The effects of the M2a macrophage-induced axonal regeneration of neurons by arginase 1

Jieyuan Zhang1, Yue Li2, Zhaoxia Duan1, Jianyi Kang1, Kuijun Chen1, Guanhua Li1,
Changmei Weng1, Dongdong Zhang1, Lu Zhang3, Jianmin Wang1*, Bingcang Li1**

1 Daping Hospital, Army Medical University; State Key Laboratory of Trauma, burn and Combined Injury, 400042 Chongqing, People’s Republic of China
2 Xinan Hospital, Army Medical University, 400038 Chongqing, People’s Republic of China
3 Department of Pediatric Research Institute, Children’s Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders; China International Science and Technology Cooperation base of Child development and Critical Disorders; Chongqing Key Laboratory of Translational Medical Research in Cognitive Development and Learning and Memory Disorders, Chongqing, 400014 China.

*Corresponding author:
Bingcang Li, Daping Hospital, Army Medical University; State Key Laboratory of Trauma, burn and Combined Injury, 400042 Chongqing, People’s Republic of China, Tel: +86-13883161538, Email: bcli1118@qq.com and drbingcanli@yeah.net.
Jianmin Wang, Daping Hospital, Army Medical University; State Key Laboratory of Trauma, burn and Combined Injury, 400042 Chongqing, People’s Republic of China, Tel:+86-13883280366, Email: jmwang@tmmu.edu.cn;

Short title: Arginase 1 induces axonal regeneration of neurons
Abstract

**Background:** Spinal cord injury (SCI) is a challenge worldwide, but there are no effective treatments or therapeutic methods in the clinic. Recent studies have shown that type I arginase (Arginase1, Arg1) is closely associated with the treatment of SCI. The classical treatment for SCI involves filling the local area of SCI with activated M2a macrophages to allow the repair and regeneration of some synapses, but the specific mechanism of action of Arg1 is not clear.

**Method:** In this study, we first induced the polarization of RAW264.7 macrophages to M2a-type cells using IL-4 and constructed an Arg1 knockout cell line through the use of shRNA; we used these cells to treat a rat model of SCI. Finally, this study explored the mechanism and pathway by which Arginase 1 regulates spinal repair by immunoblotting and immunohistochemistry.

**Result:** Suspended M2a (Arg1-/+) macrophages were transplanted into the injury site in a rat model of contusion SCI. Compared with the model group and the shArg1 group, the shScramble (shSc) group exhibited higher BBB motor function scores, more compact structures and more Nissl bodies. Immunohistochemical results showed that the shSc group expressed higher levels of NeuN (a neuronal marker) and tau (an axonal marker), as well as the upregulation of Cdc42, N-WASP, Arp2/3 and tau, as determined by western blot.

**Conclusion:** The study found that the polarization of M2a macrophages promoted the expression of Arginase 1, which restored axonal regeneration, promoted axonal regeneration, and promoted the structural and functional recovery of the contused spinal cord.

**Key words:**

Spinal cord injury; M2a macrophage; Arginase1; Cdc42/N-WASP
Introduction

SCI is a catastrophic event for patients; it can profoundly affect a patient's quality of life and has profound social and economic impacts [1]. According to the World Health Organization, it is estimated that the annual incidence of SCI globally is 15-40 million, and it increases as society develops[2]. The number of cases of spinal cord injury in China has increased tenfold in the past decade, and 60,000 new cases are added each year [3, 4]. The low rate of systemic rehabilitation in patients with spinal cord injury not only causes serious physical and psychological harm to the patient but also imposes a huge economic burden on society as a whole. The treatment and rehabilitation of spinal cord injury has therefore made spinal cord injury treatment a major clinical and research challenge worldwide [2, 5, 6].

There are two mechanisms of injury in spinal cord injury[7]: primary and secondary lesions. The mechanism of primary injury involves mechanical trauma to the spinal cord tissue, which involves the continuous compression of the spinal cord due to movement of the bone or intervertebral disc into the tissue, resulting in damage to the nerve/glial tissue and vasculature at the injury site[8]. Secondary lesions involve edema, hemorrhage, ischemia, axonal demyelination and degeneration, or the loss of whole neurons or glial cells and thereby alter the ionic homeostasis and inflammatory/immune response of the microenvironment[9]. A serious problem caused by secondary injury is cystic cavity formation at the site of the injury, which makes axonal regeneration at the center of the injury difficult[10]. However, in the microenvironment of spinal cord injury, locally activated monocytes play an important role in tissue repair [11]. The depletion of macrophages improves recovery and increases the repair phenotype of macrophages, increasing axonal growth and motor function [1, 12]. These findings provide evidence that macrophages can promote axonal regeneration to alleviate the development of SCI.

The role of macrophage activation in promoting axonal regeneration in M2a cells is clear, but the mechanism of action has not been reported in the literature. After macrophages are polarized to the M2a subtype, the expression specificity of Arg1 is increased [13]. Moreover, recent studies have reported that Arg1 is also an important switch molecule involved in axonal regeneration [14]. The role of Arg1 in promoting axonal regeneration is closely related to the promotion of the cytoskeleton by tubulin [15]. From these studies, we hypothesize that Arg1 is an important switch molecule in M2a macrophages for the promotion of neuronal axon regeneration. Therefore, this study evaluated the effect of M2a macrophages on the therapeutic effect of Arg1 on SCI. By constructing an SCI model
and knocking down M2a macrophages, the effects of Arg1 on M2a macrophages in SCI were explored by observing the therapeutic effects and changes in animal-related experiments.

**Materials and Methods**

**Reagents, Antibodies**

IL-4 was purchased from Sigma-Aldrich (St. Louis, USA). Lipofectamine 2000 was purchased from Life Technologies (Carlsbad, CA, USA). Arg1, iNOS, Cdc42, N-WASP, Arp2, NeuN and tau antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). GAP-43 and CD206 antibodies were obtained from Proteintech (Wuhan, China). GAPDH and β-actin antibodies were obtained from Santa Cruz Biotechnology.

**Animals**

All male Sprague Dawley rats (age, 6-8 weeks; weight, 150–200 g) were purchased from Animal Center of Army Medical University and approved by the ethics of Army Medical University. All animal experiments were conducted at the Experimental Animal Center of Army Medical University. All animals were housed according to the Army Medical University (AMU) guidelines, and the surgical procedures and postoperative care were conducted in accordance with protocols approved by the AMU Institutional Animal Care and Use Committee. All animals were anesthetized by sodium pentobarbital injection and sacrificed by asphyxiation in a carbon dioxide chamber.

**Cell culture**

RAW264.7 cells, a mouse macrophage cell line, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Sigma-Aldrich, St. Louis, USA) containing 10% fetal bovine serum (FBS; HyClone, UT, USA) in a humidified chamber at 37°C with 5% CO2. The cells were seeded in 6-well plates at a density of 0.5 ×10^6 cells/mL and cultured overnight.

**Preparation of recombinant viruses and cell transfection**
pGPU6/GFP/Neo lentiviral shRNA expression vectors targeting mouse Arg1 a negative control containing a sequence with no homology to the shRNA sequence of interest or to any gene sequence (shscramble, shSc) were obtained from Cyagen Biosciences (Guangzhou, China). The target sequences for Arg1 were 5'-GGACUGGACCCAUCUUUCA-3' and 5'-GAAGUAACUCGAACAGUGA-3'. An oligonucleotide sequence with a short hairpin structure was cloned into the empty pGPU6/GFP/Neo vector to construct shRNA recombinant plasmid. The cells were divided into three groups: the shSc group (transfection of the pGPU6/GFP/Neo-NC negative plasmid), the shArg1-1 group (transfection of the pGPU6/GFP/Neo-Arg1 plasmid$^1$), and the shArg1-2 group (transfection of the pGPU6/GFP/Neo-Arg1 plasmid$^2$). The recombinant plasmid was transfected into the cells with Lipofectamine™ 2000 according to the manufacturer’s instructions. Thirty-six hours after transfection, G418 (500 µg/mL) was used to screen the transfected cells, and then the monoclones were selected for culture expansion.

**Quantitative real-time PCR**

Total RNA was extracted from cells using TRizol (Invitrogen) following the manufacturer’s instructions. A total of 1 µg of RNA was converted to cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa). After a 10-fold dilution, 4 µl of cDNA was used as a template for real-time PCR with TransStart Green qPCR SuperMix (TransGen Biotech, Beijing, China). The expression of β-actin was detected as an internal control. The primer sequences used for PCR were as follows:

**Arg1 Forward:** 5'-CTCCAAGCCAAAGTCCTTAGAG-3';

**Reverse:** 5'-AGGAGCTGTCATTAGGGACATC-3'.

**Western blotting assay**

Cells and tissues were lysed using ice-cold cell lysis buffer (Beyotime Biotechnology, Haimen, China). After protein content determination via a BCA Protein Assay Kit (Beyotime Biotechnology), the protein samples were denatured at 95°C for 5 min. The protein samples (100 µg) were loaded on 8–12% SDS-PAGE gels and then electrophoretically separated. After being transferred to PVDF membranes (Millipore, USA), the proteins were blocked with 5% nonfat milk at room temperature and incubated with primary antibodies at 4°C overnight. Then, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for 1 h. The immunoreactive
bands were visualized by the enhanced chemiluminescence method.

**Animal model**

Adult female Sprague-Dawley rats (N=13/group, 180-220 g) were anesthetized with a single intraperitoneal injection of 1% sodium pentobarbiturate (30 mg/kg). A sterile laminectomy was performed at the vertebral T10 level without disrupting the dura. The spinal cord was contused using an NYU-II impactor with a weight of 10.0 g released from 25 mm above the exposed cord. The procedure resulted in hind limb paralysis in all animals. Cell transplantation was performed after spinal cord injury using a previously described method (Li, 2012 #104). Briefly, 2 µl of suspended cells (1.0x10^5 M2a (Arg1-/+) macrophages per µl) was injected under a surgical microscope using a Hamilton syringe, which remained in place for 5 min after each injection. Model animals were injected with identical volumes of DMEM at the same site. After the injection, the muscle and skin were closed with interrupted sutures. Bicillin (60,000 U/kg, intramuscular route) was administered daily for 7 days after contusion to prevent wound and bladder infections, and bladder expression was performed twice daily.

**Basso, Beattie, Bresnahan (BBB) scores**

The animals were placed on a circular platform with a diameter of 2 m, and walking and hind limb activity were observed. The scale was divided into three parts: the first part was 0-7 points, and the hind limb joint activity of the animals was judged; the second part was 8-13 points, and the gait and coordination of the hind limbs were judged; the third part was 14-21 points, and the fine movement of the paws was judged. The three parts totaled 21 points. BBB scoring was performed on the experimental animals before surgery, on the third day after surgery, 1 week after surgery, 2 weeks after surgery, and 4 weeks after surgery. The data were analyzed by Student’s t test.

**Immunofluorescence (IF)**

Cells were seeded on coverslips overnight and then fixed with 4% formaldehyde for 5 min at room temperature, followed by treatment with 1% Triton X-100 (Sigma-Aldrich) for permeabilization. After blocking with 2% bovine serum albumin for 1 h, the cells were incubated with an anti-iNOS antibody (1:500; Cell Signaling Technology) overnight and then incubated with a FITC-conjugated secondary antibody (1:200; Cell Signaling Technology) for 1 h. DAPI (Sigma-Aldrich) was used to visualize the
cell nuclei, and the fluorescence staining was examined under a microscope (Olympus, Tokyo, Japan).

**Flow cytometry**

The cells were carefully scraped with a cell scraper, centrifuged, and resuspended in 100 μL of PBS to form a single cell suspension. A CD206 antibody (1:1000) and 10 μL of negative control IgG were added. The cells were incubated for 15 min in the dark, washed with PBS to wash away the excess antibody, and resuspended in buffer. The expression level of CD206 was detected by flow cytometry.

**Immunohistochemistry (IHC)**

Immunohistochemical analysis was performed as described previously. In brief, tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were prepared for the staining with the indicated primary antibodies (tau: 1:500; NeuN: 1:400) or hematoxylin and eosin (H&E) according to standard protocols.

**Nissl staining**

After dewaxing the paraffin sections, the sections were immersed in Nissl staining solution for 5 min. They were then rinsed with distilled water, dehydrated in ethanol, and cleared in xylene. Images of the gray matter area were taken using an optical microscope (Olympus TH4200). The percentage of Nissl positive neurons relative to the total number of neurons in the same field of view was then calculated.

**Toluidine blue staining**

The paraffin sections were dewaxed, and the sections were placed in a toluidine blue staining solution for 30 min, washed with distilled water, dehydrated stepwise, cleared in xylene, and sealed with neutral resin. Images of the gray matter area were taken using an optical microscope (Olympus TH4200). The percentage of positive neurons relative to the total number of neurons in the same field of view was then calculated.

**Statistical analysis**

All values are expressed as the mean ± standard deviation. Statistical differences were determined using a 2-tailed Student’s t-test. Differences between the SCI group and control group were calculated
using an unpaired Student’s t test, and differences between the other groups were analyzed by one-way analysis of variance (ANOVA). Statistical significance was defined as \( P<0.05 \). All statistical analyses were conducted using SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA).

**Result**

1. **Identification of M2a macrophages**

Mouse RAW264.7 macrophages were first polarized to M2a cells. RAW264.7 cells showed an elliptical shape in the medium. After stimulation with 20 nmol of IL-4, the cells grew towards the periphery and became spindle-shaped (Fig. 1A). Immunofluorescence was then used to examine the expression of the iNOS protein in RAW264.7+LPS/IL-4 cells. iNOS was expressed in RAW264.7+LPS cells but hardly expressed in RAW264.7+IL-4 cells (Fig. 1B). Then, we used western blotting and flow cytometry to verify the specific protein expression of Arg1 and CD206 in RAW264.7+IL-4 cells (Fig. 1C-D). The experimental results showed that RAW264.7 cells that were stimulated by IL-4 for 24 h were polarized to M2a (iNOS-/Arg1+/CD206+) macrophages, and subsequent experiments were carried out.

2. **Verification of Arg1 knockdown in M2a macrophages**

Activated M2a cells were cultured, an Arg1 knockdown lentivirus was transfected with Lipofectamine 2000 transfection reagent and mRNA and protein expression were then verified by western blotting and real-time PCR. The results showed that both interference sequences effectively inhibited Arg1. Sequence 2 was more effective (Fig. 2A-2B), and the cells transfected with this sequence and controls were selected for related animal experiments.

3. **Knockdown of Arg1 in macrophages hinders axonal regeneration and the repair of the spinal cord**

After the construction of a rat model of spinal cord injury, M2a (Arg1+/_) macrophages were injected into the SCI site immediately, and culture medium was injected into the model group. To determine whether the functional recovery of spinal cord injury occurred after the injection of macrophages, we evaluated the animals using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale and assessed them weekly for 4 weeks after injury. All animals had a maximum score of 21 before SCI. The
animals scored 0 and <3 on days 1 and 4, respectively, after SCI (Fig. 3). Over the following few days, athletic performance improved and reached a stable level in the third week. From days 4 to 28, the BBB score of the M2a knockout control (shSc) group was higher than that of the other groups (P < 0.05); there was no significant difference between the control group and the shArg1 group.

Six weeks after cell treatment, the rats were anesthetized and sacrificed. The spinal cord tissue near the SCI site was collected, fixed and embedded, and pathological examination was performed. The results of HE staining showed (Fig. 4A) that, compared with the shSc-treated cells, shArg1-treated rat spinal cord cells showed small circular vacuoles in the leukoplakia and macrophage infiltration at the injury site. This result shows that the knockout of Arg1 blocks the repair of the tissue structure of the spinal cord by M2a macrophages.

To verify the above results, a portion of the sections from the center of the lesion were stained with toluidine blue (Fig. 4C). Diffuse demyelination was observed in the shArg1 and model groups, and a greater number of myelinated axons was diffused in the shSc group. The number of Nissl bodies in the shSc group was higher than that in the other groups (P <0.01). At the same time, we reached the same conclusions by Nissl staining.

The nerve cells in the shSc group were clear and intact, and the Nissl stain was uniformly distributed around the nucleus. The axons of the neurons were visible and clear. A significant proportion of neurons in the model group was damaged, showing a loss of cell integrity, cytoplasmic contraction, Nissl pyknosis and nuclear nucleation. The Nissl bodies in the shArg1 group were not significantly improved, the pyramidal cells were loosely arranged, the Nissl stain was small and fuzzy, and the axons disappeared (Fig. 4E).

4. Arg1 promotes axonal production through the Cdc42 pathway.

Pathological experiments showed that Arg1 is closely associated with the M2a macrophage-mediated repair of axons. We performed immunohistochemistry on sections and examined the specific protein expression of NeuN and tau (Fig. 5A-5C) to evaluate axon formation. The experimental results showed that the expression of NeuN and tau, i.e., axon formation, in the shSc group was increased relative to that in the model group and shArg1 group. In the shArg1 group, the expression levels of NeuN and tau decreased, and there was no significant difference between the shArg1 group and the model group.

To explore how Arg1 promotes axonal repair and generation, we detected the expression of
axon-related genes in experimental spinal tissues by western blotting (Fig. 6A). The results showed that the expression of Cdc42, N-WASP, Arp2 and tau was significantly increased in the shSc group compared with the model group, while in the shArg1 group, these expression levels were not specifically different from those in the model group, and the grayscale analysis also showed the same conclusion (Fig. 6B).

Discussion

In this study, a rat model of contusion SCI was used to study axonal repair of spinal cord cells by M2a macrophages and the molecular mechanism of Arg1 in spinal cord structural reconstruction.

One of the difficulties in the treatment of spinal cord injury is the difficulties with axonal regeneration, which is associated with a large number of necrotic or apoptotic neurons in the spinal cord, the difficulties with neuronal regeneration and a reduction in the levels of nutrient factors secreted by nerve cells, which disrupt beneficial microflora regeneration [15, 16]. Macrophages are inflammatory cells in the human body, and they play an important role in the repair of wounds [17, 18]. After SCI, macrophages promote secondary damage and axonal repair. These two opposing biological functions may be caused by different macrophage subpopulations, namely, proinflammatory M1 macrophages and anti-inflammatory M2 macrophages [19]. M2 macrophages, as well as activation of specific intracellular signaling cascades. After spinal cord injury, M1 macrophages are located in the lesion, and a proinflammatory signaling mechanism may persist indefinitely in the lesion. M2 macrophages are not neurotoxic and can promote long-distance axonal growth. M2-type macrophages are activated by Th-2 cytokines, such as IL-4 and IL-13, and immune complexes, and M2 macrophages inhibit inflammatory factors and play a role in inhibiting the inflammatory response and tissue repair. Based on their different roles in tissue repair, M2 macrophages can be further divided into three subtypes, namely, M2a, M2b and M2c macrophages. Among them, M2a macrophages, which are induced by IL-4, can play an immunomodulatory role and promote an M2-type immune response. This study also confirmed that M2a macrophages have a certain reparative effect on spinal cord injury in an SCI animal model. In this experiment, we further verified that M2a macrophages have a certain reparative effect on spinal cord injury and play an important role in spinal cord repair.

The role of M2a in promoting axonal regeneration is clear, but its mechanism of action has not yet been reported in the literature. After macrophages are polarized to the M2a subtype, the specific expression
of arginase I is increased [20, 21]. Moreover, recent studies have reported that Arg1 is an important switch molecule involved in axonal regeneration. Arg1 can catalyze the production of ornithine and polyamines by arginine, which is a necessary precursor for collagen synthesis and cell proliferation. Arg1 promotes cytoskeletal formation. Polyamines in the nervous system increase tubulin expression, promote cell scaffolding formation in neurons, and induce axonal extension [22-24]. Arg1 can block the inhibition of axonal regeneration by myelin-related inhibitors. Studies have further indicated that myelin-associated inhibitors that antagonize the expression of Arg1 activate the RhoA-ROCK signaling pathway, inhibit axonal regeneration, and significantly reduce regeneration and repair after spinal cord injury [25-27]. The regulation of Arg1 expression is closely related to the regeneration and repair of spinal cord tissue after injury [28, 29]. In this experiment, by knocking down the Arg1 gene in M2a cells in animal experiments, it was confirmed that the role of M2a macrophages in promoting axonal regeneration may be achieved through the regulation of Arg1.

Previous studies have shown that the role of Arg1 in promoting axonal regeneration is closely related to the promotion of cytoskeletal formation by tubulin [30]. Cdc42 performs a variety of important regulatory functions involving cell migration and skeletal changes. Wiskott Aldrich syndrome protein (WASP), including neuronal WASP (N-WASP) is a key molecule that regulates cell migration and cytoskeletal changes and is also an important effector downstream of Cdc42 [31]. Different members of the WASP family are activated by binding to different domains of Cdc42, and actin-related protein 2/3 (Arp2/3) complexes and actin monomers bind to activated WASP. The common domain of the family carboxy-terminal can synthesize actin monomers into actin filaments, thereby promoting the formation of filopodia and directly regulating the polymerization of actin monomers [32]. The Arp2/3 complex is a key molecule that regulates dynamic changes in actin, promotes the depolymerization and reflux of actin, and promotes actin filaments dynamics in the axonal growth cone, allowing it to be extended. The Arp2/3 complex promotes the extension of axons [33]. The binding of the Arp2/3 complex to WASP is an important process in the regulation of actin polymerization [34]. In this study, we demonstrated that Arg1-mediated M2a macrophages participate in axonal regeneration by activating the Cdc42 signaling pathway, promoting N-WASP activation, and binding to the Arp2/3 complex, thereby promoting actin polymerization and the repair of axons.

**Conclusion**
In conclusion, we demonstrated that Arg1 plays an important role in axonal repair in the spinal cord through the knockdown of Arg1 in M2 macrophages and control cell lines. The results showed that axonal repair in control M2 macrophages was increased compared with that in SCI model animals, but Arg1 knockout did not enhance repair. Moreover, we propose that Arg1-mediated M2a macrophages participate in axonal regeneration by activating the Cdc42/N-WASP/Arp2 signaling pathway (Fig. 7).

Conflicts of interest
The authors declare no conflicts of interest.

Funding
This work was supported by the National Natural Science Foundation of China (No. 81601676) and the Major Special Foundation of Army Logistic Research Plan (No. AWS14C003).

Author contribution statement:
BCL and JMW conceived and designed the experiments. JYZ, YL, ZXD, JYK, KJC, GHL and CMW performed the experiments and JYZ, DDZ, LZ analyzed the data. JYZ, BCL and JMW wrote the paper. All authors read and approved the final manuscript.
Reference:
[1] J.C. Gensel, B. Zhang, Macrophage activation and its role in repair and pathology after spinal cord injury, Brain Res, 1619 (2015) 1-11.
[2] A. Singh, L. Tetreault, S. Kalsi-Ryan, A. Nouri, M.G. Fehlings, Global prevalence and incidence of traumatic spinal cord injury, Clin Epidemiol, 6 (2014) 309-331.
[3] H.L. Li, H. Xu, Y.L. Li, S.W. Sun, W.Y. Song, Q. Wu, J. Ai, J.C. Sun, G.Z. Ning, S.Q. Feng, Epidemiology of traumatic spinal cord injury in Tianjin: An 18-year retrospective study of 735 cases, J Spinal Cord Med, (2018) 1-13.
[4] G.Z. Ning, Z.P. Mu, L. Shangguan, Y. Tang, C.Q. Li, Z.F. Zhang, Y. Zhou, Epidemiological features of traumatic spinal cord injury in Chongqing, China, J Spinal Cord Med, 39 (2016) 455-460.
[5] R.J. Hurlbert, M.N. Hadley, B.C. Walters, B. Aarabi, S.S. Dhall, D.E. Gelb, C.J. Rozzelle, T.C. Ryken, N. Theodore, Pharmacological therapy for acute spinal cord injury, Neurosurgery, 76 Suppl 1 (2015) S71-83.
[6] L.A. Simpson, J.J. Eng, J.T. Hsieh, D.L. Wolfe, T. Spinal Cord Injury Rehabilitation Evidence Scire Research, The health and life priorities of individuals with spinal cord injury: a systematic review, J Neurotrauma, 29 (2012) 1548-1555.
[7] M.T. Caprelli, A.J. Mothe, C.H. Tator, Hyperphosphorylated Tau as a Novel Biomarker for Traumatic Axonal Injury in the Spinal Cord, J Neurotrauma, 35 (2018) 1929-1941.
[8] B.C. Hains, J.A. Black, S.G. Waxman, Primary cortical motor neurons undergo apoptosis after axotomizing spinal cord injury, J Comp Neurol, 462 (2003) 328-341.
[9] X. Kong, J. Gao, Macrophage polarization: a key event in the secondary phase of acute spinal cord injury, J Cell Mol Med, 21 (2017) 941-954.
[10] C.H. Tator, Update on the pathophysiology and pathology of acute spinal cord injury, Brain Pathol, 5 (1995) 407-413.
[11] I. Francos-Quijorna, J. Amo-Aparicio, A. Martinez-Muriana, R. Lopez-Vales, IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury, Glia, 64 (2016) 2079-2092.
[12] P.G. Popovich, Z. Guan, P. Wei, I. Huitinga, N. van Rooijen, B.T. Stokes, Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury, Exp Neurol, 158 (1999) 351-365.
[13] K.L. Lankford, E.J. Arroyo, K. Nazimek, K. Bryniarski, P.W. Askenase, J.D. Kocsis, Intravenously delivered mesenchymal stem cell-derived exosomes target M2-type macrophages in the injured spinal cord, PLoS One, 13 (2018) e0190358.
[14] M. Ahn, C. Lee, K. Jung, H. Kim, C. Moon, K.B. Sim, T. Shin, Immunohistochemical study of arginase-1 in the spinal cords of rats with clip compression injury, Brain Res, 1445 (2012) 11-19.
[15] J. Li, Q. Wang, H. Wang, Y. Wu, J. Yin, J. Chen, Z. Zheng, T. Jiang, L. Xie, F. Wu, H. Zhang, X. Li, H. Xu, J.
Xiao, Lentivirus Mediating FGF13 Enhances Axon Regeneration after Spinal Cord Injury by Stabilizing Microtubule and Improving Mitochondrial Function, J Neurotrauma, 35 (2018) 548-559.

[16] K. Nagamoto-Combs, D.W. McNeal, R.J. Morecraft, C.K. Combs, Prolonged microgliosis in the rhesus monkey central nervous system after traumatic brain injury, J Neurotrauma, 24 (2007) 1719-1742.

[17] D.L. Schonberg, P.G. Popovich, D.M. McTigue, Oligodendrocyte generation is differentially influenced by toll-like receptor (TLR) 2 and TLR4-mediated intraspinal macrophage activation, J Neuropathol Exp Neurol, 66 (2007) 1124-1135.

[18] D. Giulian, C. Robertson, Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord, Ann Neurol, 27 (1990) 33-42.

[19] K.A. Kigerl, J.C. Gensel, D.P. Ankeny, J.K. Alexander, D.J. Donnelly, P.G. Popovich, Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord, J Neurosci, 29 (2009) 13435-13444.

[20] Y. Wang, M. Wehling-Henricks, G. Samengo, J.G. Tidball, Increases of M2a macrophages and fibrosis in aging muscle are influenced by bone marrow aging and negatively regulated by muscle-derived nitric oxide, Aging Cell, 14 (2015) 678-688.

[21] J. Bai, G. Adriani, T.M. Dang, T.Y. Tu, H.X. Penny, S.C. Wong, R.D. Kamm, J.P. Thiery, Contact-dependent carcinoma aggregate dispersion by M2a macrophages via ICAM-1 and beta2 integrin interactions, Oncotarget, 6 (2015) 25295-25307.

[22] J. Zhao, Y. Cheng, C. Yang, S. Lau, L. Lao, B. Shuai, J. Cai, J. Rong, Botanical Drug Puerarin Attenuates 6-Hydroxydopamine (6-OHDA)-Induced Neurotoxicity via Upregulating Mitochondrial Enzyme Arginase-2, Mol Neurobiol, 53 (2016) 2200-2211.

[23] R. Lucas, I. Czikora, S. Sridhar, E.A. Zemskov, A. Oseghale, S. Circo, S.D. Cederbaum, T. Chakraborty, D.J. Fulton, R.W. Caldwell, M.J. Romero, Arginase 1: an unexpected mediator of pulmonary capillary barrier dysfunction in models of acute lung injury, Front Immunol, 4 (2013) 228.

[24] L.B. Moore, A.J. Sawyer, A. Charokopos, E.A. Skokos, T.R. Kyriakides, Loss of monocyte chemoattractant protein-1 alters macrophage polarization and reduces NFkappaB activation in the foreign body response, Acta Biomater, 11 (2015) 37-47.

[25] L. Yao, S. Chandra, H.A. Toque, A. Bhatta, M. Rojas, R.B. Caldwell, R.W. Caldwell, Prevention of diabetes-induced arginase activation and vascular dysfunction by Rho kinase (ROCK) knockout, Cardiovasc Res, 97 (2013) 509-519.

[26] P.N. Hoffman, A conditioning lesion induces changes in gene expression and axonal transport that enhance regeneration by increasing the intrinsic growth state of axons, Exp Neurol, 223 (2010) 11-18.

[27] J. Chedly, S. Soares, A. Montembault, Y. von Boxberg, M. Veron-Ravaille, C. Mouffle, M.N. Benassy, J. Taxi, L. David, F. Nothias, Physical chitosan microhydrogels as scaffolds for spinal cord injury restoration and axon regeneration, Biomaterials, 138 (2017) 91-107.

[28] C.M. Dumont, M.A. Carlson, M.K. Munsell, A.J. Ciciriello, K. Strnadova, J. Park, B.J. Cummings, A.J. Anderson, L.D. Shea, Aligned hydrogel tubes guide regeneration following spinal cord injury, Acta Biomater, 86 (2019) 312-322.

[29] P. Chen, X. Piao, P. Bonaldo, Role of macrophages in Wallerian degeneration and axonal regeneration after peripheral nerve injury, Acta Neuropathol, 130 (2015) 605-618.

[30] V.E. Neubrand, M. Pedreno, M. Caro, I. Forte-Lago, M. Delgado, E. Gonzalez-Rey, Mesenchymal stem cells induce the ramification of microglia via the small RhGTPases Cdc42 and Rac1, Glia, 62 (2014) 1932-1942.

[31] D.W. Leung, C. Otomo, J. Chory, M.K. Rosen, Genetically encoded photoswitching of actin assembly through the Cdc42-WASP-Arp2/3 complex pathway, Proc Natl Acad Sci U S A, 105 (2008) 12797-12802.
[32] C. Bacon, V. Lakics, L. Machesky, M. Rumsby, N-WASP regulates extension of filopodia and processes by oligodendrocyte progenitors, oligodendrocytes, and Schwann cells-implications for axon ensheathment at myelination, Glia, 55 (2007) 844-858.

[33] M.A. Shakir, K. Jiang, E.C. Struckhoff, R.S. Demarco, F.B. Patel, M.C. Soto, E.A. Lundquist, The Arp2/3 activators WAVE and WASP have distinct genetic interactions with Rac GTPases in Caenorhabditis elegans axon guidance, Genetics, 179 (2008) 1957-1971.

[34] J.P. Myers, E. Robles, A. Ducharme-Smith, T.M. Gomez, Focal adhesion kinase modulates Cdc42 activity downstream of positive and negative axon guidance cues, J Cell Sci, 125 (2012) 2918-2929.

Figure legends

Figure 1.
RAW264.7 macrophages were polarized to M2a macrophages and characterized. (A) IL-4 induces morphological changes in M2a cells. (B) Western blot showing the Arg1 protein expression level. GAPDH was used as the loading control and for band density normalization. (C) Immunofluorescence showing the expression of the M1-type specific protein iNOS. (D) Flow cytometry analysis of the expression of the M2-specific protein CD206.

Figure 2.
Verification experiments of M2 macrophages were performed by knocking down Arg1 on day 28 after spinal cord injury. (A) The expression of the Arg1 protein in cells was evaluated by western blotting. GAPDH was used as the loading control and for band density normalization. (B) The relative mRNA expression of Arg1 was examined by qPCR. The data are presented as the mean ± SD, n = 3; ***P < 0.001 vs. the shSc (shscramble) group; two-tailed Student’s t-test.

Figure 3.
Analysis of locomotor function after SCI. Locomotor function was evaluated by the BBB locomotor rating scale in the model, shSc and shArg1 groups. The data represent the mean ± SD of three independent experiments (n = 5). **P < 0.01 vs. the model group.

Figure 4.
The effect of Arg1 knockdown in M2a macrophages on histological changes in the spinal cord (200×; 400×). (A, C, E) Twenty-eight days after the injection of the cells, spinal cord tissue sections were
subjected to hematoxylin and eosin, toluidine blue, and Nissl staining. (B, D, F) Treated spinal cord tissues from each experimental group were used for histological evaluation. The data are presented as the mean ± SD, n = 3; **P < 0.01 vs. the model group; #p < 0.01 vs. the model group; two-tailed Student’s t-test.

Figure 5.
Immunohistochemical staining for NeuN and Tau. (A) The expression of NeuN, as determined by immunohistochemical staining, following shArg1 infection on day 28 after spinal cord injury (200×, 400×). (B) Quantitative analysis of NeuN-positive cells by immunohistochemistry. (C) Cross-section results (200×, 400×) Tau expression, as determined by immunohistochemical staining. (D) Quantitative analysis of tau-positive cells by immunohistochemistry. The data are presented as the mean ± SD, n = 5; *P < 0.05, **P < 0.01 vs. the model group; two-tailed Student’s t-test.

Figure 6.
Arg1 regulates axonal formation after SCI via the Cdc42/N-WASP pathway. (A) The expression of the Arg1, Cdc42, N-WASP, Arp2 and tau proteins after infection with shArg1 lentivirus in vivo. GAPDH was used as the loading control and for band density normalization. (B) The optical density analysis of the Cdc42 protein. (C) The optical density analysis of the N-WASP protein. (D) The optical density of the Arp2 protein. (E) The optical density analysis of the tau protein. The data are presented as the mean ± SD, n = 5. *P < 0.05, **P < 0.01, ***P<0.0001 vs. the model group; #p < 0.01 vs. the model group; two-tailed Student’s t-test.

Figure 7.
Schematic illustration of treatment of SCI by Arg1 regulation of Cdc42/N-WASP pathway.
A rat model of SCI was constructed mechanically, and M2a macrophages were injected to activate the Cdc42/N-WASP pathway to promote Arg1-mediated axonal regeneration.
A

M2a

|                | shSc | shArg1<sup>1</sup> | shArg1<sup>2</sup> |
|----------------|------|-------------------|-------------------|
| Arg1           |      |                   |                   |
| β-actin        |      |                   |                   |

B

Relative Arg-1 mRNA

|                | shSc | shArg1<sup>1</sup> | shArg1<sup>2</sup> |
|----------------|------|-------------------|-------------------|
| shSc           | 1.0  | 0.2               | 0.1               |
| shArg1<sup>1</sup> | 0.2  |                   |                   |
| shArg1<sup>2</sup> | 0.1  |                   |                   |

*** P < 0.001
