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**Bachelor Degree Thesis**

# Dectin-1 與周邊神經損傷再生之關係

# The role of Dectin-1 in peripheral nerve injury and regeneration

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# 中文摘要

Dectin-1 為一存在於巨噬細胞膜上的模式辨認受體(pattern recognition receptor, PRR)，且大部分位於會促進神經再生(nerve regeneration)的 M2 巨噬細胞膜上。此受體可以辨識出真菌細胞壁上的 beta-1,3-glucan，在內生性免疫中具有重要角色。本研究發現抑制 Dectin-1 會抑制血管的生長以及延後瓦勒氏退化(Wallerian degeneration)中殘餘神經的清除。抑制 Dectin-1 也會延後感覺異常(hypesthesia)之發生時間，因此本研究推論出含有 Dectin-1 的巨噬細胞在促進周邊神經的再生上，佔有重要角色。

# Abstract

Dectin-1, a C-type lectin receptor, has been shown to play a role in nerve regeneration in the central nervous system. However, whether it plays a role in the peripheral nervous system is not well understood. Our staining showed colocalization of Dectin-1on the membrane of macrophages, and there is also innate Dectin-1 expression in intact myelinated nerves. We also used a model of sciatic nerve crush injury to demonstrate that there is a delay in nerve degeneration related processes such as breakdown of injured myelinated nerve fibers and formation of myelin ovoid in groups injected with WGPS, a Dectin-1 antagonist. There is also less blood vessels present in WGPS injected group 7 days after injury. This suggests Dectin-1(+) macrophages play a role in debris clearance, nerve degeneration, and angiogenesis.

# 目　錄

# Table of Content

# List of Figures

# List of Table

# Chapter 1 Introduction

## 1.1 Wallerian degeneration

Injuries to the peripheral nerve results in consequent processes denoted “Wallerian degeneration”. Among the very first steps of Wallerian degeneration is the clearance of injured axons by macrophages, followed by sprouting of new axons.[1] Morphologically, myelinated nerves first undergo swelling, degeneration and infolding of the Schwann cells, and then the debris of degenerated myelinated nerves are cleared, to make way for the growth of regenerated myelinated nerves smaller in diameter and more compact in distribution as compared with intact myelinated nerves.[2] Macrophages plays an important role in clearance of nerve debris. The arrival of the macrophage population is seen as early as 3 days, and peaks at 7 days after peripheral nerve injury.[3]

## 1.2 M1 and M2 macrophage subtypes

There are two major groups among macrophages that could participate in peripheral nerve injury, the pro-inflammatory M1 group, and the pro-regeneration M2 group[4], the M2 macrophages were found to encourage axonal growth when incubated with DRG cortical neurons[4] and aid in the repair of the central nervous system[5].

## 1.3 Previous studies on Dectin-1

Dectin-1, a C-type lectin receptor, has been shown to be found mainly on the M2 subtype macrophages[6]

Previous studies have shown that by stimulating Dectin-1 after retro-orbital optic nerve crush (ONC), axon regeneration was promoted[7]. In another study, however, stimulating Dectin-1 increased nerve injury and axon degeneration after spinal cord injury[8]. Another study has shown that injecting apoptotic neuron into the brain of mice induced “homeostatic” microglia to express

ApoE, TREM-2, and Dectin-1, becoming microglia that induced inflammation in the brain, which was linked to neuro-degenerative diseases[9]. This suggests that Dectin-1 played a role in neural regeneration and homeostasis in the central nervous system.

However, it remains unclear whether Dectin-1 mediates nerve regeneration or degeneration on the peripheral nervous system or not. Therefore, we used a model of sciatic nerve crush injury to study the effect of Dectin-1 on the peripheral nervous system.

# Chapter 2 Materials and Methods

## 2.1 Animals

Surgeries were performed on adult male C57BL/6J mice (8 to 9 weeks old).

## 2.2 Sciatic nerve crush surgery

Animals were anesthetized and both thighs were shaved and the skin of the right thigh was incised. The fascial plane between the gluteus maximus and the anterior head of the biceps femoris was opened to reveal the sciatic nerve. No.5 forceps (Ideal-tek, Balerna, Switzerland) were dipped with carbon, then the sciatic nerve was crushed for 30 seconds. The skin incision was closed with EZ clip wound closing kit. (Stoelting Co.,Wood Dale, IL)

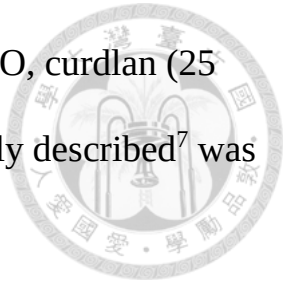
## 2.3 Sham surgery

The contralateral (left) sciatic nerve was exposed and mobilized but not crushed, leaving the sciatic nerve intact.

## 2.4 Sciatic nerve injection

Sciatic nerve was exposed as previously described; immediately after crush surgery, 32 gauge needles (Hamilton robotics, Reno, NV) was carefully inserted into the nerve to 1mm distal of the crush site. 4 ul of either PBS, solution of WGP soluble (WGPS

1mg/ml) (Invivogen, San Diego, CA)of 1mg/ml in filtered ddH2O, curdlan (25 mg/ml) (Sigma- Aldrich, Saint Louis, MO, prepared as previously described[7] was injected into the nerve.



### 2.5 Mechanical threshold test

Mice were individually placed in a Plexiglas™ container on metal mesh and allowed to habituate to the new environment. A mechanical stimulus was delivered to the plantar surface of the hind paw from below the floor of the test chamber with a dynamic plantar aesthesiometer (Ugo Basile, Comerio-Varese, Italy). A steel rod (diameter: 0.5 mm) was pushed against the hind paw with ascending forces of 0 to 50.0 g over a 20-s period. When the animal withdrew its hind paw, the mechanical stimulus stopped automatically and the force at which the animal withdrew its paw was recorded to the nearest 0.1 g, which indicates the nociceptive threshold; each hind paw was alternatively tested for 5 times with a minimal interval of 5 min between measurements. The average of the measurements was used for analysis.

### 2.6 Neurophysiological studies (CMAP and SNAP)

#### 2.6.1 Compound muscle action potential (CMAP)

Mice were anesthetized before testing, and the compound muscle action potential (CMAP) was measured using a Nicolet VikingQuest System (Nicolet Biomedical, Madison, WI). Stimulating electrodes were inserted and placed at the sciatic notch to stimulate the sciatic nerve, and recording electrodes were placed on the plantar

muscles. Amplitudes of the CMAP on both sides were recorded for analysis.

### 2.6.2 Sensory nerve action potential (SNAP)

Mice were anesthetized. Tibial and common peroneal nerves were cut leaving only the sural nerve intact. Stimulating electrodes were placed on the gastrocnemius muscle, and recording electrodes were placed on the sural nerve. Amplitudes of the SNAP on both sides were recorded for analysis.

### 2.7 Tissue preparation

Animals were anesthetized and perfused intracardiaclly first with 1% sodium nitrite solution (1 ml/g), then with 4% paraformaldehyde solution (2ml/g). Tissues were post-fixed for another 2hrs, then stored in 0.1M phosphate buffer in 4°C. Prior to sectioning, tissues were cryoprotected in 30% sucrose in 0.1M phosphate buffer. Tissues were cut into sections 8um in thickness with a microtome (Leica, Wetzlar, Germany), then mounted onto gelatin- coated slides.

### 2.8 Immunohistochemistry (IHC)

Slides were washed with Tris buffer for 5 minutes 3 times, incubated in 1% H2O2 for 30 minutes, blocked with 0.5% nonfat dry milk in Triton X-100 for one hour, and incubated with goat antibody to Dectin-1 (1: 750, R & D Systems, Minneapolis, MN) in 0.5% nonfat dry milk/Triton X-100 at 4 ℃ for 20- 22h. After rinsing in Tris buffer, sections were incubated in biotinylated horse anti-goat immunoglobulin G (1:100,

Vector, Burlingame, CA) in 0.5% nonfat dry milk/Triton X-100 at room temperature for 1 h, followed by incubation with the avidin- biotin complex (Vector) for 45 minutes. The reaction product was demonstrated by 3,3-diaminobenzidine (Sigma, St. Louis, MO). Image was acquired with a Leica DM2500 microscope (Figure 1) or Zeiss AxioImager. M1 microscope and colocolized with differential interference contrast (DIC) image. (Figure 3)

### 2.9 Immunofluorescence staining (IF)

Slides were washed with PBS for 5 minutes 3 times, then incubated in 0.05% triton in PBS for 30 minutes. Sections were then incubated in primary antibodies listed below for 20- 22hrs. After rising in PBS, sections were incubated in secondary antibodies for 1.5 hours (Cy3 conjugated donkey-anti-rabbit IgG, 488 conjugated donkey-anti-goat IgG, 488 conjugated donkey-anti-rabbit IgG, Cy3 conjugated donkey-anti-goat IgG, 488 conjugated donkey-anti-rat IgG, all from Jackson ImmunoResearch, West Grove, PA). Sections were then incubated in DAPI (Sigma-Aldrich, St. Louis, MO) solutions for 3 min, then rinsed with water and mounter in glycerol gelatin.

**Table 1.** List of primary antibodies

| Target protein | Number | Company | titration |
|---|---|---|---|
| Dectin-1 | AF1756 | R & D Systems, Minneapolis, MN, United states | 1:500 |
| Iba1 | 019-19741 | Wako, Osaka, Japan | 1:250 |
| CD206 | GTX42263 | Genetex, Taiwan, R. O. C | 1:200 |

## 2.10 Quantification of macrophage on myelinated nerves

Crush injury sites with carbon labeling were recognized, and 400X tile photos were taken from 500 um proximal to 500 um distal to the injury site with a Zeiss Cell Observer spinning disk confocal microscope. 3 slides randomly chosen, with sections at least 20 um apart, were used for each individual mouse.

Cell numbers were calculated using Metamorph. Only cells with nuclei and positive staining were included in analysis. Cell density was derived and expressed as the number of cells per square micrometre of sciatic nerve (cells/um$^2$). The ratio of dectin-1(+) macrophage is the number of dectin-1(+) Iba1(+) cells divided by the number of Iba1(+) cells; the ratio of M2 macrophage in the dectin-1(+) cells subgroup is the number of CD206(+)dectin-1(+) cells divided by dectin-1(+) cells. The average of the 3 sections of each mouse was used for statistical analysis.

## 2.11 Semi-thin section of sciatic nerves

The assessment of nerve pathology followed our established protocol.[10] The sciatic nerves were collected from 1mm proximal to the crushed site to the site of trifurcation. The most distal 2 mm were taken and then fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (PB) at 4℃ for 2 hours. The tissues were post-fixed in 2% osmic acid for 2 h at room temperature, dehydrated and embedded in Epon 812 resin (Polysciences, Philadelphia, PA). 1um thick Semi-thin sections were cut on a Reichert Ultracut E (Leica, Wetzlar, Germany) and stained with toluidine blue.

## 2.12 Quantifying myelinated nerve fibers and blood vessels

Myelinated nerve fibers were photographed under a Leica DM2500 microscope. All myelinated nerve fibers in the entire fascicle were counted first with the AxonSeg software[11], then false negatives were manually corrected using the Image-Pro PLUS software (Media Cybernetics, Silver Spring, MD). Myelinated nerve fiber density was derived and expressed as the number of nerve fiber per square micrometre of nerve fascicle (nerves/um$^2$).

Myelinated nerve fibers were counted according to the following characteristics:

(1) Myelin sheath should be of the same thickness, continuous, with clear outlines, and darker then the axons

(2) Axon should be homogenous

Blood vessels were counted according to the following characteristics: lumen surrounded by at least on endothelium cell with apparent nucleus.

Myelin ovoid were counted according to the following characteristics: homogenous, round objects as dark as myelin.

## 2.13 Statistical analysis

Kruskal-Wallis test with post-hoc Mann-Whitney test was used for the analysis of median of myelinated nerve due to its skewed distribution. ANOVA with post-hoc t-test was used for analysis of myelinated nerve density, myelin ovoid density, blood vessel density, macrophage density, and macrophage ratio. GraphPad Prism 7 was used for statistical analysis.

# Chapter 3 Results

## 3.1 Elevated expression of Dectin-1 on the sciatic nerve 7 days after crush surgery

There is an elevated expression of Dectin-1 on the crushed sciatic nerve distal to the crush site on immunohistochemical and immunofluorescence staining. (Figure 1 and 2). Moreover, there is innate expression of Dectin-1 in the axon of myelinated nerves, as shown in Figure 3.

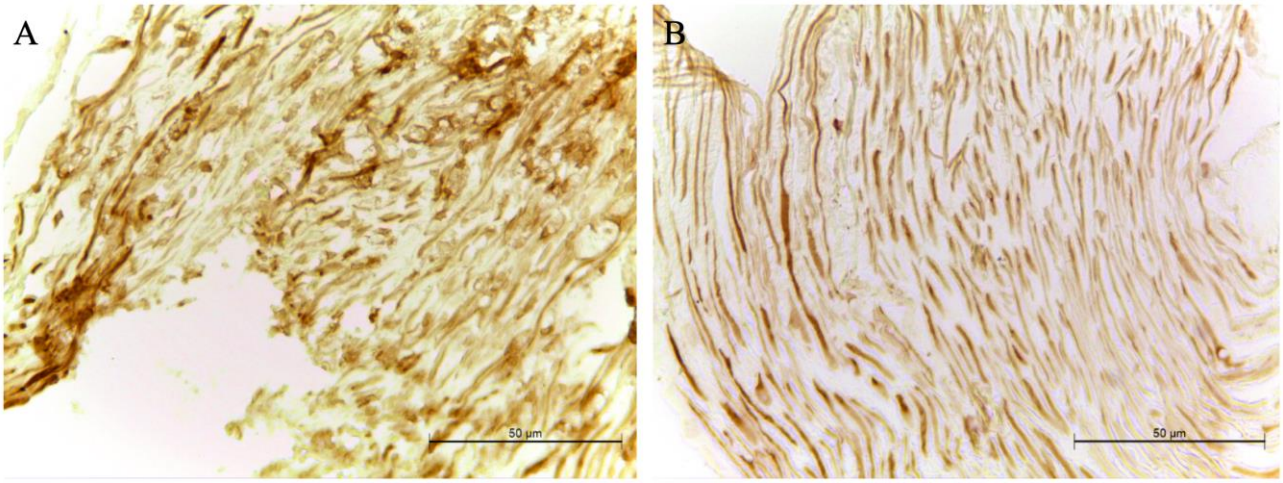



**Figure 1. Immunohistochemistry staining of Dectin-1 of sciatic nerve.** (A) Crushed side distal to the injury site. and (B) sham surgery side.

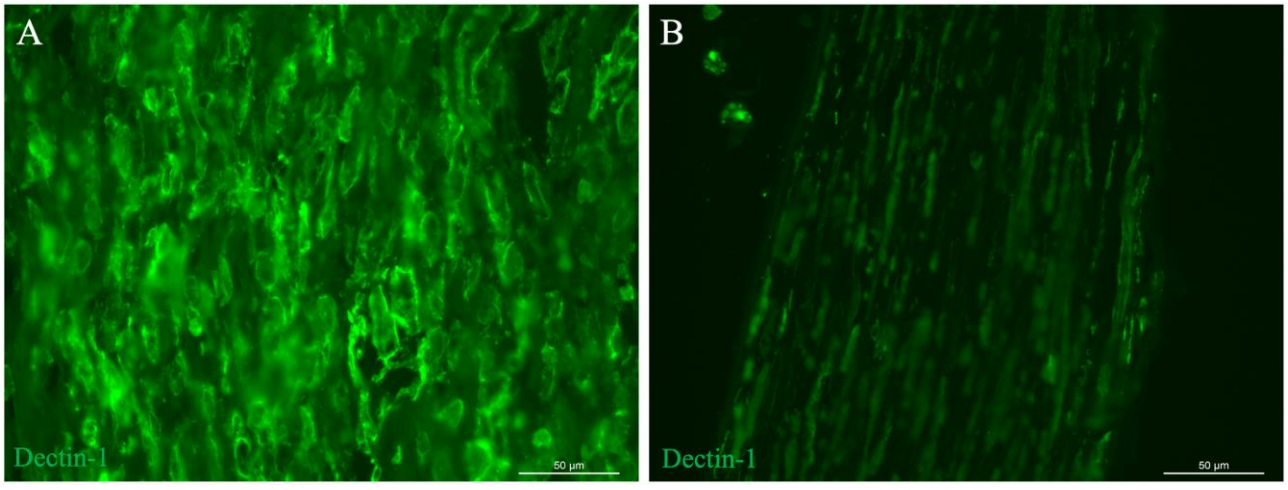


**Figure 2. Immunofluorescence staining of Dectin-1 of sciatic nerve.** (A) Crushed side distal to the injury site. and (B) sham surgery side.

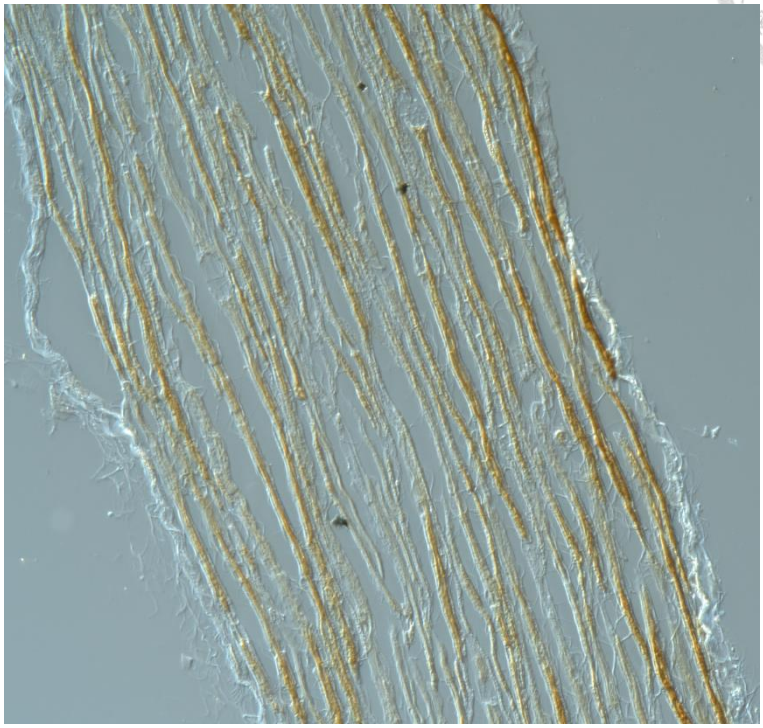


**Figure 3. Immunohistochemistry staining of Dectin-1 co-localized with differential interference contrast image of intact sciatic nerve.** Immunoistochemical staining showed positive staining of dectin-1 on myelinated axon of intact sciatic nerve (sham surgery side).

## 3.2 Dectin-1 is localized on macrophage cell membrane on the sciatic nerve

The expression of Dectin-1 co-localizes with Iba1, a pan-macrophage marker localized to macrophage membrane. The positive staining of Iba1 and Dectin-1 surrounds DAPI stained areas, indicating Dectin-1 localization on macrophage cell membrane. (Figure 4)

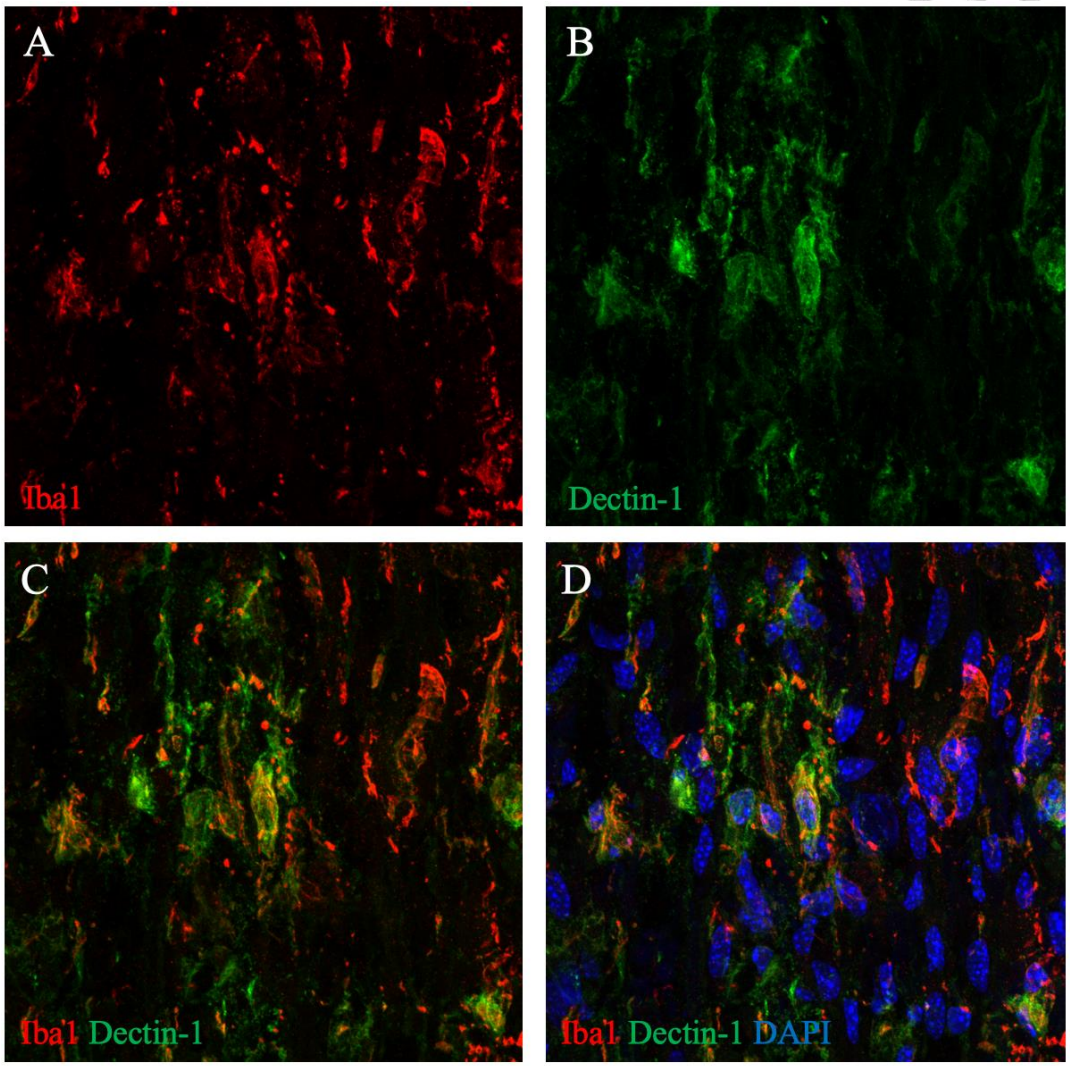



**Figure 4. Immunofluresence double staining of Iba1 and Dectin-1 of the crushed sciatic nerve distal to the injury site.** (A) Anti- Iba1 antibody was linked to Cy3 (red), (B) Anti-Dectin-1 antibody was linked to 488 (green), (C) Merging A and B shows co-localization of Dectin-1 with iba1, (D)Merging of Iba1, Dectin-1, and DAPI suggests the expression of Dectin-1 on macrophage membranes.

## 3.3 Myelinated nerve density is highest in WGPS injected groups 3 and 7 days after crush and injection surgery

The myelinated nerve density of the WGPS (Dectin-1 antagonist) injected group is significantly higher than that of the curdlan group 3 days after surgery, while 7 days after surgery, myelinated

nerve fiber density is significantly higher in WGPS treated group than the curdlan (Dectin-1 agonist) and PBS injected groups. (Figure 5 and 6) There is no significant difference in myelinated nerve density between the 3 groups 14 days after surgery. (see Supplementary figure 3)

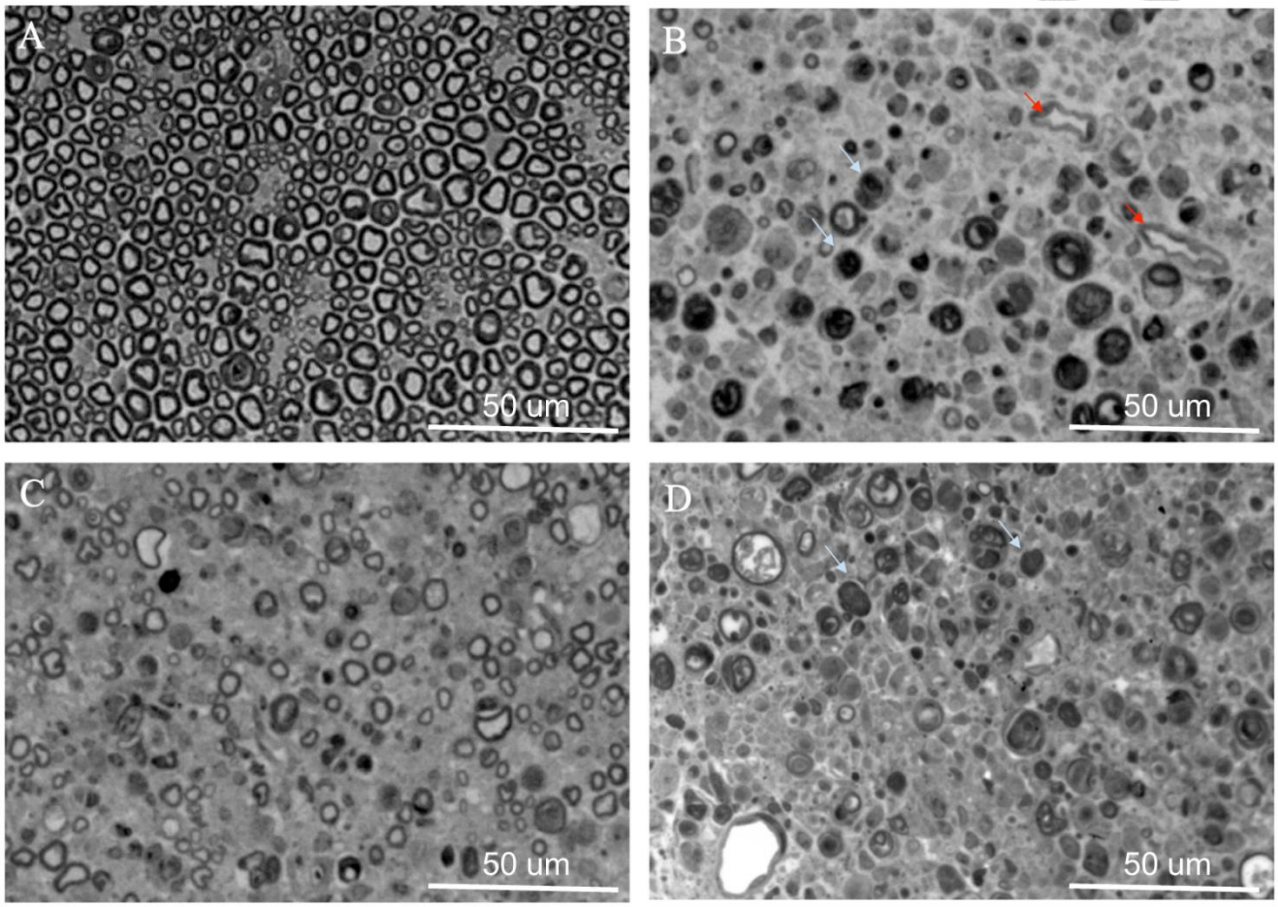



**Figure 5. Semi-thin section image of sciatic nerve.** (A) sham surgery side, (B) crush surgery plus PBS injected sciatic nerve distal to the crush site, (C) crush surgery plus WGPS injected sciatic nerve distal to the crush site, and (D) crush surgery plus curdlan injected sciatic nerve distal to the crush site 7 days after surgery. There is more myelin ovoid (blue arrows) and blood vessels (red arrows) in the PBS and curdlan groups while in the WGPS group, there is more intact myelinated nerves and less blood vessels.

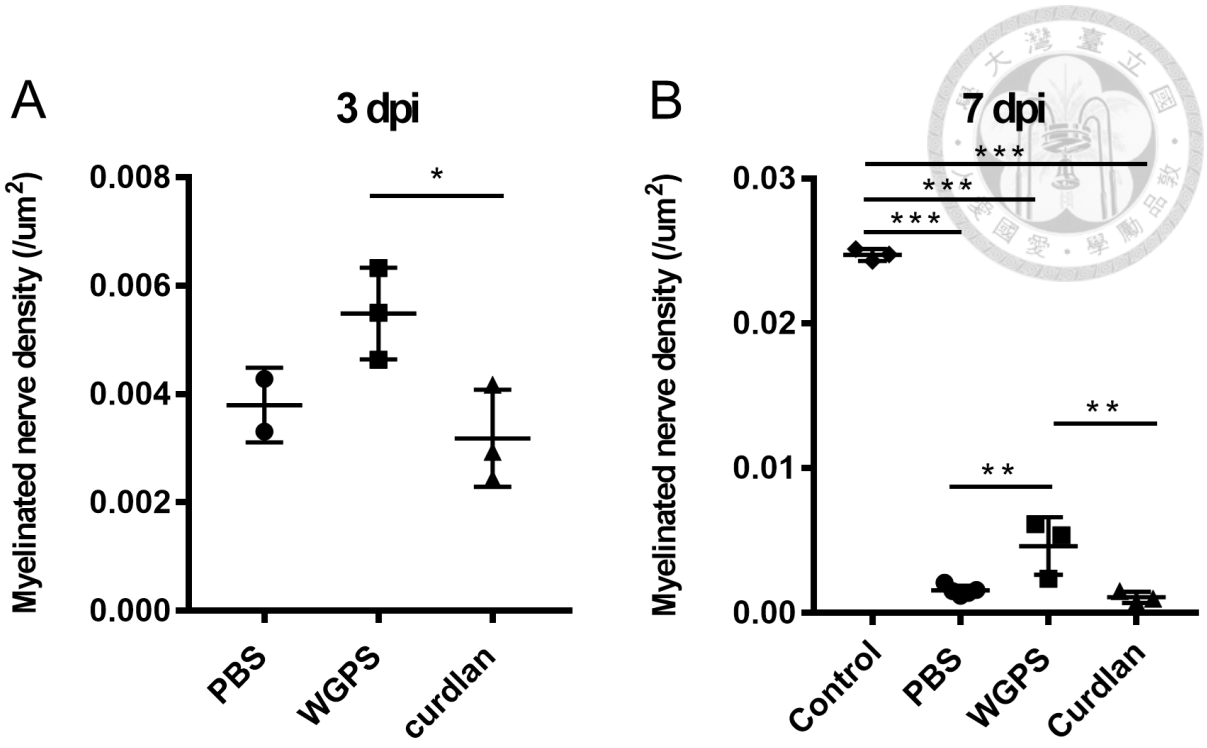



**Figure 6**. **Myelinated nerve density of sciatic nerve** (A) myelinated nerve fiber density is highest in the WGPS group 3 days after surgery. (n: PBS=2, WGPS=3, curdlan=3) (B) myelinated nerve fiber density is highest in the WGPS group 7 days after surgery.(n: control=3, PBS=6, WGPS= 3, curdlan=3).

## 3.4 Myelin ovoid density is significantly lower in WGPS injected groups 7 days after surgery, but became highest 14 days after crush and injection surgery

There is no difference in myelin ovoid density between the 3 groups (see supplementary Figure 4) 3 days after surgery. Myelin ovoid density is significantly lower in the WGPS group than the PBS group 7 days after surgery. However, 14 days after surgery, myelin ovoid density of the WGPS group is significantly higher than the PBS and curdlan groups.

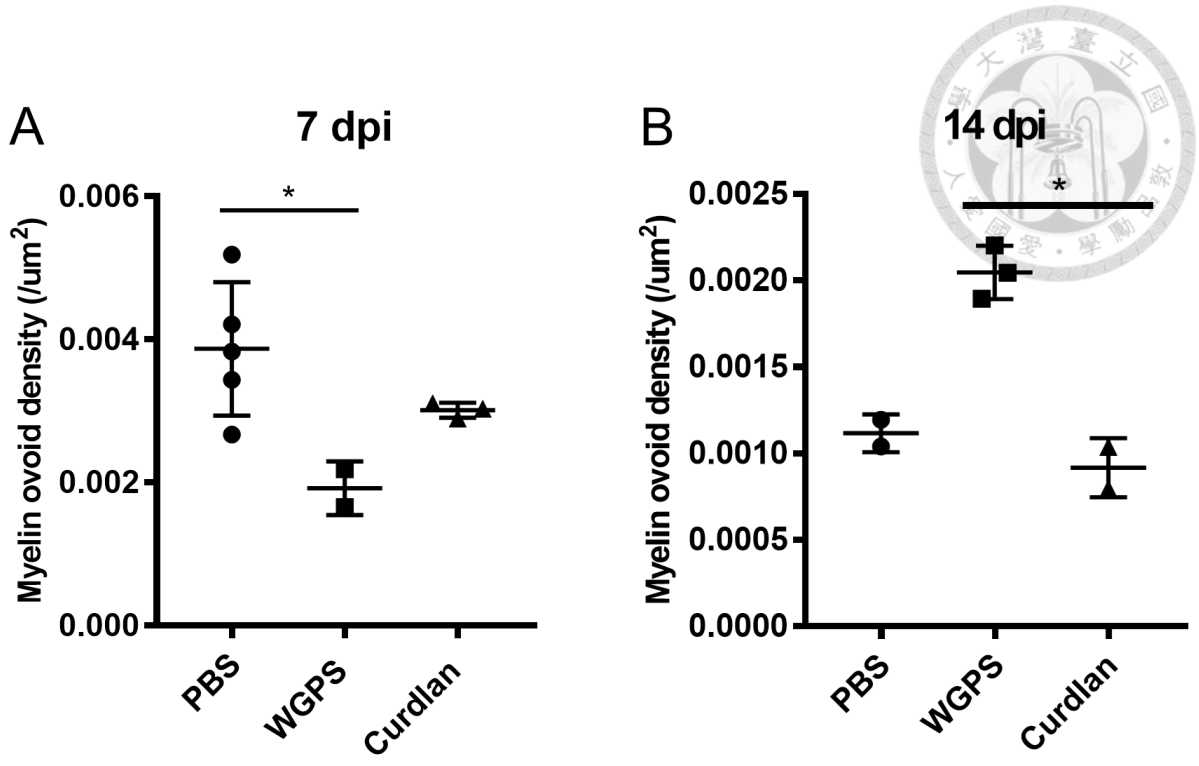



**Figure 7**. **Myelin ovoid density of sciatic nerve** (A) myelin ovoid density is lower than the PBS group 7 days after surgery. (n: PBS=6, WGPS=2, curdlan=3) (B) myelin ovoid density is highest in the WGPS group 14 days after surgery.(n: PBS=2, WGPS= 3, curdlan=2).

## 3.5 Median of myelinated nerve diameter first increased, then decreased after injury

Compared with intact myelinated nerve profiles, the median of myelinated nerve diameter in the PBS injected group increased 3 days after surgery, then continued to decrease up to 14 days after surgery. (Figure 8 and 9)

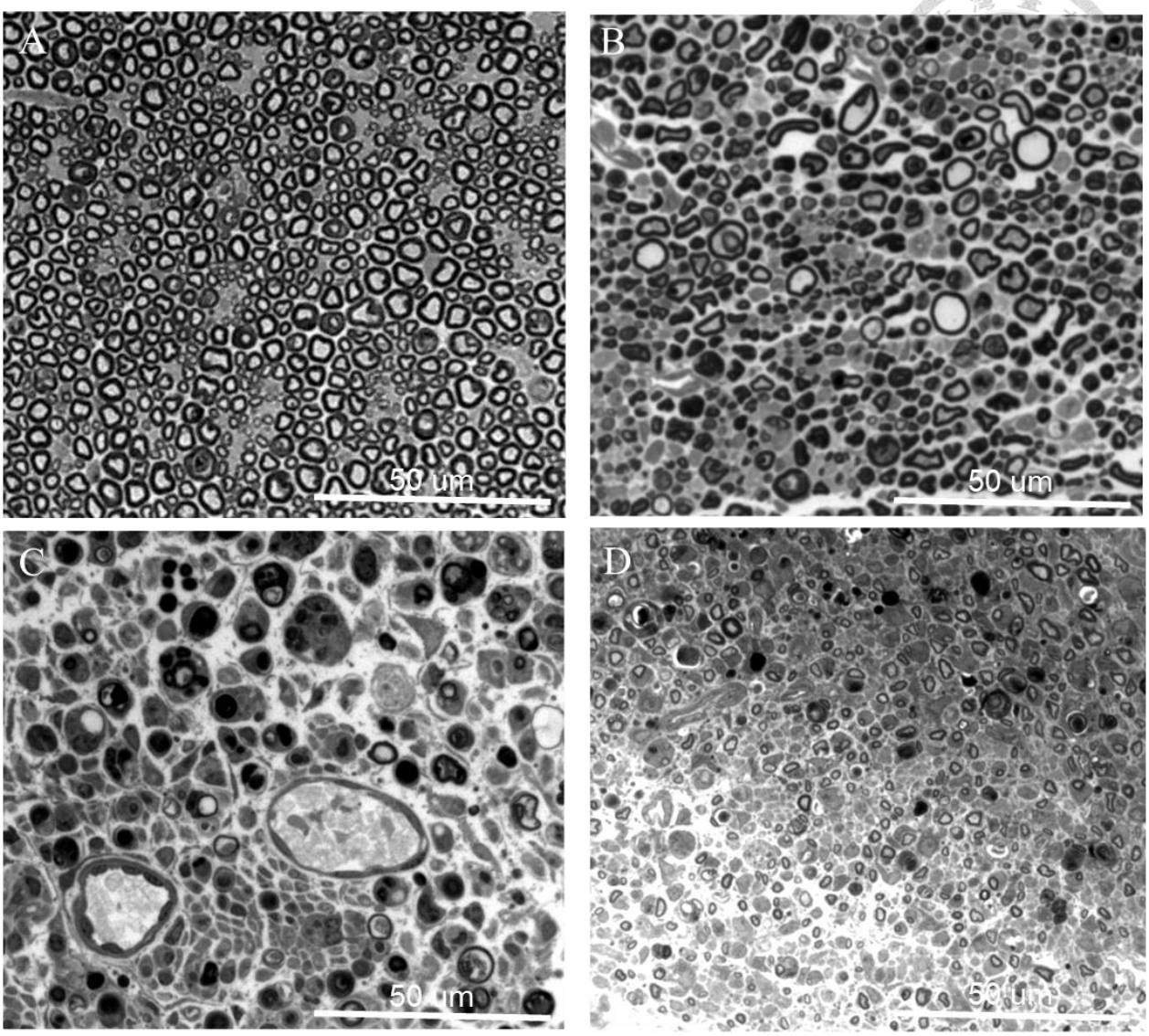



**Figure 8. Temporal change of semi-thin sections in the PBS injected group and intact sciatic nerve.** (A) intact sciatic nerve of sham surgery. (B) sciatic nerve 3 days after surgery. (C) sciatic nerve 7 days after surgery (D) sciatic nerve 14 days after surgery.

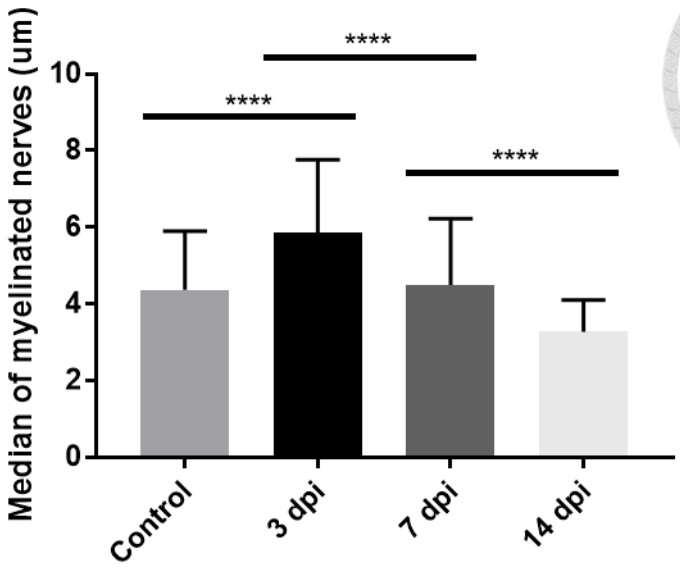



**Figure 9. Temporal change of median of myelinated nerve diameter in the PBS injected group**

Compared with intact myelinated nerves of the control group, median of myelinated nerve diameter increased at 3 days after nerve injury, but decreased in 7 and 14 days after surgery. (n: control=3, 3 DPI= 2, 7 DPI=3, 14 DPI=3)

## 3.6 Median of myelinated nerve diameter is smaller than the other groups in the WGPS group 3 and 14 days after surgery, but is greater than the other groups 7 days after surgery

3 days after surgery, the median of myelinated nerve diameter is smallest in the WGPS group; 7 days after surgery, however, the median of myelinated nerve diameter became highest in the WGPS group. 14 days after surgery, the median of myelinated nerve diameter again became smallest in the WGPS group, while curdlan is the highest. (Figure 10 and 11)

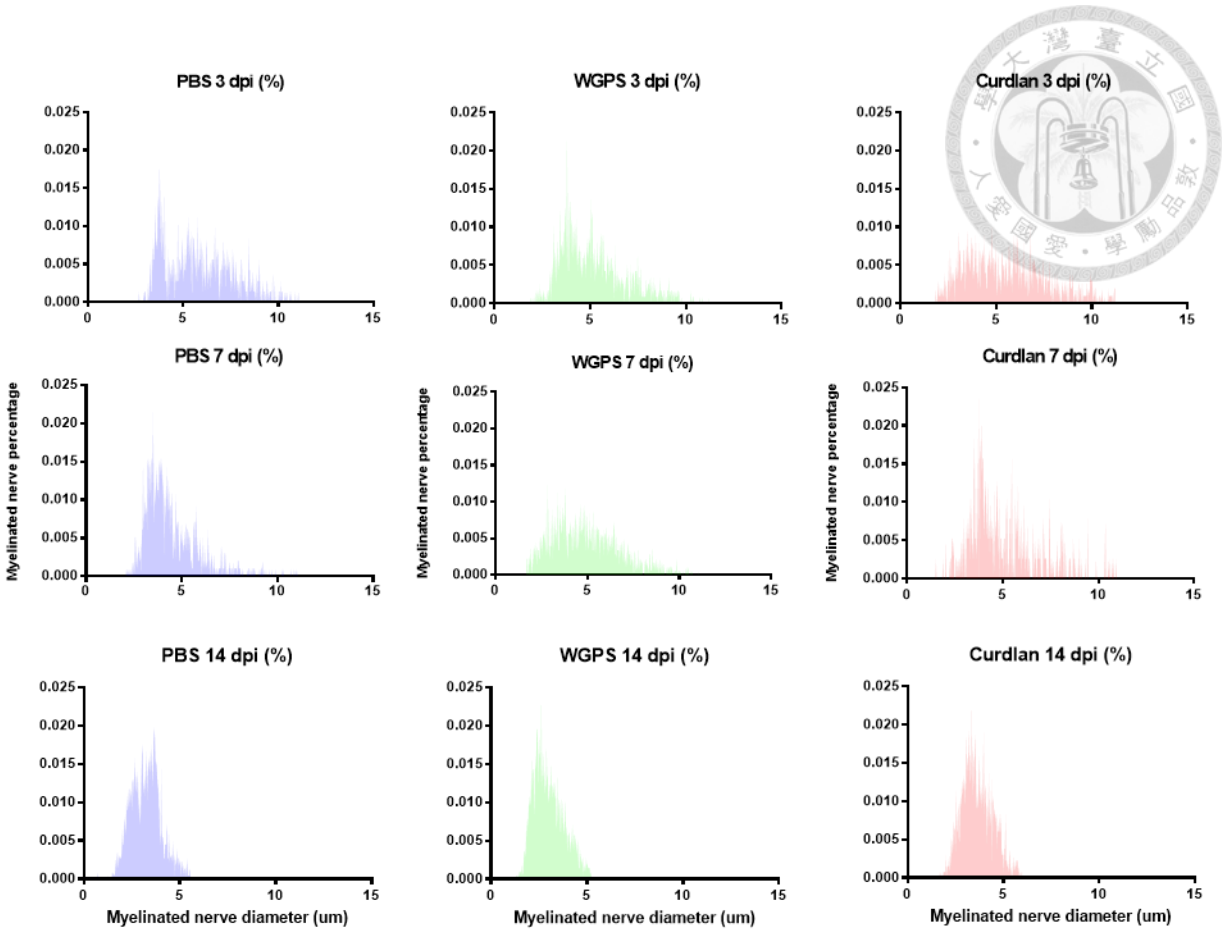



**Figure 10. Histogram of myelinated nerve distribution percentage.** Histogram of myelinated nerve distribution 3, 7, and 14 days after surgery in the PBS, curdlan, and WGPS groups. (n: 3 DPI: PBS=2, WGPS=3, curdlan=3; 7 DPI: PBS=6, WGPS=3, curdlan=3; 14 DPI: PBS=3, WGPS=3, curdlan=3)

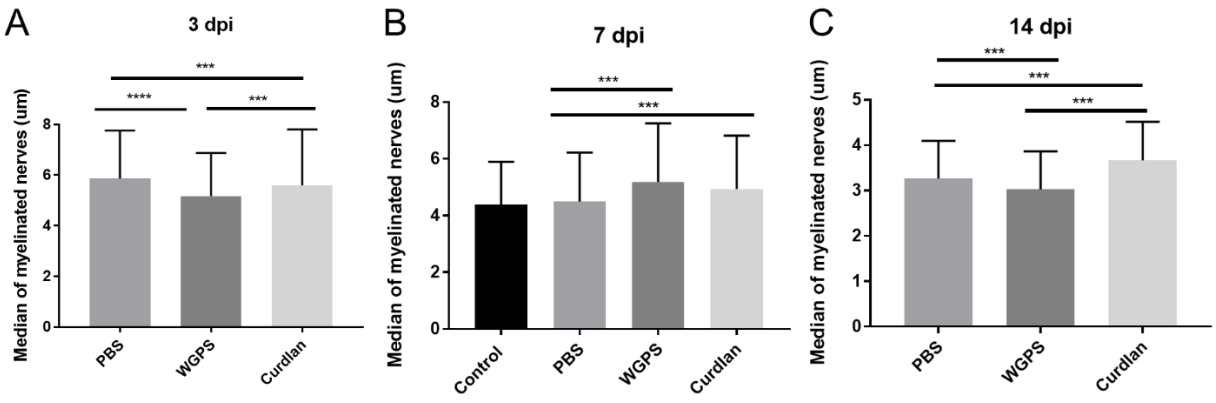


**Figure 11. Median of myelinated nerve diameter** (A) the median of myelinated nerve diameter is smallest in the WGPS injected group 3 days after surgery. (B) The median of myelinated nerve diameter is greatest in the WGPS injected group 7 days after surgery. (C) The median of myelinated nerve diameter is smallest in the WGPS injected group 14 days after surgery.
(n: 3 DPI: PBS=2, WGPS=3, curdlan=3; 7 DPI: PBS=6, WGPS=3, curdlan=3; 14 DPI: PBS=3, WGPS=3, curdlan=3)

## 3.7 Blood vessel density is significantly lower in the WGPS group 7 days after surgery

7 days after surgery, blood vessel density is significantly lower in the WGPS group (Figure 12) while there is no difference between the PBS and curdlan groups. (Supplementary figure 5)

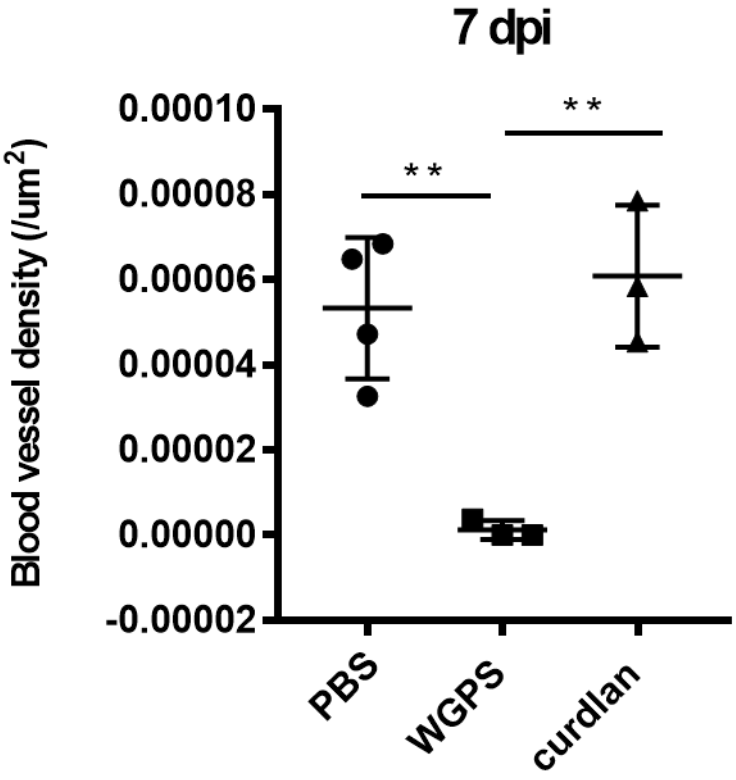


**Figure 12. Blood vessel density 7 days after surgery.** There is significantly decreased blood vessel density in the WGPS group 7 days after injury. (n: PBS=5, WGPS=3, curdlan=3)

## 3.8 Dectin-1 and Iba1 double staining revealed increased dectin-1 (+) macrophage in WGPS injected group 7 days after surgery

Iba1, a pan-macrophage marker, is used to label the total population of macrophage on injured sciatic nerves; the ratio of the number of dectin-1 (+) Iba1(+) cells to the number of Iba1(+) cells were calculated, the value of which is smaller in the PBS group than the WGPS group. (Figure 13)

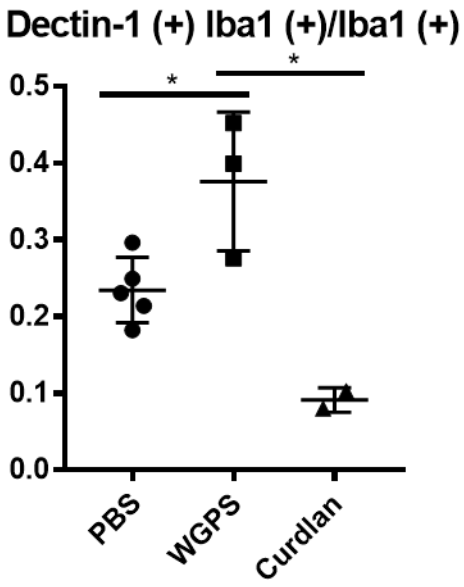



**Figure 13. Dectin-1(+)Iba1(+) to Iba1(+) ratio 7 days after surgery.** There is an increase in dectin-1 (+) macrophage (dectin-1(+) Iba1(+) cells) in the WGPS injected group 7 days after surgery. (n: PBS=5, WGPS=3, curdlan=2)

## 3.9 Dectin-1 (+) macrophage density is significantly higher in the WGPS and PBS group than the curdlan group

Dectin-1(+) Iba1(+) cells, i.e. dectin-1(+) macrophage, were significantly higher in density in the WGPS and PBS injected groups than the curdlan group. (Figure 14) However, there is no difference in macrophage density (Iba1(+) cells) between the 3 groups (see supplementary Figure 6).

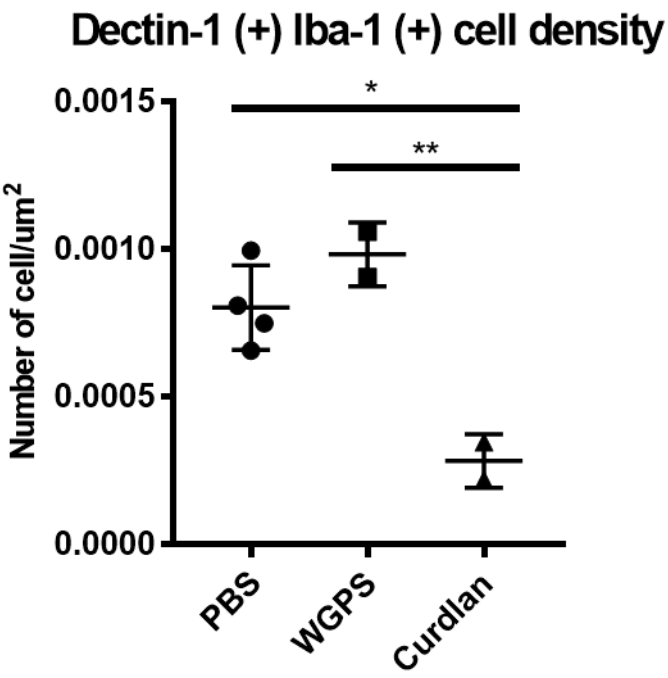



**Figure 14. Dectin-1(+)Iba1(+) cell density 7 days after surgery.** Dectin-1(+) macrophage density is significantly greater in the WGPS and PBS group than the curdlan group. (n: PBS=4, WGPS=2, curdlan=2)

## 3.10 Dectin-1 and CD206 double staining revealed increased M2 macrophage proportion in the Dectin-1 (+) macrophage population in WGPS injected group 7 days after surgery

Anti-CD206 antibody is used to label the M2 macrophages. The ratio of the number of CD206 (+) Dectin-1(+) cells to the number of Dectin-1(+) cells, i.e. the dectin-1 cells that belong to the M2 subgroup of macrophages, was calculated. The M2 macrophages ratio in dectin-1 (+) macrophages were significantly higher in the WGPS group than the curdlan group. (Figure 15)

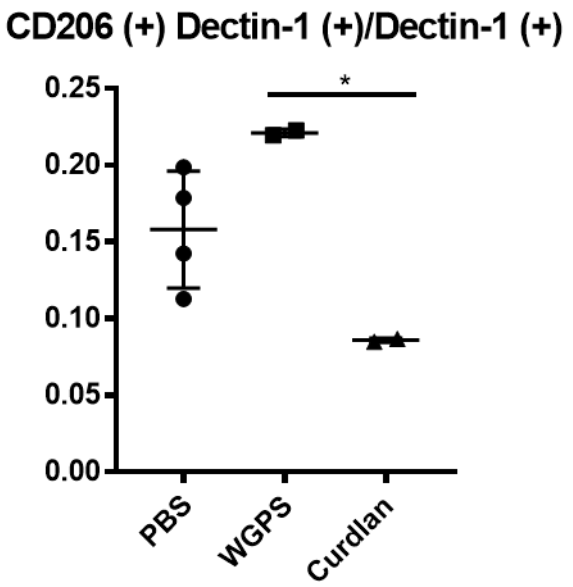



**Figure 15. CD206(+)Dectin-1(+) to Dectin-1(+) cell ratio.** The CD206 (+) cells in the Dectin-1(+) population is significantly higher in the WGPS group. (n: PBS=4, WGPS=2, curdlan=2)

## 3.11 There is delayed onset of prominent hypesthesia in WGPS injected group 6 days after surgery

The mechanical nociceptive threshold of the surgical side is mostly greater than that of the sham side. However, the mechanical threshold difference between the sham and surgery side is only significantly different from zero in the WGPS group 13 days after surgery (p=0.009). Therefore, 13 days after surgery, there is hypesthesia on the surgery side in the WGPS injected group. Comparing different groups, the mechanical threshold difference between the surgery and sham side is significantly greater in the curdlan group than the WGPS and PBS groups at 6 days after the surgery, while at 13 days after the surgery, the mechanical threshold difference is higher in the WGPS group than in the curdlan group. This indicates a trend of delay in hypesthesia in the WGPS group. (Figure 16 and 17)

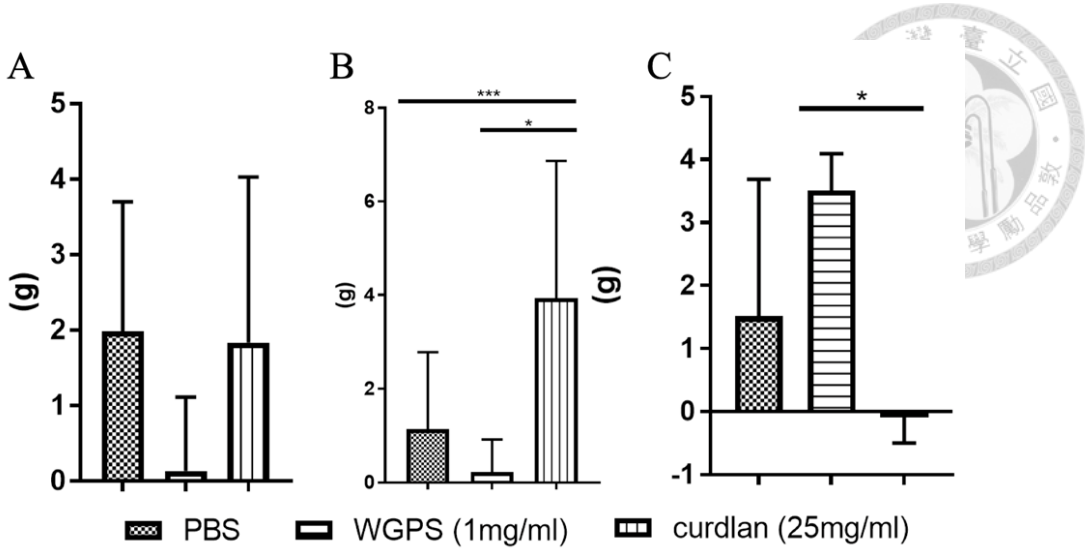



**Figure 16. Mechanical nociceptive threshold difference between the sham side and surgery side.** (A) 2, (B) 6, and (C) 13 days after the surgery. (n=3 in each group; except for PBS 6 days after surgery, n=6)

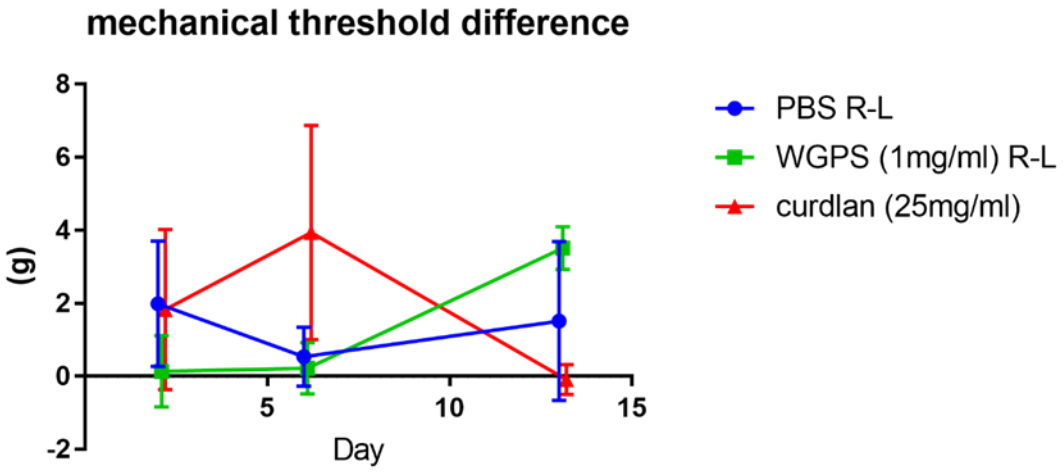



**Figure 17. Temporal pattern of mechanical threshold difference in of the 3 groups** (n=3 in each group; except for PBS 6 days after surgery, n=6)

# Chapter 4 Discussion

**Dectin-1 is localized on macrophage membrane and myelinated nerve axons**

Immunohistochemistry and immunofluorescence staining revealed an elevated expression of Dectin-1 (Figure 1 and 2) on the injured sciatic nerve. The positive staining co-localizes with the staining of Iba1 (Figure 4), a pan-macrophage membrane marker, suggesting Dectin-1 expression on the sciatic nerve is localized to the membrane of macrophages. Moreover, Figure 3. has shown that Dectin-1 is also present in intact myelinated nerve axons. To our knowledge, our study is the first to show there is presence of Dectin-1 on the axon of myelinated nerves; however, our experiments focus on the effect of Dectin-1 on macrophage, and the function of Dectin-1 on axon is still unclear.

**Semi-thin morphometry analysis showed delayed myelinated nerve degeneration in the WGPS injected group**

Semi-thin section morphology analysis of sciatic nerve distal to the injury site revealed significantly higher myelinated nerve density in the WGPS group than the curdlan group 3 days after surgery (Figure 6 (A)), and significantly higher myelinated nerve density in the WGPS group than the other groups 7 days after surgery (Figure 6 (B)). There are two possibilities: one is that WGPS promotes nerve regeneration and increased the number of regenerated nerve fibers, and the other is that WGPS delayed myelinated nerve degeneration and destruction, causing intact myelinated profiles, to be retained, seemingly increasing myelinated nerve density.

Myelin ovoid, as an indicator of complete myelinated nerve destruction[12], could differentiate between these two hypothesis. There is no significant difference between the three groups in myelin ovoid density 3 days after surgery (Supplementary Figure 4). Except for a few outliers, most of the myelin ovoid density at 3 days after surgery is smaller than the myelin ovoid density at 7 days after surgery. It is possible that by 3 days after surgery, myelin ovoid is not completely formed in all of the three groups, hence there is no difference. However, 7 days after surgery, myelin ovoid density

is significantly lower in the WGPS group than the PBS group, and 14 days after the surgery, myelin ovoid density is significantly higher in the WGPS group than the PBS group (Figure 7). The lower density of myelin ovoid and higher density of myelinated nerve at 7 days after the surgery, and the higher myelin ovoid density at 14 days after the surgery in the WGPS group suggests that there is a delayed formation of myelin ovoid, indicating delayed myelinated nerve degeneration in the WGPS injected group.

In the PBS injected groups, there is first an increase and then a decrease in the median of myelinated nerve diameter after injury (Figure 8 and 9). Shortly after nerve injury, an influx of calcium will cause axon to swell, followed by cytoskeleton breakdown.[12,13] After degeneration and myelinated nerve destruction, new sprouts of myelinated nerve with smaller diameter would form[1,12], compatible with the decrease in the median of myelinated nerve diameter 7 and 14 days after surgery. These new sprouts of myelinated nerves would then increase in size until they approach the diameter of intact myelinated fibers.[1]

However, comparing the WGPS injected group with the other groups, the median of myelinated nerve diameter is the smallest at 3 days, greatest at 7 days, and smallest again 14 days after surgery (Figure 10 and 11). This indicates the evident "swelling" of myelinated nerves is delayed from 3 days to 7 days after surgery in the WGPS groups, and the appearance of newly sprouted and smaller myelinated nerves is also delayed.

The median of myelinated nerve diameter in the curdlan group is biggest, hence the closest to intact myelinated nerve profile at 14 days after surgery, however (Figure 11), suggesting curdlan might aid in nerve regeneration 14 days after surgery. Further observation of longer than 14 days after surgery is still needed.

Therefore, WGPS is associated with a "delay" in Wallerian degeneration in that injured myelinated nerves are not readily cleared, less myelin ovoids and regenerated myelinated fibers are formed in the WGPS injected groups.

Less blood vessels are also observed in the WGPS groups (Figure 12); in a study by Agostini et al., barley beta-glucan promotes angiogenesis under oxidative microenvironment[14]. Though there is no difference of blood vessel density between our curdlan and PBS groups, there is a decrease in blood vessel density in our WGPS injected group at 7 days after surgery, indicating that by inhibiting downstream pathway of Dectin-1, not only Wallerian degeneration was delayed, but angiogenesis was also inhibited.

Except for the median of myelinated nerve diameter, we did not observe a change in blood vessel density (Supplementary Figure 5), myelinated nerve fiber density (Figure 6), or myelin ovoid density (Figure 7) between the curdlan and PBS group. (Supplementary figure 5) Krassmen et.al has shown that injection of apoptotic neuron in mice brain recruits Dectin-1 positive microglia, which suggests that in the injured nervous system, endogenous ligand might be present for Dectin-1[9]. It is possible that the Dectin-1 pathway is already activated in our PBS injected group, and thus trying to activate it does not create significantly different effects morphologically.

Moreover, one immediate injection after crush injury is probably not enough to retain its biological effects 14 days after surgery, therefore there is no significant difference between the 3 groups in blood vessel or myelinated nerve density 14 days after surgery.

Curdlan, a dectin-1 agonist, is associated with activation of macrophages[15] and induce down-stream immune response and cytokine release. Moreover, macrophage recruitment typically peaks at around 7 days after injury[12] However, our study has shown that at 7 days after nerve crush and injection surgery, there is an increased proportion of dectin-1(+) macrophage (Figure 13) and increased density of dectin-1(+) macrophage (Figure 14) in the WGPS injected group, but there is no significant difference in the density of macrophage between the 3 groups (Supplementary Figure 6.) Since our 1 dose of curdlan or WGPS is given immediately after nerve crush injury, it is possible that dectin-1 (+) macrophage is recruited at an earlier than 7 days in the curdlan group, but with delayed recruitment to 7 days in the WGPS group.

Our result has also shown that there is more M2 macrophage (CD206(+)) in the dectin-1 (+) subgroup of macrophage in the WGPS injected group 7 days after surgery. (Figure 15) Since M2 macrophage differentiation occurs at a later time than macrophage recruitment[12], it is reasonable to infer that the there is also a delayed recruitment or differentiation of the M2 subgroup of macrophage 7 days after nerve injury in the WGPS group.

Mechanical nociceptive threshold also suggests a trend of delayed hypesthesia in the WGPS injected group. However, there is no significant difference in our electrophysiological study in any groups, suggesting that 14 days is not enough for injured nerve to generate normal action potential, regardless of injected material.

# Chapter 5 Conclusions

1. There is an elevated expression of Dectin-1 on the sciatic nerve distal to the nerve crush site 7 days after the surgery, as compared with sham surgery side.
2. Dectin-1 is localized on macrophage cell membrane on the sciatic nerve distal to the crush site 7 days after sciatic nerve crush surgery.
3. Dectin-1 is innately present on the axon of myelinated nerves.
4. By injecting Dectin-1 antagonist WGPS immediately into the sciatic nerve after crush surgery was performed, clearance of injured myelinated nerves and formation of myelin ovoid was delayed and angiogenesis inhibited. This suggests Dectin-1(+) macrophage's involvement in debris clearance and angiogenesis after peripheral nerve injury.
5. The density of dectin-1 (+) macrophage and ratio of M2 macrophage in the dectin-1 (+) macrophage population are both significantly higher in the WGPS group 7 days after nerve injury, suggesting delayed recruitment of dectin-1(+) macrophage and delayed differentiation to the M2 macrophage in the WGPS group.
6. There is a trend of more prominent hypesthesia in the curdlan injected group 7 days after the surgery suggesting more neuronal injury; however, hypesthesia is more prominent in the WGPS injected groups 14 days after surgery, further supporting delayed clearance and inhibitory effects to macrophage activity of WGPS.

# References


1. Gaudet, A. D., Popovich, P. G. &Ramer, M. S. Wallerian degeneration: Gaining perspective on inflammatory events after peripheral nerve injury. *Journal of Neuroinflammation* vol. 8 (2011).
2. Guilbaud, G. *et al.* Time course of degeneration and regeneration of myelinated nerve fibres following chronic loose ligatures of the rat sciatic nerve: can nerve lesions be linked to the abnormal pain-related behaviours? *Pain* **53**, 147–158 (1993).
3. Chen, P., Piao, X. &Bonaldo, P. Role of macrophages in Wallerian degeneration and axonal regeneration after peripheral nerve injury. *Acta Neuropathologica* vol. 130 605–618 (2015).
4. Kigerl, K. A. *et al.* Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J. Neurosci.* **29**, 13435–13444 (2009).
5. Cherry, J. D., Olschowka, J. A. &O'Banion, M. K. Neuroinflammation and M2 microglia: The good, the bad, and the inflamed. *Journal of Neuroinflammation* vol. 11 (2014).
6. Martinez, F. O., Gordon, S., Locati, M. &Mantovani, A. Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression. *J. Immunol.* **177**, 7303–7311 (2006).
7. Baldwin, K. T., Carbajal, K. S., Segal, B. M. &Giger, R. J. Neuroinflammation triggered by β-glucan/dectin-1 signaling enables CNS axon regeneration. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 2581–2586 (2015).
8. Gensel, J. C. *et al.* Toll-Like receptors and Dectin-1, a C-type lectin receptor, trigger divergent functions in CNS macrophages. *J. Neurosci.* **35**, 9966–9976 (2015).
9. Krasemann, S. *et al.* The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity* **47**, 566-581.e9 (2017).
10. Chiang, H. Y. *et al.* Reinnervation of muscular targets by nerve regeneration through

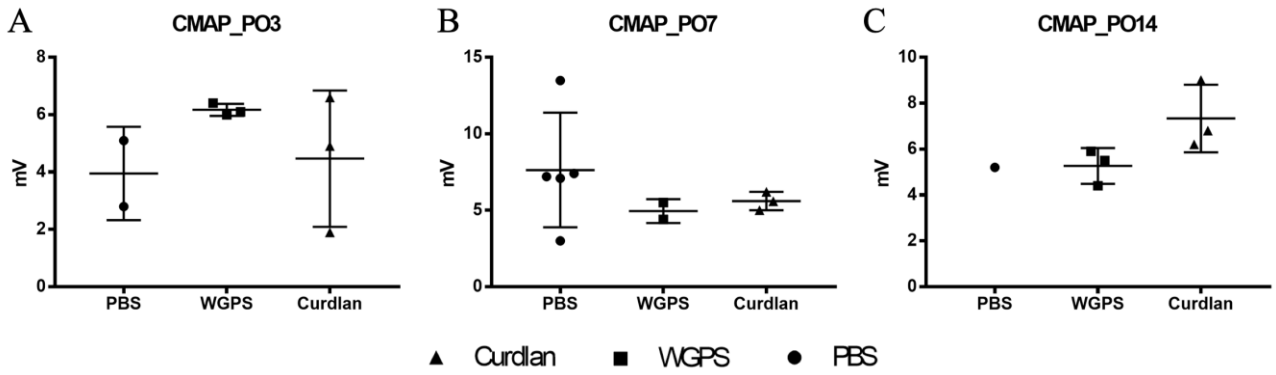
guidance conduits. *J. Neuropathol. Exp. Neurol.* **64**, 576–587 (2005).

11. Zaimi, A. *et al.* AxonSeg: Open Source Software for Axon and Myelin Segmentation and Morphometric Analysis. *Front. Neuroinform.* **10**, (2016).
12. Tricaud, N. &Park, H. T. Wallerian demyelination: chronicle of a cellular cataclysm. *Cellular and Molecular Life Sciences* vol. 74 4049–4057 (2017).
13. Hutton, E. J. *et al.* C-Jun expression in human neuropathies: A pilot study. *J. Peripher. Nerv. Syst.* (2011) doi:10.1111/j.1529-8027.2011.00360.x.
14. Agostini, S. *et al.* Barley beta-glucan promotes MnSOD expression and enhances angiogenesis under oxidative microenvironment. *J. Cell. Mol. Med.* **19**, 227–238 (2015).
15. Rosas, M. *et al.* The Induction of Inflammation by Dectin-1 In Vivo Is Dependent on Myeloid Cell Programming and the Progression of Phagocytosis. *J. Immunol.* **181**, 3549–3557 (2008).

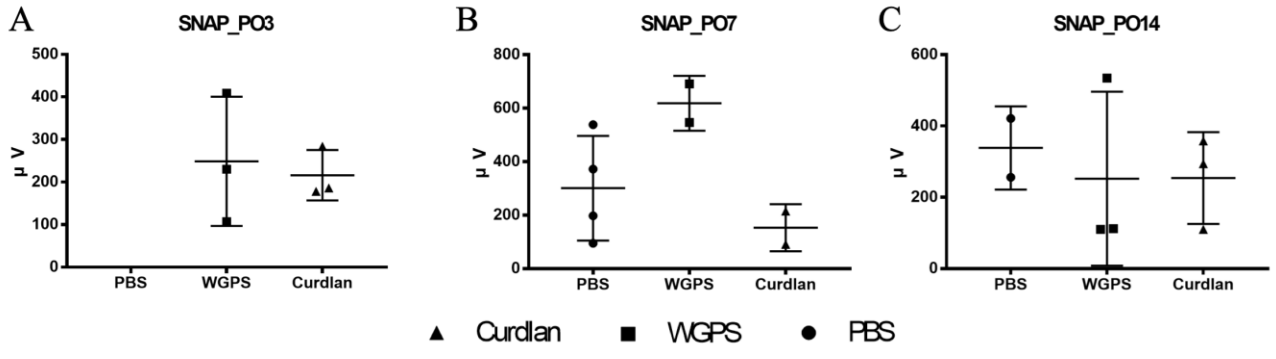
# Supplementary Materials

## S1 Injured nerve is unable to generate normal action potential in 14 days

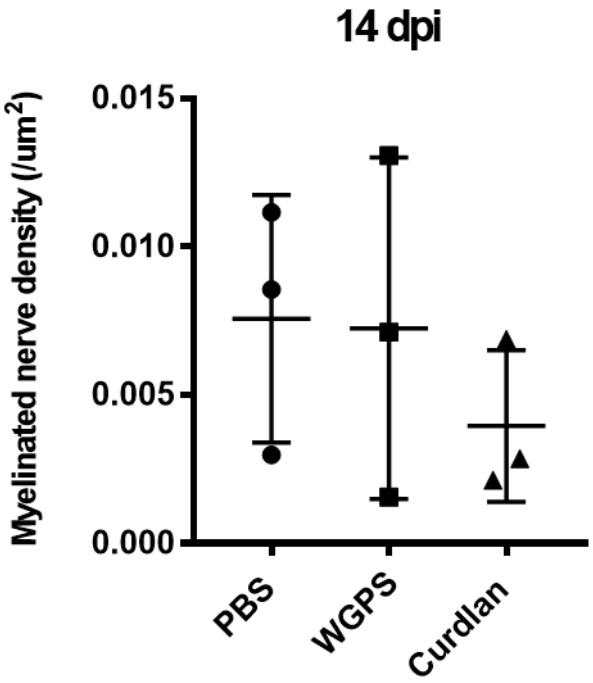
We calculated the difference of action potential between the sham side and surgery side, to use the sham side as internal control. There is no statistical difference between the compound muscle action potential difference and sensory nerve action potential difference any groups at 3, 7, and 14 days after the surgery. (Supplementary Figure 1 and 2)



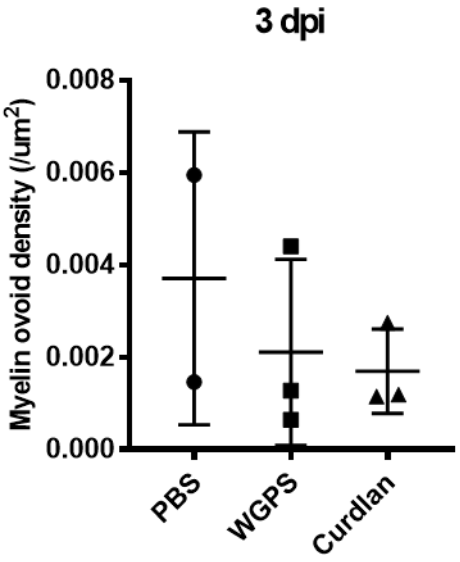

**Supplementary Figure 1. Compound muscle action potential (CMAP) difference of the PBS, WGPS, and curdlan injected groups**. (A) 3, (B) 7, and (C) 14 days after the surgery; there is no significant difference between any groups. Each point on the graph corresponds to n=1.



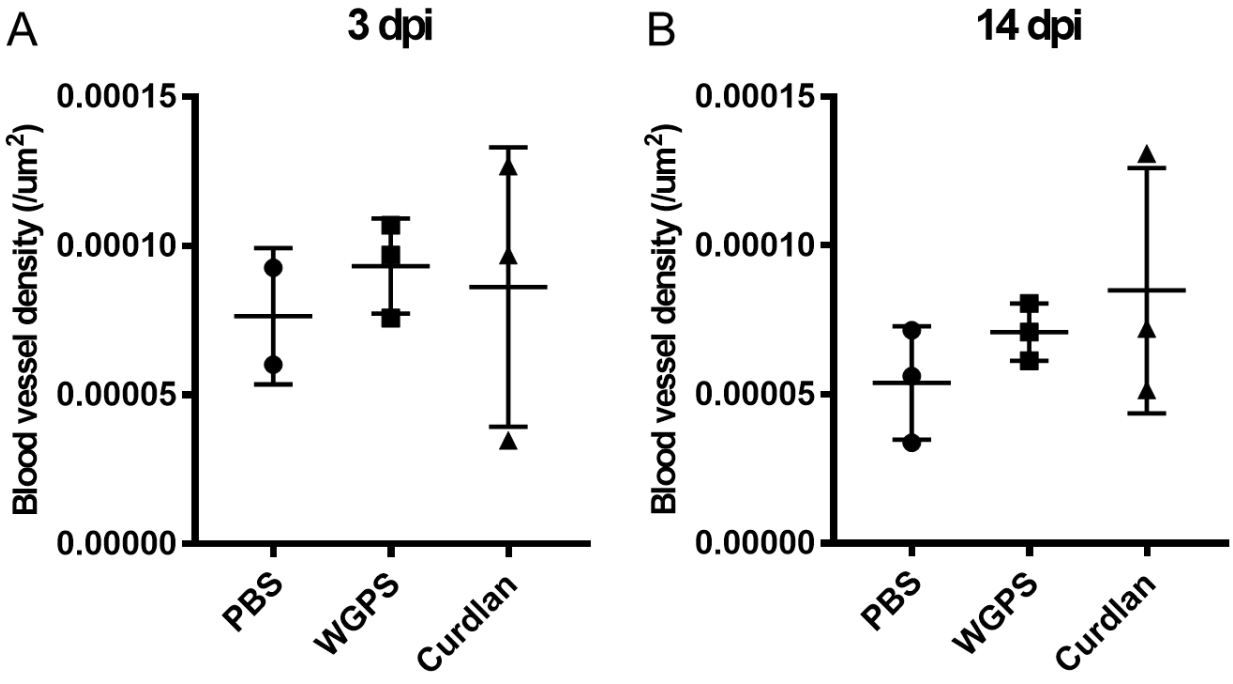
**Supplementary Figure 2. Sensory nerve action potential (SNAP) difference of the PBS, WGPS, and curdlan injected groups** (A) 3, (B) 7, and (C) 14 days after the surgery; there is no significant difference between any groups. Each point on the graph corresponds to n=1.



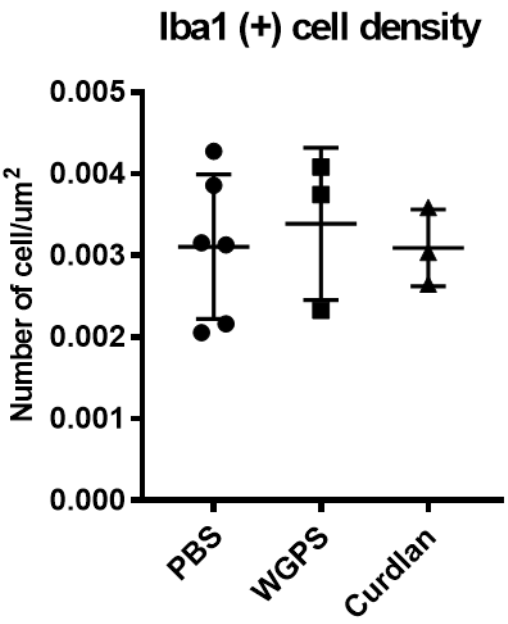

**Supplementary Figure 3. Myelinated nerve density 3 days after surgery.** There is no significant difference between the 3 groups in myelinated nerve density 14 days after surgery. Each point on the graph corresponds to n=1.




**Supplementary Figure 4. Myelin ovoid density 3 days after surgery.** There is no significant difference between the 3 groups in myelin ovoid density 3 days after surgery.




**Supplementary Figure 5. Blood vessel density.** There is no difference in blood vessel density in the PBS, WGPS, and curdlan injected groups 3(A) and 14(B) days after surgery,




**Supplementary Figure 6**. **Iba1 (+) cell density**. There is no significant difference between the 3 groups in macrophage density.