objective of this study is to establish a clearer understanding of the role of TLR3 in Wallerian degeneration after a

peripheral nerve injury. It was found that sciatic nerve crush injury reduced the number of degenerating myelin axons in TLR3 knock-out mice. After 7 days, TLR3 knock-out mice showed delayed sciatic nerve degeneration compared with WT mice. In addition, macrophage infiltration into injury site was significantly increased in WT mice, but not in TLR3 knock-out mice. The nerve injury-induced expression of macrophage infiltrated-related chemokines such as CC-chemokine ligand (CCL)2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES was compromised in Schwann cells of TLR3 knock-out mice *in vitro* and *in vivo*. Similarly, the TLR3 ligands-induced chemokine expression was reduced in Schwann cells derived from TLR3 KO mice. Finally, polyinosinic-polycytidylic acid (poly(I:C)), a synthetic TLR3 agonist, injection into the sciatic nerve of the rat induced macrophage infiltration *in vivo*. Taken together, these data show that TLR3 is required for the inflammatory Schwann cell activation and contributes to Wallerian degeneration after peripheral nerve injury.

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Key Words :

Toll-like receptor 3, Schwann cells, Wallerian degeneration, Sciatic nerve injury, chemokine, macrophage recruitment

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CONTENTS

Ⅰ. Introduction

After nerve injury, two processes are involved in nerve repair, Wallerian degeneration and nerve regeneration. Wallerian degeneration occurs when the nerve fibers are damaged by crush, traumatic, transection, chemical and/or toxic events (Coleman, 2005). Wallerian degeneration in peripheral nerve system and central nerve system differ with regard to the types of cells involved. Contrary to oligodendrocytes in central nerve system, Schwann cells in peripheral nerve system play important role in Wallerian degeneration after nerve injury (Vargas and Barres, 2007). Upon peripheral nerve injury, the axon stump distal to the injury site are getting degraded and produce axonal debris. It works as an axonal outgrowth inhibitor, thus, rapid and efficient clearance of axonal debris is an essential prerequisite for successful nerve regeneration to proceed (Grados-Munro and Fournier, 2003). Activated Schwann cells clean up the axonal debris by phagocytosis. In addition, Schwann cells recruit macrophage to the injury site by secreting macrophage-recruiting chemokines. Infiltrated macrophages also participate in the clearance of axonal debris during Wallerian degeneration after peripheral nerve injury (Stoll et al., 1989) Likewise, the function of Schwann cells during Wallerian degeneration has been well characterized. However, until now, the mechanism of Schwann cell activation is remained unknown.

Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR) and recognize the conserved molecular structures found in pathogens called pathogen-associated molecular patterns (PAMPs) (Akira and Sato, 2003). So far, there are many PAMPs have been identified, such as tri-acyl lipopeptides from mycoplasma, peptidoglycan from Gram positive bacteria, viral double-strand RNA,

lipopolysaccharide from Gram-negative bacteria (Hoshino et al., 1999; Hemmi et al., 2000; Takeuchi et al., 2002; Shimizu et al., 2007). In addition to exogenous ligands for TLRs, recently, additional endogenous ligands also have been reported. These include necrotic cells and amyloid beta for TLR2, heat shock proteins such as Hsp60, Hsp70 and Gp96 for TLR2/4, mRNAs or RNAs with hairpin structures for TLR3, and fibrinogen for TLR4 (Ohashi et al., 2000; Li et al., 2001; Smiley et al., 2001; Asea et al., 2002; Kariko et al., 2004; Jana et al., 2008). These endogenous ligands activate TLRs and lead to inflammatory response after tissue or nerve damage. Therefore, it appears that TLRs are involved in Wallerian degeneration after peripheral nerve injury. However, the roles and mechanisms of the TLRs during Wallerian degeneration are not fully understood.

It has been reported that TLR4 and TLR2- deficient mice had a decreased recruitment of macrophages, persisted myelin debris in the distal nerve stump, and delayed Wallerian degeneration during the nerve regeneration process (Boivin et al., 2007). The impaired nerve regeneration was mainly attributed to the delayed axonal demyelination in TLR2 and TLR4 knock-out mice (Wu et al., 2013). Likewise, it has been well-studied that TLR2 and TLR4 have a role in Wallerian degeneration after peripheral nerve injury. Furthermore, Schwann cells treated with necrotic neuronal cells (NNC) become activated and express proinflammatory genes through TLR2 and TLR3 activation (Lee et al., 2006). It has been previously tested that not only TLR2 and TLR4 but also TLR3 are expressed in rodent Schwann cells (Lee et al., 2007). Based on these studies, I postulated that TLRs in Schwann cell might activate Schwann cells and thereby promote Wallerian degeneration. Specifically, I investigate the role of TLR3 in Schwann cells activation and wallerian degeneration by comparing pathological processes in WT with TLR3 knock-out mice after peripheral nerve injury.

Ⅱ. Materials and Methods

Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University. All mice and Rat were housed in an animal facility with a specific pathogen-free barrier at a temperature of 23±2℃. They experienced a 12 h light-dark cycle, and they were allowed to access food and water *ad libitum*. Adult TLR3 knock-out mice of C57BL/6 background and C57BL/6 mice aged 8 to 10 weeks were used for all experimentation. Adult male Sprague-Dawley rats (250-300 g) were used for histology.

Sciatic nerve crush injury

Animals were deeply anesthetized for surgery by pentobarbital sodium (50 mg/kg) via intraperitoneal injection. Hairs were carefully shaved using surgical clippers then skin was gently dissected from the underlying musculature. The right sciatic nerve at the mid-thigh level was exposed and then was crushed by fine Dumon #5 forcep for consistent 30 seconds. For sham operated mice, the right sciatic nerve was exposed, but left untouched. After release of the forcep, the surgical site was carefully closed to protect it from contamination. Finally, the skin incision was also closed using surgical staples. The all animal awaked and remained healthy in postoperative care room. 1, 3 days after surgery, nerves were collected.

Injection

PBS or poly(I:C) was introduced into rat sciatic nerve subepineurally as described elsewhere (Dyck et al., 1982; Deretzi et al., 2000). Briefly, adult male SD rats (250−300 g) were anaesthetized with pentobarbital sodium. At the mid-thigh, the right sciatic nerve was surgically exposed and fixed with a pair of forceps. A 30G needle attached to a 10 μL Hamilton syringe was inserted subepineurally in the sciatic nerve, and 5 μl PBS or poly(I:C) (50 μg) was carefully injected for a period of 5 min, then the skin was sutured. After 3 and 7 days, the sciatic nerves were removed for experiments.

Toluidine blue staining

Toluidine blue staining was performed as previously described (Lee et al., 2007) with the following little modification. Three and seven days post sciatic nerve crush injury, WT and TLR3 knock-out mice were perfused with 0.1 M PB (pH 7.4), and fixed with a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB. The sciatic nerves were removed and immersed in the same fixative overnight. Then, the fixed sciatic nerves were rinsed and cut both at the midpoint of the injected site and at a position 3 mm distal to the first cut. The segments were postfixed in 2% phosphate-buffered osmium tetroxide solution for 2 h, dehydrated in an ethanol series, passed through propylene oxide, and embedded in epoxy resin. Transverse semi-thin sections (1.0 μm-thick) were picked up onto glass slides, dried on the hot plate, and stained with 1% toluidine blue. The stained sections were then viewed with a light microscope (BX51; Olympus) using an image analysis program (DP70 controller; Olympus), and pictures were taken with a digital camera (DP70; Olympus).

Immunohistochemistry

Adult male SD rats (250-300 g) were anaesthetized and perfused transcardially with 4% paraformaldehyed in 0.1 M phosphate buffer (Ph 7.4) on 3, 7 days. The sciatic nerve were removed and postfixed in 4% PFA at 4°C overnight, and rinsed and transferred to 30% sucrose in PBS for 2 days. Sciatic nerve sections (10 μm thick) were prepared on gelatin-coated slide glass using a cryocut microtome. The sections were blocked in solution containing 5% normal donkey serum (Jackson ImmunoResearch, Bar Harbor, ME, USA), 2% BSA (Sigma, St. Louis, MO, USA) and 0.1% Triton X-100 (Sigma) for 1 h at room temperature. The sections were then incubated overnight at 4°C with primary antibody for mouse-anti–rat ED-1 (1:100, AbD Serotec, Oxford, United Kingdom). The sections were then incubated for 1 h at room temperature with a mixture of Cy3-conjugated secondary antibodies (1:200, Jackson ImmunoResearch). The sections were mounted, and fluorescent images were obtained using a confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

Primary Schwann cell culture

Primary mouse Schwann cells were cultured from sciatic nerves of WT and TLR3 knock-out mice. After dissection, 2 mm sciatic nerves were removed from 8 to 10 weeks-old mice. These nerve fragments were placed onto 6-well culture dishes (5~6 explants/well) with Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After allowing fibroblasts to grow out for 3 weeks, nerves were enzymatically and mechanically dissociated. A pellet of sciatic nerves was incubated in 1 ml of 0.25% collagenase A/dispase and 0.1ml of 0.2% DNase I at 37°C

for 30 min. The nerves were then treated again with 1 ml of 0.25% trypsin-EDTA for 30 min. Subsequently, the cells were dissociated by repeated pipetting with a Pasteur pipette in culture medium containing DMEM, 10% heat-inactivated FBS, 2 mM L-glutamine, 0.5% glucose, and antibiotics. After filtering in a 70 μm cell strainer, the cells were resuspended in culture medium, plated on precoated poly-D-lysine (20 μg/ml) dishes and maintained in a 5% CO_2 incubator at 37°C. After 1 day, 10 μm cytosine arabinoside (Ara-C) was added to the medium to suppress fibroblast proliferation, and forskolin (2 μM) and neuregulin-1 (10 ng/ml) was added to the medium after 2 days. The primary Schwann cells were used for experiments after second passage.

Reverse transcription polymerase chain reaction and real-time RT-PCR

Total RNA was isolated by using TRI reagent according to the manufacturer's instruction, treated with RNase-free DNase I for 30 min, and then heat-inactivated for 30 min at 65°C. The cDNA was synthesized from 1 μg of the total RNA by incubating for 1 h at 37°C in a reaction mixture containing 0.5 μg of oligo $(dT)_{15}$, 0.5 Mm dNTP mix, 1 x first-strand buffer, RNase inhibitor (5 U), 5 mM DTT, and M-MLV reverse transcriptase (5 U).

Real-time RT-PCR was performed using the SYBR Green PCR Master Mix and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The following PCR primer sequences were used: mouse/rat GAPDH (forward), 5'-AGG TCA TCC CAG AGC TGA ACG-3'; mouse/rat GAPDH (reverse), 5'-CAC

CCT GTT GCT GTA GCC GTA T-3'; mouse CD68 (forward), 5'-GGG GCT CTT GGG AAC TAC AC-3'; mouse CD68 (reverse), 5'-GTA CCG TCA CAA CCT CCC TG-3'; mouse F4/80 (forward), 5'-AGC ACG TCC TAT TTC AAC GGT-3'; mouse F4/80 (reverse), 5'-CTG TGT TTA CTG CTT GAT GGG T-3'; mouse CCL2 (forward), 5'-TCA GCC AGA TGC AGT TAA CG-3'; mouse CCL2 (reverse), 5'-GAT CCT CTT GTA GCT CTC CAG-3'; mouse CCL3 (forward), 5'-ACT GCC TGC TGC TTC TCC TAC A-3'; mouse CCL3 (reverse), 5'- AGG AAA ATG ACA CCT GGC TGG -3'; mouse CCL4 (forward), 5'-TCC CAC TTC CTG CTG TTT CT-3'; mouse CCL4 (reverse), 5'-GAA TAC CAC AGC TGG CTT GG-3'; mouse CCL5 (forward), 5'-GCA AAA AGC TGA AGA GCG TG-3'; mouse CCL5 (reverse), 5'-TGC AGC ATA GTG AGC CCA GA-3'. The level of each gene was normalized to the levels of the rat GAPDH or mouse GAPDH gene and represented as a fold induction. The fold induction was calculated using the $2^{-\Delta\Delta CT}$ method as previously described (Livak and Schmittgen, 2001). All real-time RT-PCR experiments were performed at least three times, and the mean ± S.E.M values have been presented unless otherwise noted.

Statistical analysis

Data are represented as mean ± standard errors of the mean (SEMs). The statistical significance of differences in different groups was analyzed using the unpaired Student's *t* test with a threshold of *, $p < 0.05$; **, $p < 0.01$, versus sham control in each mice group, $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$, versus TLR3 knock-out mice

Ⅲ. Results

Peripheral nerve injury-induced Wallerian degeneration is impaired in TLR3 knock-out mice

To identify the role of TLR3 during Wallerian degeneration after nerve injury, sciatic nerve crush injury, one of the most common rodent peripheral nerve injury models, was done on the right sciatic nerve of the WT and TLR3 knock-out mice. Then semi-thin cross sections of uninjured and injured sciatic nerves were performed with toluidine blue staining. The degenerating axons were more abundant and more widely distributed in the WT mice than in the TLR3 knock-out mice at 3 days after the crush injury (Fig. 1). After 7 days, all of the axons undergoing degeneration were clearly removed in the WT mice, whereas, remaining degenerating axons were still detected in TLR3 knock-out mice. These data show that Wallerian degeneration is delayed in TLR3 knock-out mice.

Toll-like receptor 3 signaling is required for nerve injury-induced macrophage infiltration

It is well known that the Schwann cells contribute to wallerian degeneration by recruiting macrophages to the injured site. Infiltrated macrophages also mediate axonal debris clearance via phagocytosis (Stoll et al., 1989). To test whether TLR3 signaling is required for macrophage infiltration, sciatic nerves were removed from WT and TLR3 knock-out mice at 1 day following a sciatic nerve crush injury. CD68 and F4/80 are widely used as macrophage-specific markers. CD68 mRNA expression

levels in the WT mice increased more than 8-fold compared to the sham-operated control mice, while that of TLR3 knock-out mice decreased remarkably (Fig. 2A). Similarly, the nerve injury-induced F4/80 mRNA expression in nerve of the WT mice increased 3.5-fold, while that of the TLR3 knock-out mice was upregulated only 1.5-fold (Fig. 2B). These data indicate that macrophage infiltration is decreased in TLR3 knock-out mice after peripheral nerve injury.

Toll-like receptor 3 is required for macrophage-recruiting chemokine expression following sciatic nerve injury

After peripheral nerve injury, lymphocytes have been known to accumulate at sites of immune and inflammatory reactions, and the chemokine receptors and chemokines that induce these responses have been previously documented (Table 1). In particular, CCR1, CCR2 and CCL5 are expressed on the surface of macrophage (Sallusto and Baggiolini, 2008). Previous studies have reported that a peripheral nerve injury induces chemokines such as CCL2/MCP-1 (Perrin et al., 2005), CCL3/MIP-1α, CCL4/MIP-1β (Saika et al., 2012) and CCL5/RANTES, and that these chemokines are involved in macrophage infiltration into the injured region. To determine whether TLR3 signaling is required for nerve injury-induced macrophage infiltration, I determined the macrophage-recruiting chemokine gene expression levels in injured sciatic nerve by real-time RT-PCR. CCL2, CCL3, CCL4 and CCL5 mRNA expression in the WT mice were remarkably induced by 10-, 80-, 120-, and 2.5- fold, respectively (Figs. 3 A-D). However, the induction levels of these genes were significantly decreased in the TLR3 knock-out mice. These data indicate that nerve injury-induced

macrophage recruiting chemokine is abolished in the sciatic nerve, leading to decreased macrophage infiltration into the injury site in TLR3 knock-out mice.

Toll-like receptor 3 in Schwann cells is required for macrophage- recruiting chemokine expression and *in vitro*

The primary Schwann cells from the WT and TLR3 knock-out mice were cultured and then stimulated with poly(I:C) to test if macrophage recruiting chemokine expression is regulated in the Schwann cells. After 3 h, CCL2, CCL3, CCL4 and CCL5 mRNA expression in the WT mice were increased by 10-, 6-, 40-, and 12,000-fold, respectively (Figs. 4 A-D). On the other hand, the induction levels of these genes were significantly decreased in the TLR3 knock-out mice by more than 90%, with the exception of the CCL3 gene which was similar to that of the WT mice. These results suggest that TLR3 agonist-induced macrophage infiltration related-chemokine gene expressions require TLR3 signaling in the Schwann cells.

TLR3 stimulation induces macrophage infiltration in sciatic nerve

In order to test if TLR3 stimulation induces macrophage infiltration in sciatic nerve *in vivo,* poly(I:C), a synthetic TLR3 agonist, was directly introduced into rat's sciatic nerve. Three days after the injection, ED-1 positive macrophage was induced in injection site. After 7 days, a significant increase of infiltrated macrophage was detected at the site of injection (Fig. 5). A few infiltrating macrophage was shown in the PBS-injected sciatic nerve, which could have been caused by the needle itself. These data show that TLR3 activation induces macrophage infiltration in sciatic nerve.

Table 1. Chemokine receptors and their ligands.

Chemokine Receptors	Chemokines
CCR1	RANTES, MIP-1α, MCP-2, MCP-3
CCR2	MCP-1, MCP-2, MCP-3, MCP-4
CCR3	Eotaxin, eotaxin-2, RANTES, MCP-2, MCP-3, MCP-4
CCR4	TARC, RANTES, MIP-1α, MCP-1
CCR5	RANTES, MIP-1α, MCP-1β
CCR6	LARC/MIP-3α /exodus
CCR7	ELC/MIP-3β
CCR8	I- 309

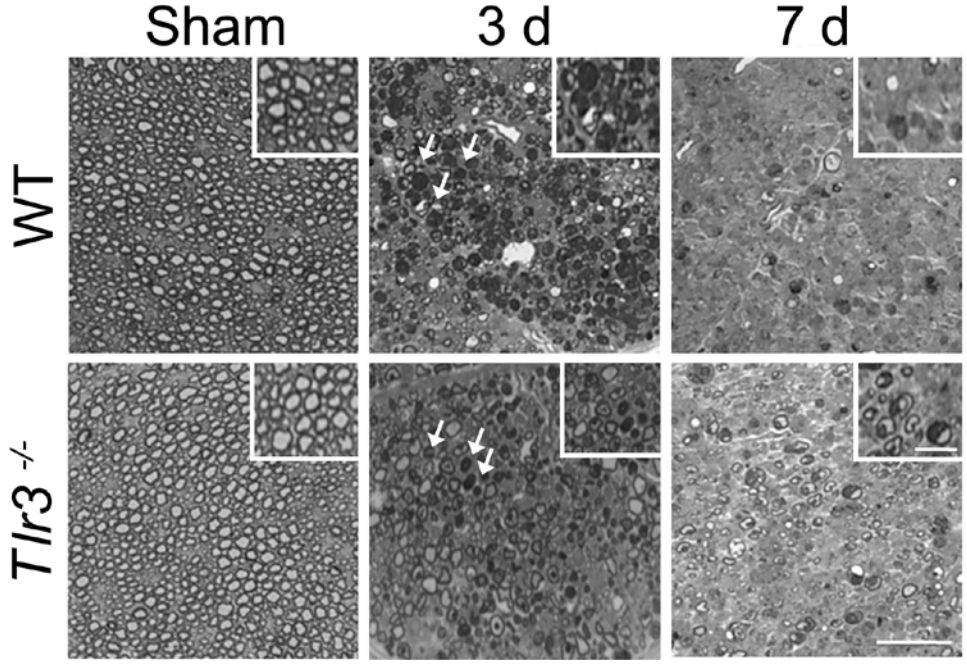
Sham
3 d
7 d
WT
Tlr3 -/-

Fig. 1. Axonal degeneration is delayed in TLR3 knock-out mice after sciatic nerve injury.

After sciatic nerve crush injury, nerve segment distal to the injury site from WT and TLR3 knock-out mice were subjected to cross section and stained with toluidine blue (scale bar: 25 μm and 100 μm for insets). Left panels: semi-thin cross sections of uninjured sciatic nerves from WT and TLR3 knock-out mice. Middle panels: three days after sciatic nerve crush injury. There are degenerating axons were observed in site of crush injury (arrows). Right panels: seven days after sciatic nerve crush injury.

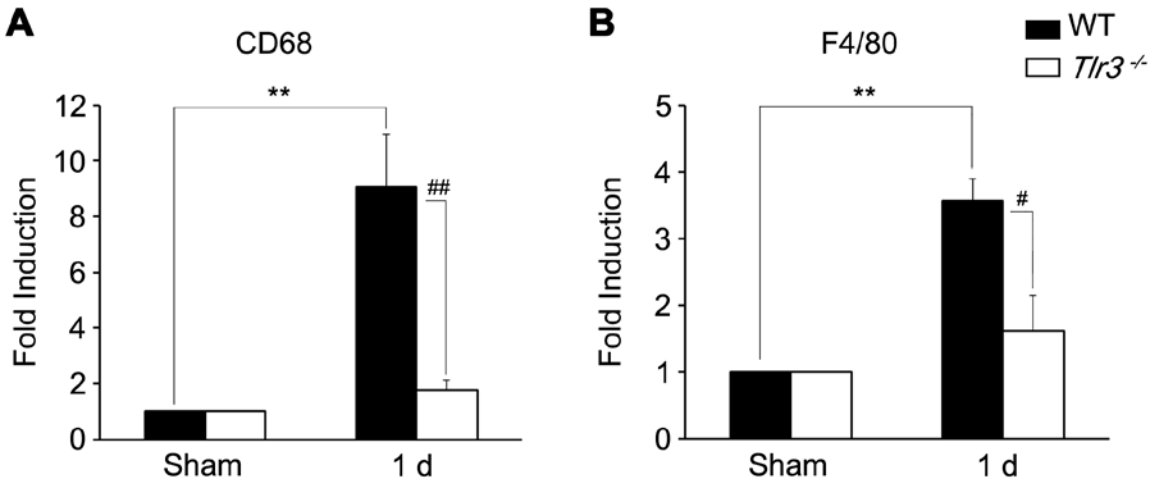
A
CD68
Fold Induction
12
10
8
6
4
2
0
**
##
Sham
1 d
B
F4/80
WT
Tlr3 -/-
Fold Induction
5
4
3
2
1
0
**
#
Sham
1 d

Fig. 2. Sciatic nerve crush injury-induced CD68 and F4/80 mRNA expression level is decreased in TLR3 knock-out mice.

(A) CD68 and (B) F4/80 mRNA expression in sciatic nerve after nerve injury was measured by real-time RT-PCR. Total RNA was isolated from sciatic nerve of uninjured control mice and sciatic nerve crush injured mice (each group, n=3) at 1 day. Data are expressed as mean ± SEM (Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$, versus sham control in each mice group, $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$, versus TLR3 knock-out mice).

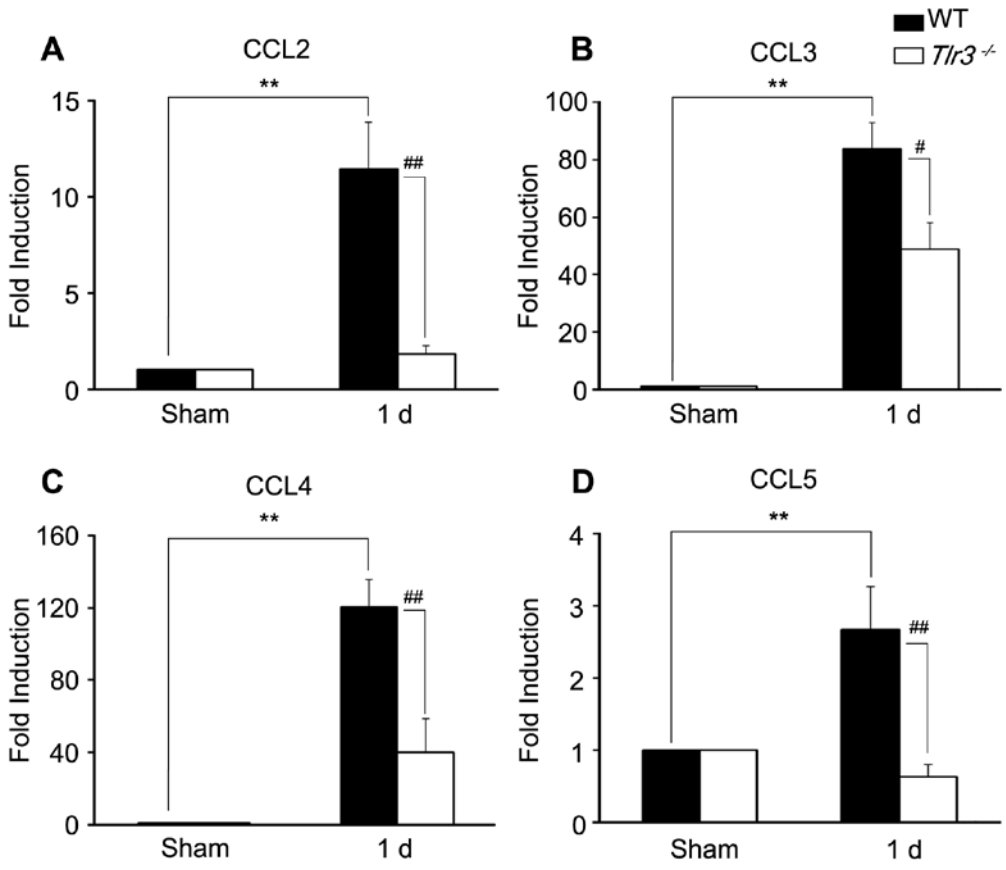
A
CCL2
B
CCL3
WT
Tlr3 -/-
C
CCL4
D
CCL5
Fold Induction
Sham
1 d
**
##
#
15
10
5
0
100
80
60
40
20
160
120
4
3
2
1

Fig. 3. Expression of macrophage-recruiting chemokine is abolished in the TLR3 knock-out mice.

Total RNA was isolated from sciatic nerve of uninjured control mice and nerve injured mice (each group, n=3). Each sample used to determine CCL2 (A), CCL3 (B), CCL4 (C) and CCL5 (D) gene expressions using real-time RT-PCR. Data are expressed as mean ± SEM (Student's t-test, *, $p < 0.05$; **, $p < 0.01$, versus sham control in each mice group, $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$, versus TLR3 knock-out mice).

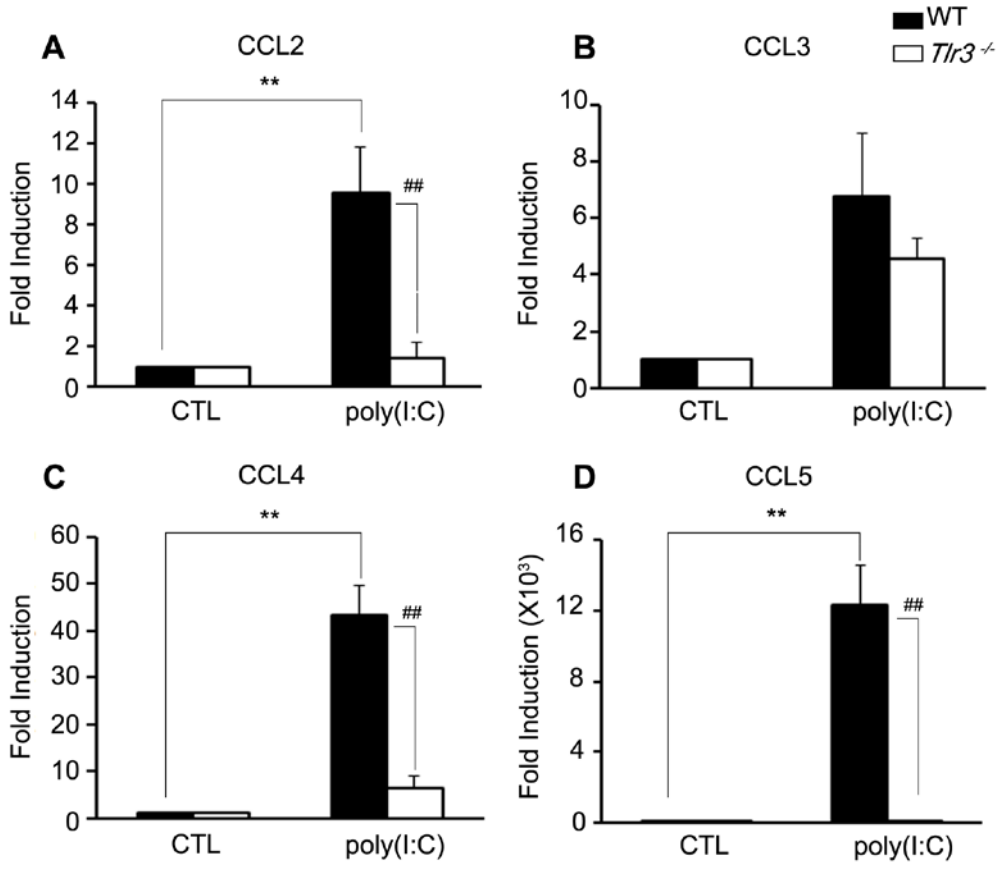
A
CCL2
B
CCL3
WT
Tlr3 -/-
C
CCL4
D
CCL5
Fold Induction
Fold Induction (X10³)
CTL
poly(I:C)
**
##

Fig. 4. TLR3 agonist-induced macrophage infiltration related-chemokine gene expression is ameliorated in primary Schwann cells of TLR3 knock-out mice.

Primary Schwann cells prepared from WT and TLR3 mice were activated with poly(I:C) (20μg/ml) for 3 h. Total RNA was isolated from each sample and used to determine CCL2 (A), CCL3 (B), CCL4 (C) and CCL5 (D) gene expressions using real-time RT-PCR. Three independent experiments were performed using primary Schwann cells from WT and TLR3 knock-out mice. Data are expressed as mean ± SEM (Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$, versus sham control in each mice group, $^{\#}$, $p < 0.05$; $^{\#\#}$, $p < 0.01$, versus TLR3 knock-out mice).

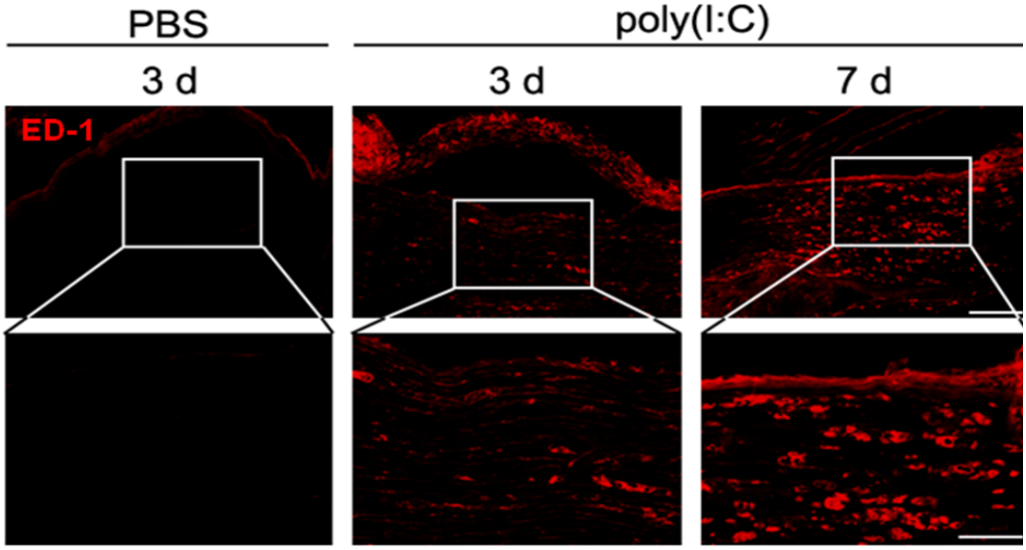
PBS
poly(I:C)
3 d
3 d
7 d
ED-1

Fig. 5. TLR3 agonist poly(I:C) induces macrophage infiltration *in vivo*.

The rat sciatic nerve was injected with 50 μg poly(I:C) in PBS (5 μL) or PBS alone. After 3 and 7 days, the injected sciatic nerves, as well as the uninjected control nerves, were removed and used for immunohistochemistry. Cy3-conjugated anti-ED-1 antibody was used for immunostaining of macrophage.

Ⅳ. Discussion

Following peripheral nerve injury, Schwann cells in the distal portion of the axon are activated. Then the activated Schwann cells recruit macrophages to the site of injury by secreting macrophage-recruiting chemokines, which consequently promotes Wallerian degeneration. However, the mechanism of Schwann cell activation is not clearly defined. In this study, I found that the nerve injury-induced Wallerian degeneration is delayed in TLR3 knock-out mice. In addition, macrophage infiltration and macrophage-recruiting chemokine expression are significantly decreased in TLR3 knock-out mice. Additionally, I showed that poly(I:C), a synthetic TLR3 agonist, injection into the sciatic nerve significantly induced macrophage infiltration.

It can be speculated that delayed Wallerian degeneration in TLR3 knock-out mice may lead to impaired nerve regeneration after nerve injury. In fact, previous studies found that nerve regeneration of injured sensory axons was impaired on Wallerian degeneration is delayed in C57BL/Ola mice (Bisby and Chen, 1990). Therefore, I compared the phenotypes of the nerve segment distal to the injury site of WT and TLR3 knock-out mice during nerve regeneration. Interestingly, however, there were no significant differences between WT and TLR3 knock-out mice 14 and 21 days after crush injury (data not shown). Furthermore, axons of both showed a normal nerve regeneration phenotype, which means both mice underwent normal nerve regeneration. It is reasonable to speculate that more macrophages infiltrate the injury site and clean up the axonal debris effectively via phagocytosis in TLR3 knock-out mice during days 7-14. In order to explain this hypothesis, I tested CD68 mRNA levels and show a tendency to increased macrophage infiltration in TLR3 knock-out mice compare with

WT at 7 days (data not shown). Furthermore, the population of infiltrated macrophages during nerve regeneration, especially, 7 and 14 days must be tested using immunostaining.

In this study, I found that Wallerian degeneration was delayed in TLR3 knock-out mice through semi-thin toluidine blue staining (Fig. 1). It can be also speculated that delayed Wallerian degeneration may lead to impaired recovery of locomotor function in TLR3 knock-out mice after nerve injury. Therefore, I tested footprinting behavior test to evaluate the recovery of locomotor function between WT and TLR3 knock-out mice. Unexpectedly, there was no significantly difference between two groups.

I observed that CD68 and F4/80, a macrophage marker, as well as macrophage infiltration-related chemokine mRNA levels were increased in WT, but not in TLR3 knock-out mice (Fig. 2). However, protein expression level of macrophage infiltration after sciatic nerve injury was untested. So far studies have only tested mRNA levels, Therefore, future studies are needed to test the level of macrophage infiltration by immunohistochemistry *in vivo*.

In my *in vitro* data, Schwann cells induce macrophage-recruiting chemokine expression via TLR3 activation (Fig. 4). Previous studies have shown that CCL2/MCP-1 and CCL3/MIP-1α were expressed in DRG neurons after peripheral nerve injury (Kim et al., 2011). Thus, it needs to be determined if Schwann cells induce macrophage-recruiting chemokines *in vivo*.

Furthermore, it is well known that macrophages express TLRs, which sense pathogens and lead to inflammatory responses (Applequist et al., 2002). Injection of

TLR2 or TLR4 ligands into rat sciatic nerve lesion leads to increase induction of myelin debris-ingested macrophage, respectively (Boivin et al., 2007). This implies that the phagocytic function of macrophages is affected via TLRs. Recently, it was reported that TLR3 activation in mouse macrophages enhances their ability to uptake bacterias via IFR3 activation, and inhibits apoptotic cell phagocytosis via NF-κB activation (Deng et al., 2013). I used TLR3 knock-out mice in which the TLR3 gene is deleted in all tissues. This suggests that the phagocytic function of macrophages is affected in TLR3 knock-out mice. Therefore, it is possible that TLR3 in macrophages partly contribute to the delayed Wallerian degeneration. This issue can be resolved in the future study using Schwann cell-specific TLR3 conditional knockout mice. Likewise, TLR3 activation could regulate the phagocytosis of Schwann cells. To test if the TLR3 on Schwann cells effect the ability of phagocytosis, it could be test via phagocytosis assay between WT and TLR3 knock-out mice.

In previously study, injection of poly(I:C) into rat sciatic nerve induce axonal degeneration in Wallerian degeneration (Lee et al., 2007). To test whether macrophage infiltration is increased during Wallerian degeneration after poly(I:C) injection, I treated poly(I:C) into sciatic nerve, then performed immunostaining for macrophage infiltration. I found that a significant increase of infiltrated macrophage was detected at the site of injection (Fig. 5). However, expression level of macrophage infiltration between WT and TLR3 knock-out mice was untested. Therefore, it is needed to test that if macrophage infiltration is decreased in TLR3 knock-out mice.

In conclusion, I found that TLR3 is a critical receptor for inflammatory Schwann cell activation and promotes Wallerian degeneration after peripheral nerve injury.

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Ⅵ. 국문초록

말초 신경 손상 이후 나타나는 Wallerian degeneration에서 슈반세포의 역할이 중요하다는 사실은 매우 잘 알려져 있다. 최근 톨유사수용체 (Toll-like receptor) 신호기전이 Wallerain degeneration에서 중요하다는 것이 보고되었다. 또한, 선행연구에서는 톨유사수용체 리간드에 의해 유도된 염증성 슈반세포의 활성화가 톨유사수용체 2와 3을 통한다는 것을 검증하였다. 그러나 말초 신경 손상 이후 나타나는 Wallerian degeneration에서의 톨유사수용체 3의 역할과 그 기전에 대해서는 아직까지 명확하게 밝혀지지 않았다. 본 연구에서는 말초 신경 손상 이후 유도되는 Wallerian degeneration에서의 톨유사수용체 3의 역할에 대해 조사하였다. 먼저, 좌골 신경 손상 후 톨유사수용체 3 유전자가 적중된 생쥐의 좌골 신경에서 대조군에 비해 퇴행 중인 axon의 수가 크게 감소한 것을 확인하였다. 또한, 말초 신경 손상 이후 7 일째에는 톨유사수용체 3 유전자가 적중된 생쥐에서 좌골 신경 퇴행 과정이 지연되고 있는 것을 관찰하였다. 말초 신경 손상 이후 1 일째에는 대조군의 좌골 신경에서 대식세포의 유입이 증가하였지만, 반면 톨유사수용체 3 유전자가 적중된 생쥐에서는 대식세포의 유입이 크게 감소하였다. 뿐만 아니라, 체 내에서 신경 손상에 의해 대식세포의 이동에 관여하는 CCL2/MCP-1, CCL3/MIP-1α, CCL4/ MIP-1β 그리고□CCL5/RANTES와 같은 유전자들의 발현이 톨유사수용체 3 유전자가 적중된 생쥐에서 현저하게 줄어드는 것을 확인하였다. 또한,

대식세포의 이동에 관여하는 유전자들의 발현이 슈반세포에서 TLR3 활성화에 의해 유도되는 것을 슈반세포 배양을 통해 확인하였다. 마지막으로 톨유사수용체 3의 자극제로 잘 알려진 poly(I:C)를 쥐의 좌골 신경에 주사했을 경우 대식세포의 유입이 크게 증가하는 것을 확인하였다. 결론적으로 본 연구 결과는, 좌골 신경 손상 이후 발생하는 Wallerian degeneration과 염증성 슈반세포의 활성화에 톨유사수용체 3가 기여한다는 것을 보여준다.

주요어:

톨유사수용체 3, 슈반세포, Wallerian degeneration, 좌골 신경 손상, chemokine, 대식세포 유입

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