Aldose reductase regulates microglia/macrophages polarization through cAMP-responsive binding element after spinal cord injury in mouse

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Abstract

Inflammatory reactions are the most critical pathological processes occurring after spinal cord injury (SCI). Activated microglia/macrophages have either detrimental or beneficial effects on neural regeneration based on their functional polarized M1/M2 subsets. However, the mechanism of microglia/macrophages polarization to M1/M2 at the injured spinal cord environment remains unknown. In this study, wild type (WT) or aldose reductase (AR) knockout (KO) mice were subjected to SCI by spinal crush injury model. The expression pattern of AR, behavior tests for locomotor activity, and lesion size were assessed at between 4 hours and 28 days after SCI. we found that the expression of AR is upregulated in microglia/macrophages after SCI in WT mice. In AR KO mice, SCI led to smaller injury lesion areas compared to WT. AR deficiency-induced microglia/macrophages induce the M2 rather than the M1 response and promote locomotion recovery after SCI in mice. *In vitro* experiments, microglia cell lines (N9 or BV_2) were treated with AR inhibitor (ARI), Fidarestat. AR inhibition caused 4-Hydroxynonenal (HNE) accumulation, which induced the phosphorylation of cAMP-responsive binding element (CREB) to promote Arg1 expression. KG501, the specific inhibitor of phosphorylated CREB could cancel the upregualtion of Arg 1 by ARI or HNE stimulation. Our results suggest that AR works as a switch which can regulate microglia by polarizing cells to either the M1 or the M2 phenotype under M1 stimulation based on its states of activity. We suggest that inhibiting AR may be a promising therapeutic method for SCI in the future.

41 Keyword: Aldose reductase — spinal cord injury — locomotor recovery —
42 microglia/macrophages—M1/M2 polarization—cAMP-responsive binding element

Introduction

Spinal cord injury (SCI) is one of the most debilitating diseases with poor prognosis worldwide. Traumatic damage triggers a complex local inflammatory response, a critical pathophysiological process following a secondary injury after SCI. At the spinal cord lesion site, microglia/macrophages derived from both residential microglia and hematogenous macrophages or from activated microglia and infiltrated macrophages, respectively, cannot be distinguished based on their morphology or specific molecular markers [13]. Microglia/macrophages have either detrimental or beneficial effects on neural regeneration based on their two extreme functional polarized subsets, “classically activated” pro-inflammatory (M1) or “alternatively activated” anti-inflammatory (M2) cells [6-8, 18, 23]. In response to lipopolysaccharide (LPS) and the pro-inflammatory cytokine interferon-γ (IFN-γ), macrophages undergo M1 polarization characterized by the expression of pro-inflammatory cytokines [interleukin (IL)-12, IL-1β and tumor necrosis factor (TNF)-α] and cytotoxic mediators (reactive oxygen and nitrogen species), as well as increase their phagocytic and antigen-presenting capacity. In contrast, activating macrophages in the presence of IL-4 or IL-13 undergo M2 polarization characterized by the expression of anti-inflammatory cytokines such as transforming growth factor (TGF)-β and IL-10, which contribute to the termination of inflammation [13, 28, 45]. Kigerl et al. reported that most microglia/macrophages are polarized to M1 cells, with only a transient and small number in M2 polarization [23]. *In vitro*, they showed that M1 macrophages are neurotoxic and possess only moderate axon growth-promoting

effects. In contrast, M2 macrophages can promote long-distance axon growth, even in the present of potent growth inhibitory substrates [23].

One of the well-characterized pathological processes occurring early after SCI is the formation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) [9, 30]. RNS and ROS cause lipid oxidation as well as nitrative and oxidative damage to proteins and nucleic acids, leading to neuronal loss by necrosis or apoptosis [30, 54]. Microglia/macrophages are the major cells that produce neurotoxic RNS and ROS after SCI [14]. Oxidative stress-induced inflammation is a major contributor to secondary injury after SCI [9]. ROS-mediated activation of redox-sensitive transcription factors, such as nuclear factor-kappa B (NF-κB) and activator protein (AP)-1, as well as subsequent expression of pro-inflammatory cytokines, chemokines, and growth factors, are characteristics of inflammatory diseases [5, 48]. The pro-inflammatory cytokines TNF-α and IL-1β, which are up-regulated immediately after SCI combining with the ligands of pattern recognition receptors (PRRs), such as high mobility group box protein (HMBG1) and LPS, can cause microglia/macrophages to enter an overactive state and begin releasing ROS [13, 14, 23]. Cytokines and LPS cause oxidative stress to form toxic lipid aldehydes such as 4-hydroxynonenal (HNE), which triggers multiple signaling cascades that variably affect cell growth, differentiation, and apoptosis through lipid peroxidation [36].

Aldose reductase (AKR1B1, AR) belongs to the aldo-keto reductase (AKR) superfamily, which includes several enzymes that catalyze oxidation and reduction reactions involved in various cellular processes [48]. AR is the only enzyme

responsible for reducing glucose to sorbitol in the polyol pathway of glucose metabolism and identified as the chief facilitator of hyperglycemic injury in secondary diabetic complications such as cataractogenesis, retinopathy, neuropathy, nephropathy, and cardiovascular diseases [39]. Recent studies have suggested that glucose may be an incidental substrate of AR, which appears to be good at catalyzing the reduction of a wide range of aldehydes generated from lipid peroxidation. Several reports showed that AR, in addition to reducing glucose, efficiently reduces the lipid peroxidation-derived aldehydes such as HNE, were thought to be the preferred physiological substrates with higher affinity than glucose [48]. AR catalyzes the reduction of lipid aldehydes and their reduced glutathione (GSH) metabolites both *in vitro* and *in vivo* [47]. Pharmacological inhibition or small interfering RNA (siRNA) ablation of AR prevented the biosynthesis of TNF-α, IL-1β, IL-6, macrophage-chemoattractant protein (MCP)-1, cyclooxygenase-2 (Cox-2), and prostaglandin E2 (PGE2) in LPS-activated RAW264.7 murine macrophages. AR inhibition or ablation significantly attenuated LPS-induced activation of protein kinase C (PKC) and phospholipase C (PLC), nuclear translocation and phosphorylation of NF-κB, and proteolytic degeneration of IκBα in macrophages [38]. These results indicate that AR can promote LPS-induced inflammatory signals in macrophages by promoting NF-κB-dependent expression of inflammatory cytokines and chemokines. Whether AR plays a role in M1 or M2 polarization of microglia/macrophages and in neuroinflammation after SCI remains unclear. In this study, we found that mRNA and protein levels of AR increased in a time-dependent

manner after SCI. In $AR^{-/-}$ mice, activated microglia/macrophages at the injured site were prone to M2 rather than M1 polarization during early stages of SCI. HNE, the substrate of AR, accumulated in microglia treated with AR inhibitor inducing transcript factor cAMP-responsive element-binding protein (CREB) upregulation and phosphorylation, which promoted microglia polarization to M2.

Materials and Methods

Animals and SCI model

$AR^{-/-}$ mice generated previously and backcrossed to C57BL/6 mice for more than 10 generations, which are considered to be congenic with C57BL/6 mice, were used (n=53) [21]. Age-matched normal C57BL/6 (n=55) (SLAC, Shanghai, China) were used as control wild-type mice ($AR^{+/+}$). Mice were housed in a specific pathogen-free (SPF) environment at the animal facility of Department of Neurobiology, the Fourth Military Medical University. All procedures were conducted under guidelines approved by the Fourth Military Medical University Animal Care and Use Committee. Anesthetized mice with 1% sodium pentobarbital (Sigma-Aldrich, 40 mg/kg) received a severe spinal crush injury as described previously with certain modifications [33]. Briefly, a laminectomy was performed at vertebral midthoracic level T8-T10 leaving dura intact. SCI was made at the level of T8 by compressing the cord laterally from both sides for 20 s with Number 5 Dumont forceps (Fine Science Tools) modified with a spacer of 0.25 mm to produce severe injury. Sham group was generated by only performing laminetomy but no compressing the cord.

Postoperatively, mice received manual bladder evacuation twice a day to prevent urinary tract infections until normal voiding reflexes returned.

Behavioural assessment

Gross locomotor recovery after SCI was assessed using the Basso Mouse Scale (BMS) hindlimb locomotor test for mice [2]. For 1 week before surgery, mice were acclimated to the testing field. At 0, 3, 5, 7, 14 and 28 day post injury (dpi), mice were placed in this field and observed with video record for 4 min. Each hind limb was scored by two investigators blinded to the treatment protocol.

Estimation of lesion area

Spaced serial 10 μm thick sections 100 μm apart were stained with Luxol Fast Blue (LFB, Sigma-Aldrich) [4]. Briefly, the sections were dehydrated and incubated in 0.1% LFB dissolved in 95% ethanol at 60°C overnight. Stained sections were cooled at RT and differentiated in 0.05% lithium carbonate and 70% ethanol. Sections were then counterstained with 0.1% Cresyl Violet (Sigma-Aldrich) at 60°C for 10 min and mounted in neural resin . Digital images were acquired using a DP70 digital camera (Olympus) mounted on a BX51 Olympus microscope equipped with a 10× objective. Five random sections were quantified per mouse and three mice of each kind were measured per group. Quantification of LFB stained areas was performed using Photoshop CS3 software.

Cell cultures

Microglial cell lines culture

The murine microglial cell line N9 (keeping in our lab) or BV2 was cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) supplemented with 5% Fetal Bovine Serum (FBS; Gibco), 2 mM L-Glutamine 100 U/ml penicillin and 100 μg/ml streptomycin. 5×10^5 cells were seeded onto 12-well plates. To detect the polarization of N9 microglia, cells were treated with LPS (100 ng/ml; Sigma-Aldrich) or IL-4 (20 ng/ml; R&D Systems), respectively, combined with Fidarestat 20 μM (Lot. 308002, Sanwa Kagaku Kenkyusho, Nagoya, Japan) following two strategies. One was first to stimulate N9 cells with the above cytokines for 24 h, respectively, and then add ARI for another 24 h. The other was first to treat N9 cells with ARI for 24 h and then add the cite cytokines, respectively, for another 24 h. After those treatments, N9 cells was washed with D-Hank's solution for 3 times and harvested for further analysis with qPCR or Western blot.

Primary cortical neurons cultures

Primary cortical neurons were prepared from wild type (C57BL/6) and AR deficient mouse at E15 [15]. Using aseptic technique, the fetal brains were removed and transferred into ice-cold D-Hank's medium without Ca^{2+} and Mg^{2+}. After, bilateral cortices were mechanically separated and the meninges were carefully removed using fine forceps (Sigma-Aldrich, style #7). Then the cerebral cortex was minced using sterile iridectomy scissors. These pieces were incubated in Ca^{2+} and Mg^{2+} free D-Hank's balanced salt solution with 0.125 % trypsin and 0.1% DNase at 37 °C for

15 min. The enzymatic digestion was followed by a brief mechanical trituration using polished Pasteur pipettes and centrifuged at 800 rpm for 5 min. Cells were suspended in Neurobasal A (Gibco) supplemented with B27 (Gibco) and 2 mM L-glutamine. Cell suspension were seeded in poly-L-lysine (25 μg/ml, Sigma) coated 6-well culture plates at a density of 4×10^4 cells/cm^2 at 37 °C/5% CO_2 for 7-14 d culture. The cell populations consisted of >95% neurons before used, which was determined by immunocytochemical stain with anti Neuron- specific β-tubulin III (data not shown) [46].

Primary astrocytes cultures

Primary astrocytes were cultured from the cerebral cortex of 1-2 d postnatal mouse pups [17]. The cerebral cortices were dissected and trypsinized as indicated above for the primary neurons cultures. Cells were suspended in DMEM medium supplemented with 10% FBS (Gibco), 2 mM glutamine, 50 U/ml Penicillin and 50 μg/ml Streptomycin (Glibco). Cells were plated on PLL coated 75 cm^2 culture flasks at a density of 1×10^5 cells/cm^2 and cultured in 5% CO_2 at 37 °C. After 7-10 d incubation, cultures were purified by shaking for 20 h on an orbital shaker (180 rpm) at 37°C, resulting in cultures of 95% GFAP-positive cells. Purified astrocytes were digested by trypsinization, and cells were again plated at the density of 5×10^5/cm^2 onto PLL coated 6-well plates and incubated in 5% CO_2 at 37 °C for two days before used.

Oligodendrocyte cultures

Oligodendrocyte precursor cells (OPC) were generated from primary mixed glial cultures above mentioned as described previously [49, 55]. Briefly, after ten days culture in DMEM medium with 10% FBS (Gibco) and supplemented with 2 mM glutamine, 50 μg/ml Streptomycin and 50 U/ml Penicillin. Oligodendrocyte precursor cells were shaking off based on the different adhesion properties of different glial cell types. Cell cultures were shaken first at 200rpm, 37°C for 30min and rinced with fresh DMEM medium with 10% FBS (Gibco) and supplemented with 2 mM glutamine, 50 μg/ml Streptomycin and 50 U/ml Penicillin. After cells were cultured for another 14-16 h, cell cultures were shaken at 280 rpm, 37°C for 18-20 h to collect the OPC. Precursor cells were obtained by sequential dislodging procedure. Purified OPC were plated onto PLL coated 6-well plates at the density of 4×10^4 cells/cm^2 and cultured in Satomedium (DMEM/F12 supplemented with 4 mM Glu, 0.1 mM sodium pyruvate, 0.1% BSA, 50μg/ml transferrin, 5μg/ml Insulin, 30 nM sodium selenate, 10 nM Vitamin H and 10 nM hydrocortisone) supplemented with 10 ng/ml PDGF-AA and 10 ng/ml bFGF to promote cell growth. OPC were cultured in above expansion medium for 2-3 d and passaged with DMEM/F12 supplemented with 0.01% EDTA , 0.2 mg/ml DNase and 5 μg/ml insulin. The supernatant was collected and $NG2^+$ cells were plated onto PLL coated 6-well plates at the density of 8×10^4 cells/cm^2 in differentiation medium which contains Satomedium supplemented with 15 nM triiodothyronine (T3), 10 ng/ml ciliary neurotrophin factor (CNTF), and 5 μg/ml N-acetyl-l-cysteine (NAC) for 6 days before used.

Primary microglia cultures

Primary microglia cultures were also obtained from the cerebral hemispheres of newborn mouse brains (P1-2) as previously described [3, 24]. In brief, mixed glial cells were seeded onto PLL coated culture flasks at a density of 1×10^5 cells/cm^2. Two weeks later, microglia were shaken off at 200 rpm for 30min. Highly enriched microglial suspension were collected and filtered through 41 μm cell strainer. After centrifugation at 800 rpm for 5 min, cells were resuspended in DMEM medium with 10% FBS, and plated onto 6-well plates at a density of 2×10^5 cells/cm^2. The purity of cells obtained was harvested > 95% based on staining with Iba-1 antibody before used.

When the purified neural cells reached 80% confluence, TNF-α+IFN-γ or LPS were pulsed into culture medium for another 24 h incubation. Purified neural were harvested for AR level detection with qPCR.

Immunohistochemistry

Mice were killed by injection of an overdose of 2% sodium pentobarbital. The animals were transcardially perfused with ice-cold saline followed by 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for at least 15 min at room temperature (RT). After perfusion, the spinal cords were dissected out and postfixed 4-6 h at 4°C in the same solution used for perfusion. Spinal cord tissue was then immersed into 20% sucrose solution in 0.1 M phosphate buffer, pH 7.3, at 4°C for cryoprotection. Tissues were embedded in Tissue Tek (Sakura Finetek, Zoeterwoude, The Netherlands), cut transversely or longitudinally into 10-μm slices in a cryostat,

mounted onto glass slides, and store at -20 °C for further processing.

The sections were blocked with 1% bovine serum albumin containing 0.3% triton X-100 for 1 h. Sections were then incubated with primary antibodies (see Table 1) overnight at 4 °C. Then sections were incubated with appropriate secondary antibody for 2 h at room temperature. Images were acquired by using FV 1000 confocal system (Olympus, Japan).

To detect the accumulation of HNE after given ARI, we treated cultured BV2 and N9 microglia with Fidarestat at 0, 20, 30, and 40 μM for 24 h. After washing 3 times with PBS, the cells were fixed with 4% formaldehyde for 10 min at RT. Cells were incubated with rabbit anti-HNE pAb (abcam) overnight at 4 ºC. After washing with PBS 3 times, the FITC labeled anti-rabbit IgG were incubated for 2 h. The density of fluorescence were judged under BX53 microscopy (Olympus, Japan) with same expose time or with fluorescent density scan（excitation wavelength 493 nm and emission wavelength 518 nm） by Infinite M200 PRO (TECAN, Swiss). All antibodies and their dilutions used in IHC in this study were listed in Table 1.

Table 1. Antibodies for IHC and WB

			Dilution	
Antibodies	Host	Vendor	IHC	WB
AR	Mouse	Santa	1:200	1:1000
β-actin	Mouse	Sigma	N/A	1:8000
iNOS	Rabbit	abcam	1:500	1:500
Arginase 1	Goat	Santa	1:100	1:1000

NF-κB	Rabbit	Anbo	N/A	1:1000
pNF-κB	Rabbit	Cell Signaling	N/A	1:1000
CREB	Rabbit	Epitomics	N/A	1:1000
pCREB	Rabbit	Epitomics	N/A	1:500
F4/80	Rabbit	AbD	1:100	N/A
Iba 1	Rabbit	Wako	1:1000	N/A
GFAP	Rabbit	Dako	1:1000	N/A
NeuN	Mouse	Chemicon	1:200	N/A
NG2	Mouse	Millipore	1:400	N/A
MBP	Rat	Millipore	1:400	N/A
HNE	Rabbit	abcam	1:300	N/A

Quantitative Real-time PCR

The method of QRT-PCR was carried out as our previously description with slight modifications [56]. Brifely, mice were sacrificed 1, 3, 7, 14 and 21 days after SCI with an overdose of 2% pentobarbital sodium intraperitoneal injection. An 1cm spinal cord segment containing the injured site in the middle was removed quickly for RNA isolation. Controled mice were sacrificed after the operation as well.

Total RNA was extracted using TRizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration of RNA was measured by UV absorbance at 260 and 280 nm (260/280) while the quality was checked by gel

electrophoresis. Equal amount of RNA from each group was used for Poly(A) tailing and quantitative reverse transcription consisted of 4 µl 5 × reaction mix, 2 µl 10 × SuperScript enzyme mix, and 200 ng total RNA in a final volume of 20 µl. Then real time PCR was performed using the NCode™ EXPRESS SYBR GreenER™ microRNA qRT-PCR Kit (Invitrogen) containing 1 µl of the RT product, 10 µl EXPRESS SYBR green qRT-PCR SuperMix, 0.4 µl microRNA-specific forward primer (10 µM), and 0.4 µl universal qPCR primer (10 µM) in a final volume of 20 µl. PCR cycling began with template denaturation and hot start Taq activation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min performed in a CFX96 Real-Time PCR System (BioRad). Beta-actin was used as the internal standard reference and normalized expressions of targeted genes were calculated using the comparative CT method and fold changes were derived from the equation $2^{-\Delta\Delta Ct}$ for each gene. All primers were used in this study are list in Table 2.

Table 2. Primer sequences for QRT-PCR analysis

Gene	Accession number	Forward primer (5'-3')	Reverse Primer (5'-3')
AR	NM_009658.3	ACGGCTATGGAACAACTA	TGTGGCAGTATTCAATCAG
Arg 1	NM_007482.2	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTG
iNOS	NM_010927.2	CCCTTCAATGGTTGGTACATGG	ACATTGATCTCCGTGACAGCC
NF-κB	NM_008689.2	CCTACGGTGGGATTACATTC	CTCCTCGTCATCACTCTTGG
CREB	NM_001037726.1	AGAAGCGGAGTGTTGGTGAGT	GGTTACAGTGGGAGCAGATG
CD86	NM_019388	TTGTGTGTGTTCTGGAAACGGAG	AACTTAGAGGCTGTGTTGCTC

CD206	NM_008625.1	TCTTTGCCTTTCCCAGTCTCC	TGACACCCAGCGGAATTTC
β-actin	NM_007393.3	AGAAGGACTCCTATGTGGGTGA	CATGAGCTGGGTCATCTTTTCA

Western blotting

Tissue samples about 1 cm of spinal cord with the injured site in middle were lysed by homogenizing per 100 mg tissues in 1ml RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) buffer or the cells in 6-well plates were lysed in 300 μl RIPA buffer containing 1 mM PMSF. Lysates were centrifuged at 12,000 rpm for 10 min, and the supernatant was collected and added same volume sample buffer. The protein amount was determined using BSA Protein Assay Kit (Pierce). Samples with equal amounts of protein were then separated by 10% SDS-PAGE, and electrotransferred onto (300mA for 60min) Polyvinylidene Fluoride (PVDF) Membranes (Millipore, 0.45 μm) with Transblot Turbo (Bio-Rad, USA). Membranes were incubated with 5% nonfat milk in Tris buffer containing Tween 20 (TBST; 10 m M Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 2h at RT and incubated with the primary antibodies which listed in Table 1 at 4°C overnight. Membranes were then washed three times for 10 min with TBST and probed with relative 2nd antibodies conjugated with horseradish peroxidase (abcam) at RT for 2 h. Membranes were finally washed three times for 10 min with TBST to remove unbound 2nd antibodies and visualized using enhanced chemiluminescence (Thermo Scientific). The density of specific bands was measured using ImageJ densitometry software and normalized against a loading control (β-actin).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software). All data are expressed as group mean ± SEM unless otherwise noted. Comparison of the two groups were performed using independent t tests. Results were considered statistically significant at $p < 0.05$.

Results

Time-course changes in mRNA and protein levels of AR after SCI

We analyzed time-dependent changes in the expression profiles of AR transcript and protein between 4 hours and 14 days postinjury (hpi or dpi) using qPCR and WB in C57BL/6 mice. Starting at 4 hpi, the AR mRNA level began to increase, with a significant increase 1 dpi and was maintained until 14 dpi according to qPCR results (Fig. 1a). This result was subsequently confirmed by WB (Fig. 1b, c). The AR protein was also upregulated in a time-dependent manner after injury (Fig. 1b). The densitometric ratio of AR/β-actin showed that AR protein clearly increased from 1 dpi to 14 dpi (Fig. 1c). These results suggest that AR expression was temporally upregulated after SCI in normal mice.

We next investigated the types of neural cells showing upregulated expression of

313 AR following SCI in mice. Based on the above results showing that both mRNA and
314 protein levels of AR reached a peak 3 dpi in the injured spinal cord, we used the
315 immunoflorescence double-labeling method to immunostain AR bound to other neural
316 cell markers, such as NeuN (for neuron), GFAP (for astrocyte), Iba-1 (for
317 microglia/macrophages), and MBP (for oligodendrocyte), respectively. Neurons
318 adjacent to the lesion site presented both AR- and NeuN-positive signals (Fig. 1d).
319 Microglia/macrophages that were Iba-1 positive were also highly expressed AR
320 adjacent to the lesion site [10, 23] (Fig. 1e). The distal area from the lesion site of the
321 spinal cord, however, expressed a low level of AR and Iba-1 (Fig. 1e). We also
322 evaluated AR expression in astrocytes and oligodendrocytes, but no double-labeled
323 astocytes or oligodendrocytes were observed in injured spinal cord (data not shown).
324 These results suggest that an injured, and not a healthy spinal cord, induces AR
325 expression in microglia, macrophages, and neurons, but not in astrocytes or
326 oligodendrocytes.

327 M1 polarized microglia/macrophages, which mainly secret pro-inflammatory
328 cytokines, such as INF-γ and TNF-α, are dominant at early stages in SCI [10, 23]. We
329 suspected that the pro-inflammatory cytokine environment was likely to cause neural
330 cells to upregulate AR. We isolated primary neural cells, including astrocytes,
331 oligodendrocytes, neurons, and microglia, and cultured these cells with cytokines
332 IFN-γ+ TNF-α stimulation for 24 h *in vitro*, respectively. Next, we extracted RNA
333 from the cells and measured AR expression using qPCR. Primary neurons and
334 microglia (Fig. 1h and i), but not astrocytes or oligodendrocytes (Fig. 1f and g),

expressed high levels of AR transcripts under proinflammatory cytokine stimulation (Fig. 1f). In addition, under LPS stimulation, microglia expressed much higher levels of AR mRNA compared to that under proinflammatory cytokine stimulation (Fig. 1i). A previous study showed that both IFN-γ and LPS can induce macrophage polarization to the M1 phenotype via IFN-γR and TLR4, respectively [13, 25, 45]. Thus, our results suggest that AR may play a role in polarization of microglia/macrophages after SCI.

AR deficiency promoted locomotor recovery after SCI

AR-deficient mice showed no apparent development, reproductive, or nervous system abnormalities except for a partially defective urine-concentrating ability [21]. However, whether AR plays a role in recovery after SCI remained unclear. We evaluated locomotor recovery based on the BMS locomotor rating scale after SCI with a severe crush model in $AR^{-/-}$ mice [22, 33]. AR-deficient mice exhibited significant higher BMS scores beginning at 3 dpi (Fig. 2a). After 21 dpi, hind limb locomotion appeared to reach a plateau in both $AR^{+/+}$ and $AR^{-/-}$ mice. At the end of the 28 dpi assessment period, $AR^{+/+}$ mice showed a mean score of approximately 4 (n = 10), while the $AR^{-/-}$ mice had a score of 6.5 (n =8) on the BMS scale (Fig. 2a). To evaluate the lesion sizes after SCI, we performed LFB staining in spinal cord sections of $AR^{+/+}$ and $AR^{-/-}$ mice, respectively. First we compared the lesion areas, which was evidenced by reduced LFB staining, between $AR^{+/+}$ and $AR^{-/-}$ mice at 3, 7, and 14 dpi

(Fig. 2b). At all three time-points, $AR^{-/-}$ mice showed much smaller lesion areas compared to $AR^{+/+}$ mice after SCI (Fig. 2c). Thus, these results suggest that AR deficiency can promote functional recovery after SCI in mice.

M1/M2 ratio decreased significantly during early stages of SCI

Microglia/macrophages are critical inflammatory cells which significantly contribute to the pathological environment in SCI. They can be detrimental or beneficial to functional recovery after SCI based on their polarized subsets, M1 or M2 [10, 13, 44]. In fact, there are both M1 and M2 microglia/macrophages present at the injured spinal cord despite whether it is an early stage or late stage in SCI. The M1/M2 ratio determines the outcome of microglia/macrophages, which is detrimental or beneficial for recovery after SCI [10, 23]. We stained injured spinal cord sections with antibodies of the M1-specific marker iNOS and M2-specific marker Arg1 combined with microglia and macrophage-specific marker F4/80 or Iba1 in $AR^{+/+}$ and $AR^{-/-}$ mice, respectively. In $AR^{-/-}$ mice, the number of $iNOS^+/F4/80^+$ cells were less than those in $AR^{+/+}$ mice after SCI (Fig. 2d). In contrast, there was a larger number of $Arg1^+/Iba1^+$ cells in $AR^{-/-}$ mice compared with $AR^{+/+}$ mice after SCI (Fig. 2e). These results show that more $Arg1^+$, but not $iNOS^+$, microglia/macrophages were present at the injured site in $AR^{-/-}$ mice after SCI.

In $AR^{+/+}$ mice, the iNOS mRNA increased rapidly and reached its highest level

377 at 1 and 3 dpi. At 14 dpi, iNOS mRNA had nearly returned to normal levels in $AR^{+/+}$
378 mice (Fig. 3a). In $AR^{-/-}$ mice, however, iNOS mRNA showed no significant change
379 at all time-points compared to the sham group after SCI (Fig. 3a). Comparison of
380 iNOS mRNA level at 4 hpi and 1, 3, and 7 dpi in $AR^{+/+}$ with $AR^{-/-}$ mice showed that
381 iNOS mRNA was clearly higher in $AR^{+/+}$ mice than in $AR^{-/-}$ mice (Fig. 3a). Another
382 M1 cell marker, CD86, was also detected after SCI by qPCR. The CD86 mRNA level
383 differed from the iNOS transcription profile at 14 dpi in $AR^{+/+}$ mice, which displayed
384 clear upregulation compared to $AR^{-/-}$ mice (Fig. 3b). In $AR^{+/+}$ mice, Arg1 mRNA
385 level increased rapidly over a short period of time post-injury and reached its highest
386 expression at 3 dpi (Fig. 3c). Arg1 mRNA level decreased suddenly at 7 dpi and
387 recovered to its normal expression level at 14 dpi in $AR^{+/+}$ mice Fig. 3c). The Arg1
388 expression pattern in $AR^{-/-}$ mice was similar to that in $AR^{+/+}$ after SCI, in which Arg1
389 mRNA was upregulated rapidly after SCI, reaching its highest expression level at 1
390 dpi and followed by a decrease in expression; however, the pattern was quite different
391 for Arg1 mRNA expression between the two types of mice (Fig. 3c). In $AR^{-/-}$ mice,
392 the Arg1 mRNA level was also decreasing, but it decreased slowly comparing to in
393 $AR^{+/+}$ mice at same time-point after SCI (Fig. 3c). The mRNA of CD206, another M2
394 cell marker, also increased significantly at 1, 3, 7, and 14 dpi in $AR^{-/-}$ mice compared
395 to in $AR^{+/+}$ mice after SCI (Fig. 3d).

396 iNOS protein level was downregulated rapidly after SCI in $AR^{-/-}$ mice, while it
397 was upregulated post-injury in $AR^{+/+}$ mice (Fig. 3e and f). In contrast, Arg1 was
398 rapidly upregulated and then decreased at 14 dpi both in $AR^{+/+}$ and $AR^{-/-}$ mice (Fig.

399 3e and g). However, $AR^{-/-}$ mice expressed more Arg 1 than $AR^{+/+}$ mice at same
400 checkpoint post-injury (Fig. 3g). Interestingly, we found that both iNOS and Arg1
401 levels were higher in $AR^{-/-}$ mice than in $AR^{+/+}$ mice in the sham groups. It is unclear
402 whether these results suggest that AR-deficient microglia indicate higher basic
403 activity. Densitometric analysis showed that iNOS/β-actin was much lower in $AR^{-/-}$
404 mice than in $AR^{+/+}$ mice at same checkpoint post-injury (3f). While densitometic
405 analysis Arg1/β-actin and the ratio of Arg1/iNOS protein expression was much
406 higher in $AR^{-/-}$ mice than in $AR^{+/+}$ mice at same checkpoint post-injury (Fig. 3g and
407 h). The characteristic markers of M2 macrophages, such as Arg1 and CD206, were
408 upregulated rather than M1 cell markers, such as, iNOS and CD86, after SCI in $AR^{-/-}$
409 mice. This suggests that macrophages and microglia may be polarized to M2 cells at
410 the lesion site after SCI in $AR^{-/-}$ mice.
411

412 ## AR deficiency caused NF-κB downregulation and CREB
413 ## upregulation after SCI in mice

414 Several key transcription factors that translate signals in the microenvironment into a
415 polarized macrophage phenotype are produced according to several reports [25, 45].
416 TLR engagement can activate NF-κB and produce inflammation mediators associated
417 with M1 macrophages [25, 45]. In addition, the CREB-C/enhancer binding protein
418 (EBP) axis plays an important role in macrophage polarization and induces M2
419 macrophage-specific gene expression [43]. AR-mediated LPS-induced inflammatory

signals in macrophages promote NF-κB dependent expression of inflammatory cytokines and chemokines [37-40]. AR also regulates LPS-mediated inflammation through the cAMP/PKA/CREB pathway [42]. Thus, we focused on the expression profiles of NF-κB and CREB after SCI in $AR^{+/+}$ and $AR^{-/-}$ mice. The mRNA of the p65 subunit of NF-κB increased gradually postinjury, with $AR^{+/+}$ mice showing higher mRNA levels of p65 at 1, 3, and 7 dpi compared with $AR^{-/-}$ mice at same time-point after SCI (Fig. 4a). Additionally, mRNA levels of CREB were higher at 4 hpi and 1, 3, and 7 dpi in $AR^{-/-}$ mice (Fig. 4b). At 14 dpi, however, the mRNA level of CREB was lower in $AR^{-/-}$ mice compared to in $AR^{+/+}$ mice (Fig. 4b). In $AR^{+/+}$ mice, the protein level of p65 gradually increased at 1, 3, 7, and 14 dpi (Fig. 4c and e). In $AR^{-/-}$ mice, however, the profile of p65 continued decreasing after SCI (Fig. 4d and e). The other transcription factor studied, CREB, also showed a different expression pattern after SCI in $AR^{+/+}$ mice and $AR^{-/-}$ mice. In $AR^{+/+}$ mice, the peak of CREB expression occurred at 14 dpi, while CREB level was highest at 3 dpi and decreased at 14 dpi in $AR^{-/-}$ mice (Fig. 4d and f). Amounts of pCREB were always higher at same time-point after SCI in $AR^{-/-}$ mice compared with in $AR^{+/+}$ mice (Fig. 4g). Consistent with the higher baseline level of iNOS and Arg 1 (Fig. 3e), the levels of NF-κB, CREB, and pCREB were also higher at baseline in $AR^{-/-}$ mice (Fig. 4c and d). These results showed that there were lower levels of the p65 subunit of NF-κB and higher CREB and pCREB during early stages after SCI in $AR^{-/-}$ mice compared to those in $AR^{+/+}$ mice.

HNE and CREB were upregulated in microglia following AR inhibition.

HNE is one of the products of lipid peroxidation. It undergoes glutathione S-transferase (GST)-catalyzed conjugation to form GS-HNE, which is further reduced to GS-DHN (1, 4-dihydroxynonene) by AR [36]. To confirm whether HNE increases after ARI Fidarestat treatment, we measured the concentration of HNE in the BV2 microglia cell line based on fluorescence using *in vitro* immunohistochemistry. Compared with untreated ARI cells, HNE significantly increased in cells following ARI treatment (Fig. 5A). The concentration of ARI from 20 μM to 40 μM did not obviously increase the HNE level in a dose-dependent manner (Fig. 5a). We next detected changes in HNE levels of under 20 μM Fidarestat over time using fluorescent density scanning with an Infinite M200 PRO (Swiss). HNE level was significantly increased at 12 h after Fidarestat treatment (Fig. 5b). From 12 h to 48 h after Fidarestat stimulation, HNE levels were stable at a high level (Fig. 5b). We also confirmed this phenomena using the microglial cell line N9 (data not shown). These results suggest that AR inhibition can cause HNE accumulation in microglia.

Our results described above also showed that AR plays a role in the polarization process of microglia/macrophages after SCI. To further detect the mechanism of AR in the regulation of microglia polarization, we used the murine microglial cell line N9 *in vitro*. N9 cells were treated with dimethylsulfoxide as controls (Fig. 5c, d). Following LPS stimulation, N9 cells were polarized to the M1 phenotype characterized by high iNOS and low Arg1 levels with p-NF-κB upregulation (Fig. 5c).

In contrast, N9 cells following IL-4 stimulation were polarized to the M2 phenotype characterized by low iNOS and high Arg1 levels similarly to macrophage polarization (Fig. 5d) [25, 45]. N9 cells were treated with HNE, a substrate of AR, resulting in upregulation of Arg1 and p-CREB and downregulation of iNOS (Fig. 5c). The ARI, Fidarestat, which has been examined in phase III clinical trials for diabetic neuropathy, may also effectively upregulate Arg1 and downregulate iNOS in N9 cells, similarly to HNE (Fig. 5c) [26, 39, 41, 50]. When N9 cells were stimulated with LPS+ARI, iNOS and p-NF-κB levels were significantly downregulated, while Arg1 was upregulated (Fig. 5c). N9 cells were pulsed with a combination of LPS+HNE, and Arg1 and p-CREB were clearly upregulated, while iNOS was downregulated (Fig. 5c). Thus, these results suggest that HNE and ARI could induce microglia to upregulate Arg1 and downregulate iNOS, which may occur via the p-CREB pathway.

N9 cells that had been treated with a combination of LPS+ARI, LPS+HNE, and LPS+HNE+ARI all showed an increase in Arg1 expression. iNOS levels slightly increased following stimulation with these compounds compared to HNE or ARI stimulation alone, but decreased significantly compared with LPS stimulation (Fig. 5c). Additionally, the level of p-CREB was clearly increased. However, the level of p-NF-κB was clearly decreased following stimulation with a combination of these compounds (Fig. 5c). These results further indicate that HNE and ARI can induce Arg1 expression through the CREB pathway.

To confirm whether HNE- and ARI-induced Arg1 expression occurred via the p-CREB pathway, we used the p-CREB-specific inhibitor KG501, which can block

phosphorylation of CREB, to treat N9 cells *in vitro*. In N9 cells treated with KG501, the expression of Arg1 and p-CREB were similar to in cells treated with dimethylsulfoxide (Fig. 5d). The upregulation of Arg1 induced by HNE and ARI was stopped in presence of KG501. The p-CREB expression level was also decreased in the presence of KG501 compared with treatment with HNE or ARI alone (Fig. 5d). IL-4, which is the prototypical cytokine that polarizes macrophages into the M2 phenotype, significantly induced Arg1 upregulation and slight iNOS downregulation in N9 cells (Fig. 5d). Combining IL-4 with KG501 to stimulate N9 cells, however, a slight decrease of Arg1 was observed compared with IL-4 stimulation alone (Fig. 5d). These results suggest that the HNE and ARI induced Arg1 expression in microglia occurred through a p-CREB transcriptional factor, but IL-4 did not stimulate expression through this mechanism.

Discussion

Inflammation appears to be the most important pathological process during secondary injury after SCI. Residential microglia and hematogenous macrophages, which are the main inflammatory subpopulations, from surrounding tissues arrive at the injury site within hours after the primary injury [14, 34]. The inflammatory response after SCI is thought as a ‘double-edged sword’, with both neuroprotective and neurotoxic properties based on the polarized states of microglia/macrophages [10, 23, 44]. In the first few days after injury, iNOS and pro-inflammatory cytokines such

as TNFα, IL-1β, and IL-6 are upregulated by microglia/macrophages in SCI-induced aseptic conditions [27, 32]. These proinflammatory cytokines and other mediators including HMBG1, which are released from dead cells, activating microglia/macrophages through Toll-like receptors (TLRs), result in NF-κB-mediated production of proinflammatory cytokines and iNOS activation, the hallmarks of M1 macrophage and microglia activation during early stages of SCI [13, 23, 44]. The reports of M2 polarization in SCI are controversial. In the contused spinal cord of mouse, most microglia/macrophages are M1 cells, with only a transient and small number showing M2 polarization. The expression of one of the classic M2 markers, Arg1, was transient and returned to normal levels by 14 days post-injury [23]. A recent study reported accumulation of two distinct subsets of macrophages with different kinetics. The Ly6c^{hi}*Cx3cr1*^{lo} macrophages, which express characteristic M1 markers (IL-1β and TNF-α), are present at 1 dpi and peaked at 3 dpi at the injury site after SCI. Subsequently, Ly6c^{lo}*Cx3cr1*^{hi} macrophages, which express characteristic M2 surface markers (Dectin 1, IL-4Rα and CD206), infiltrated beginning at 3 dpi and peaked at 7 dpi at the injury site after spinal cord contused injury [44]. Our results also showed that both mRNA and protein levels of Arg1 and iNOS are upregulated in early stages after SCI in AR$^{+/+}$ mice. However, mRNA and protein levels of Arg1 and iNOS were expressed with diverging patterns; Arg1 was upregulated, while iNOS was downregulated during early stages after SCI in AR-deficient mice. The M2-type microglia/macrophages were dominant at the injured spinal cord in AR$^{-/-}$ mice, whereas M1 cells dominated in AR$^{+/+}$ mice at early stages after SCI. This suggests

529 that AR plays a critical role in the polarization of microglia/macrophages after SCI in
530 mice.

531 AR was described in 1956 by Hers to have glucose-reducing activity [19].
532 Recent several reports have shown that AR mediates LPS-induced inflammatory
533 signals in macrophages [36, 38, 40, 42, 48, 50]. Inhibition of AR by several ARIs
534 such as sorbinil, tolrestat, and zopolrestat suppresses LPS-induced production of
535 inflammatory cytokines such as TNF-α, IL-6, IL-1β, IFN-γ, and chemokine MCP-1 in
536 murine peritoneal macrophages. Inhibition of AR also prevents the production of NO,
537 and prostaglandin E2 (PGE2) and expression of iNOS and Cox-2 mediated by
538 inhibiting phosphorylation of IκB-α, IKKα/β, and protein kinase C (PKC) [40]. These
539 studies demonstrate that AR inhibition or ablation can prevent macrophages
540 polarization into the M1 phenotype. In this study, we confirmed that AR deficiency
541 decreases the number of microglia/macrophages with the M1 phenotype after SCI.

542 AR mediates inflammatory signals in macrophages mainly through regulating
543 oxidative stress responses [48]. Macrophages are polarized to the M1 response by
544 either producing pro-inflammatory cytokines or increasing the generation of ROS [29].
545 ROS-induced lipid peroxidation-derived compounds such as HNE and their
546 glutathione-conjugates (e.g. GS-HNE) are produced in large amounts in cells [39, 48].
547 AR efficiently catalyzes the reduction of HNE and GS-HNE to 1,4-dihydroxynonene
548 (DHN) and GS-DHN, respectively with much lower *K*m values in the micromolar
549 range (10–30 μM) than the *K*m value for glucose (50–100 mM) [36, 39].
550 Pharmacological inhibition or genetic ablation of AR prevents PLC, PKC, and NF-κB

551 activation caused by HNE and GS-HNE, but not by GS-DHN [36]. Thus, inhibiting
552 AR prevent macrophages from polarizing to M1, which may be due to the reduction
553 in HNE/GS-HNE to DHN/GS-DHN; this can active the redox-sensitive transcriptional
554 factors NF-κB and AP-1 to upregulate expression of genes characteristic of M1
555 macrophages [36, 39] (Fig 6a). AR inhibition can prevent LPS-induced
556 downregulation of cAMP response element modulator (CREM), phosphorylation of
557 CREB, and DNA-binding of CREB in macrophages [42]. Another report showed that
558 HNE could induce ATF-2 and CREB-1 phosphorylation to regulate the secretion of
559 some cytokines [53]. These data show that HNE and GS-HNE can trigger multiple
560 signaling cascades to modulate inflammatory responses [36, 53]. In this study, we
561 found that AR deficiency not only decreased M1 polarization, but also increased the
562 M2 phenotype of microglia/macrophages after SCI. *In vitro* experiments showed that
563 inhibiting AR with fidarestat upregulated HNE in microglia. HNE accumulation
564 induced Arg1 upregulation by activating CREB, which could be specifically inhibited
565 by KG501 in microglia. However, the upregulation of Arg1 induced by IL-4 and
566 transcribed by STAT6 was not inhibited by KG501 [45]. Thus, we hypothesize that
567 AR controls the switch resulting in accumulation of HNE in cells. If AR is sufficient,
568 HNE/GS-HNE will be reduced to DHN/GS-DHN, which activates NF-κB and AP-1
569 to polarize microglia to M1 type [38]. However, if AR is inhibited or ablated,
570 HNE/GS-HNE will be accumulated in the cytoplasm and activate CREB to polarize
571 microglia to the M2 type [42, 53] (Fig. 6a, b).

572 CREB is a pivotal transcriptional factor in macrophage polarization that

promotes activation of M2-associated genes with repressing M1 activation [1, 16, 43]. CREB induces C/EBPβ expression, which specifically regulates M2-associated genes [43]. Both STAT6 and C/EBPβ have recently been shown to be essential for Arg1 expression in macrophages, but in a stimulus-specific manner due to the selective use of a distinct *cis*-acting element in the *Arg1* promoter [16]. C/EBPβ is responsible for Arg1 expression in response to TLR ligands, whereas STAT6 regulates Arg1 expression in response to IL-4 and/or IL-13 stimulation [16, 45]. CREB also shows anti-inflammatory cytokine expression in macrophage in response to LPS, which is mediated by the p38 mitogen-activated protein kinase (MAPK) and mitogen- and stress-activated kinase 1 (MSK1) and MSK2 [1]. As it was shown previously, the M1-polarized microenvironment with high pro-inflammatory cytokines (e.g. IL-1β and IL-6) and TLR ligands (e.g. HMGB1) are dominant, which is preferred for polarizing microglia/macrophages into the M1 type during early stages of SCI [13, 27, 32]. In this study, we found that AR deficiency could switch microglia/macrophages polarized to the M2 type rather than the M1 type even at early stages of an M1-polarized microenvironment, such as in injured spinal cord. Thus, it is reasonable that AR deficiency causes accumulation of HNE in microglia, which stimulates CREB to regulate the expression of characteristic M2 genes with the repression of M1-associated genes (Fig. 6 b).

AR can be detected in a number of human and animal tissues including various regions of the eyes, testis, liver, placenta, ovary, kidney, erythrocyte, cardiac and skeletal muscle, and the brain [51]. AR is located in the cytoplasm of most cells but is

595 not uniformly distributed in all cell types of an organ. For example, in the kidney, AR
596 is present in the loop of Henle, collecting tubules, outer and inner medulla, but not in
597 the cortex [52]. In this study, we also found that although the levels of mRNA and
598 protein of AR are both gradually upregulated after injury in time-dependent manner,
599 AR is only distributed in microglia, macrophages, and neurons, but not in astrocytes
600 or oligodendrocytes near the injured site after SCI. Quantitative proteomic analysis of
601 purified *in vivo* retinal ganglion cells (RGCs) showed that AR is one of the most
602 significantly elevated proteins in experimental glaucoma [12]. The roles of AR in the
603 nervous system primarily result from diabetic complications, such as retinopathy and
604 neuropathy [12]. Previous studies demonstrated that hyperglycemia-induced oxidative
605 stress leads to activation of MAPK, which may contribute to neuronal pathogenesis.
606 Fidarestat, an AR inhibitor, was shown to prevent activation of MAPK and nerve
607 conduction velocity deficits in diabetes [20, 35]. Studies using AR knockout mice [21]
608 also demonstrated that AR deficiency could prevent diabetes-induced oxidative stress
609 in retinal neurons [11]. Moreover, both AR deficiency and AR inhibition reduced
610 oxidative stress in the peripheral nerves and markedly protected mice from
611 diabetes-induced functional deficits [20]. However, there have been few reports
612 regarding the role of AR in neuronal cells in the CNS. In this study, we found that AR
613 was upregulated in neurons near the lesion site of the spinal cord after injury. The
614 significance of AR upregulation in neurons after SCI remains unclear. However, we
615 also found that AR deficiency promotes axonal regeneration of primary cultured
616 cortical neurons *in vitro* (data not shown). We suggest that AR plays multiple roles in

neural and non-neural cells.

AR inhibition is emerging as a promising therapeutic approach in understanding the cellular and molecular mechanisms of AR [31]. Although ARI-targeted therapies are currently being evaluated in phase I/II and III studies for diabetes and endotoxin-related inflammatory diseases, yet they have not achieved worldwide clinical use in disease management because of limited efficacy and/or unfavorable adverse effects [31]. To explore the AR-mediated signaling pathway in different tissues and cells may be critical to increase efficacy and decrease side effects of ARIs in future. In this study, we found that AR has a critical role in switching microglia/macrophages polarization after SCI. We suggest that AR inhibition may be a promising therapeutic method for treating SCI in future.

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Conflict of interest

The authors declare that they have no conflict of interest.

638

639 Figure legends

640 **Fig 1**. The expression pattern of AR *in vivo* and *in vitro*. a, Time-course of AR mRNA
641 level after SCI. b, Time-course of AR protein level after SCI. c. The densitometric
642 ratio of AR in Fig. b. d, The co-localization of AR (green)- and Iba1 (red)-positive
643 cells in the sagittal section of injured spinal cord was stained by IHC. In the first row
644 panel, the boxed area 1, located near the epicenter of injured spinal cord, is enlarged
645 in second row panel 1 and the boxed area 2, located at the distal area of injured spinal
646 cord, is enlarged in third row panel 2. Bars are equal to 500 μm and 50 μm,
647 respectively. e, The co-localization of AR (green) - and NeuN(red)-positive cells in
648 the coronal section of injured spinal cord was stained by IHC. The boxed area in left
649 panel is enlarged in the right panel. Bars are equal to 200 μm and 50 μm. f, The
650 mRNA level of AR in primary cultured astrocytes with stimulation of TNF-α + INF-γ.
651 g, The mRNA level of AR in primary cultured oligodendrocytes with stimulation of
652 TNF-α + INF-γ. h, The mRNA level of AR in primary cultured neurons with
653 stimulation of TNF-α + INF-γ. i, The mRNA level of AR in primary cultured
654 microglia with stimulation of TNF-α + INF-γ and LPS, respectively. *$p < 0.05$, **$p <$
655 0.01. Iba1= ionized calcium binding adapter molecule 1; NeuN= Neuronal Nuclei.

656

657 **Fig 2**. AR deficiency promoted locomotion recovery after SCI and induced
658 microglia/macrophages polarized to M2 in mice. a, BMS score to evaluate locomotion
659 recovery at different time post injury in $AR^{+/+}$ and $AR^{-/-}$ mice. b and c, Injured areas
660 were judged in sagittal sections of injured spinal with LFB staining (b) and calculated

with statistical analysis in (c) in WT and $AR^{-/-}$ mice. Bar = 500 μm. d, Representative figures showing the amount of iNOS (green)- and F4/80 (red)-positive cells in the sections of injured spinal cord in WT and $AR^{-/-}$ mice. Boxed area in each pannel was enlarged at corner of each panel. Bar = 200 μm. e, The representative figures showing that the amount of Arg1 (green)- and Iba1 (red)-positive cells in the sections of injured spinal cord in WT and $AR^{-/-}$ mice. Boxed area in each panel was enlarged at corner of each panel. Bar = 200 μm. iNOS= inducible nitric oxide synthase; Arg 1=Arginase 1.

Fig 3. Time-course of the characteristic genes of M1 and M2 expression after SCI in WT and $AR^{-/-}$ mice. a, Time-course of iNOS mRNA levels after SCI in WT and $AR^{-/-}$ mice. b, Time-course of CD86 mRNA levels after SCI in WT and $AR^{-/-}$ mice. c, Time-course of Arg1 mRNA levels after SCI in WT and $AR^{-/-}$ mice. d, Time-course of CD206 mRNA levels after SCI in WT and $AR^{-/-}$ mice. e, Time-course of iNOS and Arg1 proteins levels after SCI in WT and $AR^{-/-}$ mice. f, iNOS normalized by β -actin. g, Arg 1normalized by β -actin. h, Time-course of the ratio of Arg1/iNOS in protein level after SCI in WT and $AR^{-/-}$ mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Fig 4. Time-course of NF-κB and CREB mRNA and protein levels after SCI in WT and $AR^{-/-}$ mice. a, Time-course of NF-κB mRNA levels after SCI in WT and $AR^{-/-}$ mice. b, Time-course of CREB mRNA levels after SCI in WT and $AR^{-/-}$ mice. c, Time-courses of NF-κB, CREB, and p-CREB protein levels after SCI in WT mice. d,

683 Time-courses of NF-κB, CREB, and p-CREB protein levels after SCI in $AR^{-/-}$ mice. e,
684 NF-κB normalized by β -actin. f, CREB normalized by β -actin. g, pCREB
685 normalized by β -actin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
686
687 **Fig 5**. Mechanism of AR in regulating M1/M2 polarization in microglia *in vitro*. a,
688 Representative fluorescent photos of HNE levels in BV2 microglial cells under
689 different dosages of AR inhibitor Fidarestat stimulation *in vitro.* Bar = 50 μm. b,
690 Time-course changes of density of fluorescence of HNE in BV2 microglial cells under
691 20-μM Fidarestat stimulation *in vitro*. *$p < 0.05$. c, analysis of the protein levels of
692 Arg1, iNOS, pCREB, CREB, pNF-κB, and NF-κB in N9 microglial cells under LPS
693 stimulations combined with ARI and HNE *in vitro* by western blotting. d, Analysis of
694 the protein levels of Arg1, iNOS, pCREB, CREB, pNF-κB, and NF-κB in N9
695 microglial cells under ARI and HNE stimulations combined with KG501 *in vitro* by
696 western blotting. Numeric values accompany the representative samples of western
697 blot indicate the relative expression level obtaining in control cells (treated with
698 DMSO), normalized by β -actin.
699
700 **Fig 6**. Schematic presentation of the mechanism of AR in regulation of M1/M2
701 polarization. a, AR typically reduces HNE/GS-HNE to DHN/GS-DHN which
702 activates NF-κB and AP1 to induce the M1-associated genes expression. b, In AR
703 inhibition or AR deficiency, the reduction of AR is decreased and causes the
704 accumulation of HNE/GS-HNE, which activated CREB to induce the M2-associated

genes expression.

References:

1. Ananieva O, Darragh J, Johansen C, Carr JM, McIlrath J, Park JM, Wingate A, Monk CE, Toth R, Santos SG, Iversen L, Arthur JS (2008) The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. Nat Immunol 9:1028-1036
2. Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635-659
3. Beutner C, Linnartz-Gerlach B, Schmidt SV, Beyer M, Mallmann MR, Staratschek-Jox A, Schultze JL, Neumann H (2013) Unique transcriptome signature of mouse microglia. Glia 61:1429-1442
4. Blanchard B, Heurtaux T, Garcia C, Moll NM, Caillava C, Grandbarbe L, Klosptein A, Kerninon C, Frah M, Coowar D, Baron-Van EA, Morga E, Heuschling P, Nait OB (2013) Tocopherol derivative TFA-12 promotes myelin repair in experimental models of multiple sclerosis. J Neurosci 33:11633-11642
5. Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8:57-69
6. Busch SA, Hamilton JA, Horn KP, Cuascut FX, Cutrone R, Lehman N, Deans RJ, Ting AE, Mays RW, Silver J (2011) Multipotent adult progenitor cells prevent macrophage-mediated axonal dieback and promote regrowth after spinal cord injury. J Neurosci 31:944-953
7. Busch SA, Horn KP, Cuascut FX, Hawthorne AL, Bai L, Miller RH, Silver J (2010) Adult NG2+ cells are permissive to neurite outgrowth and stabilize sensory axons during macrophage-induced axonal dieback after spinal cord injury. J Neurosci 30:255-265
8. Busch SA, Horn KP, Silver DJ, Silver J (2009) Overcoming macrophage-mediated axonal dieback following CNS injury. J Neurosci 29:9967-9976
9. Carrico KM, Vaishnav R, Hall ED (2009) Temporal and spatial dynamics of peroxynitrite-induced oxidative damage after spinal cord contusion injury. J Neurotrauma 26:1369-1378
10. Chan CC (2008) Inflammation: beneficial or detrimental after spinal cord injury? Recent Pat CNS Drug Discov 3:189-199
11. Cheung AK, Fung MK, Lo AC, Lam TT, So KF, Chung SS, Chung SK (2005) Aldose reductase deficiency prevents diabetes-induced blood-retinal barrier breakdown, apoptosis, and glial reactivation in the retina of db/db mice. Diabetes 54:3119-3125
12. Crabb JW, Yuan X, Dvoriantchikova G, Ivanov D, Crabb JS, Shestopalov VI (2010) Preliminary quantitative proteomic characterization of glaucomatous rat retinal ganglion cells. Exp Eye Res 91:107-110
13. David S, Kroner A (2011) Repertoire of microglial and macrophage responses after spinal cord injury. Nat Rev Neurosci 12:388-399
14. Donnelly DJ, Popovich PG (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378-388
15. Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. J Neurosci 8:1454-1468

16. El KK, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, Basaraba RJ, Konig T, Schleicher U, Koo MS, Kaplan G, Fitzgerald KA, Tuomanen EI, Orme IM, Kanneganti TD, Bogdan C, Wynn TA, Murray PJ (2008) Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol 9:1399-1406
17. Fellner L, Irschick R, Schanda K, Reindl M, Klimaschewski L, Poewe W, Wenning GK, Stefanova N (2013) Toll-like receptor 4 is required for alpha-synuclein dependent activation of microglia and astroglia. Glia 61:349-360
18. Gensel JC, Nakamura S, Guan Z, van Rooijen N, Ankeny DP, Popovich PG (2009) Macrophages promote axon regeneration with concurrent neurotoxicity. J Neurosci 29:3956-3968
19. HERS HG (1956) [The mechanism of the transformation of glucose in fructose in the seminal vesicles]. Biochim Biophys Acta 22:202-203
20. Ho EC, Lam KS, Chen YS, Yip JC, Arvindakshan M, Yamagishi S, Yagihashi S, Oates PJ, Ellery CA, Chung SS, Chung SK (2006) Aldose reductase-deficient mice are protected from delayed motor nerve conduction velocity, increased c-Jun NH2-terminal kinase activation, depletion of reduced glutathione, increased superoxide accumulation, and DNA damage. Diabetes 55:1946-1953
21. Ho HT, Chung SK, Law JW, Ko BC, Tam SC, Brooks HL, Knepper MA, Chung SS (2000) Aldose reductase-deficient mice develop nephrogenic diabetes insipidus. Mol Cell Biol 20:5840-5846
22. Joshi M, Fehlings MG (2002) Development and characterization of a novel, graded model of clip compressive spinal cord injury in the mouse: Part 1. Clip design, behavioral outcomes, and histopathology. J Neurotrauma 19:175-190
23. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG (2009) 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
24. Kim JB, Sig CJ, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS, Lee JK (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 26:6413-6421
25. Lawrence T, Natoli G (2011) Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol 11:750-761
26. Lo AC, Cheung AK, Hung VK, Yeung CM, He QY, Chiu JF, Chung SS, Chung SK (2007) Deletion of aldose reductase leads to protection against cerebral ischemic injury. J Cereb Blood Flow Metab 27:1496-1509
27. Longbrake EE, Lai W, Ankeny DP, Popovich PG (2007) Characterization and modeling of monocyte-derived macrophages after spinal cord injury. J Neurochem 102:1083-1094
28. Murray PJ, Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 11:723-737
29. O'Neill LA, Hardie DG (2013) Metabolism of inflammation limited by AMPK and pseudo-starvation. Nature 493:346-355
30. Oyinbo CA (2011) Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. Acta Neurobiol Exp (Wars) 71:281-299
31. Pandey S, Srivastava SK, Ramana KV (2012) A potential therapeutic role for aldose reductase inhibitors in the treatment of endotoxin-related inflammatory diseases. Expert Opin Investig Drugs 21:329-339
32. Pineau I, Lacroix S (2007) Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. J Comp Neurol

500:267-285
33. Plemel JR, Duncan G, Chen KW, Shannon C, Park S, Sparling JS, Tetzlaff W (2008) A graded forceps crush spinal cord injury model in mice. J Neurotrauma 25:350-370
34. Popovich PG, Wei P, Stokes BT (1997) Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J Comp Neurol 377:443-464
35. Price SA, Agthong S, Middlemas AB, Tomlinson DR (2004) Mitogen-activated protein kinase p38 mediates reduced nerve conduction velocity in experimental diabetic neuropathy: interactions with aldose reductase. Diabetes 53:1851-1856
36. Ramana KV, Bhatnagar A, Srivastava S, Yadav UC, Awasthi S, Awasthi YC, Srivastava SK (2006) Mitogenic responses of vascular smooth muscle cells to lipid peroxidation-derived aldehyde 4-hydroxy-trans-2-nonenal (HNE): role of aldose reductase-catalyzed reduction of the HNE-glutathione conjugates in regulating cell growth. J Biol Chem 281:17652-17660
37. Ramana KV, Chandra D, Srivastava S, Bhatnagar A, Srivastava SK (2003) Aldose reductase mediates the mitogenic signals of cytokines. Chem Biol Interact 143-144:587-596
38. Ramana KV, Fadl AA, Tammali R, Reddy AB, Chopra AK, Srivastava SK (2006) Aldose reductase mediates the lipopolysaccharide-induced release of inflammatory mediators in RAW264.7 murine macrophages. J Biol Chem 281:33019-33029
39. Ramana KV, Srivastava SK (2010) Aldose reductase: a novel therapeutic target for inflammatory pathologies. Int J Biochem Cell Biol 42:17-20
40. Ramana KV, Srivastava SK (2006) Mediation of aldose reductase in lipopolysaccharide-induced inflammatory signals in mouse peritoneal macrophages. Cytokine 36:115-122
41. Ramunno A, Cosconati S, Sartini S, Maglio V, Angiuoli S, La Pietra V, Di Maro S, Giustiniano M, La Motta C, Da SF, Marinelli L, Novellino E (2012) Progresses in the pursuit of aldose reductase inhibitors: the structure-based lead optimization step. Eur J Med Chem 51:216-226
42. Reddy AB, Srivastava SK, Ramana KV (2010) Aldose reductase inhibition prevents lipopolysaccharide-induced glucose uptake and glucose transporter 3 expression in RAW264.7 macrophages. Int J Biochem Cell Biol 42:1039-1045
43. Ruffell D, Mourkioti F, Gambardella A, Kirstetter P, Lopez RG, Rosenthal N, Nerlov C (2009) A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. Proc Natl Acad Sci U S A 106:17475-17480
44. Shechter R, Miller O, Yovel G, Rosenzweig N, London A, Ruckh J, Kim KW, Klein E, Kalchenko V, Bendel P, Lira SA, Jung S, Schwartz M (2013) Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus. Immunity 38:555-569
45. Sica A, Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122:787-795
46. Sisnaiske J, Hausherr V, Krug AK, Zimmer B, Hengstler JG, Leist M, van Thriel C (2014) Acrylamide alters neurotransmitter induced calcium responses in murine ESC-derived and primary neurons. Neurotoxicology 43:117-126
47. Srivastava SK, Ramana KV, Bhatnagar A (2005) Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options. Endocr Rev 26:380-392
48. Srivastava SK, Yadav UC, Reddy AB, Saxena A, Tammali R, Shoeb M, Ansari NH, Bhatnagar A, Petrash MJ, Srivastava S, Ramana KV (2011) Aldose reductase inhibition suppresses oxidative stress-induced inflammatory disorders. Chem Biol Interact 191:330-338

836 49. Sypecka J, Sarnowska A, Gadomska-Szablowska I, Lukomska B, Domanska-Janik K (2013)
837 Differentiation of glia-committed NG2 cells: the role of factors released from hippocampus and spinal
838 cord. Acta Neurobiol Exp (Wars) 73:116-129
839 50. Takahashi K, Mizukami H, Kamata K, Inaba W, Kato N, Hibi C, Yagihashi S (2012)
840 Amelioration of acute kidney injury in lipopolysaccharide-induced systemic inflammatory response
841 syndrome by an aldose reductase inhibitor, fidarestat. PLoS One 7:e30134
842 51. Tang WH, Martin KA, Hwa J (2012) Aldose reductase, oxidative stress, and diabetic mellitus.
843 Front Pharmacol 3:87
844 52. Terubayashi H, Sato S, Nishimura C, Kador PF, Kinoshita JH (1989) Localization of aldose and
845 aldehyde reductase in the kidney. Kidney Int 36:843-851
846 53. Vaillancourt F, Morquette B, Shi Q, Fahmi H, Lavigne P, Di Battista JA, Fernandes JC,
847 Benderdour M (2007) Differential regulation of cyclooxygenase-2 and inducible nitric oxide synthase
848 by 4-hydroxynonenal in human osteoarthritic chondrocytes through ATF-2/CREB-1 transactivation
849 and concomitant inhibition of NF-kappaB signaling cascade. J Cell Biochem 100:1217-1231
850 54. Xu W, Chi L, Xu R, Ke Y, Luo C, Cai J, Qiu M, Gozal D, Liu R (2005) Increased production of
851 reactive oxygen species contributes to motor neuron death in a compression mouse model of spinal
852 cord injury. Spinal Cord 43:204-213
853 55. Zhao X, Wu J, Kuang F, Wang J, Ju G (2011) Silencing of Nogo-A in rat oligodendrocyte
854 cultures enhances process branching. Neurosci Lett 499:32-36
855 56. Zhao Y, Bian G, Yu C, Liu F, Liu L, Guo H, Guo J, Ju G, Wang J (2012) Cross-reactivity of
856 anti-programmed death ligand 2 polyclonal antibody in mouse tissues. Sci China Life Sci 55:940-947

857