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

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18

19

Abstract

20
21 Inflammatory reactions are the most critical pathological processes occurring after
22 spinal cord injury (SCI). Activated microglia/macrophages have either detrimental or
23 beneficial effects on neural regeneration based on their functional polarized M1/M2
24 subsets. However, the mechanism of microglia/macrophages polarization to M1/M2 at
25 the injured spinal cord environment remains unknown. In this study, wild type (WT)
26 or aldose reductase (AR) knockout (KO) mice were subjected to SCI by spinal crush
27 injury model. The expression pattern of AR, behavior tests for locomotor activity, and
28 lesion size were assessed at between 4 hours and 28 days after SCI. we found that the
29 expression of AR is upregulated in microglia/macrophages after SCI in WT mice. In
30 AR KO mice, SCI led to smaller injury lesion areas compared to WT. AR
31 deficiency-induced microglia/macrophages induce the M2 rather than the M1
32 response and promote locomotion recovery after SCI in mice. *In vitro* experiments,
33 microglia cell lines (N9 or BV₂) were treated with AR inhibitor (ARI), Fidarestat. AR
34 inhibition caused 4-Hydroxynonenal (HNE) accumulation, which induced the
35 phosphorylation of cAMP-responsive binding element (CREB) to promote Arg1
36 expression. KG501, the specific inhibitor of phosphorylated CREB could cancel the
37 upregulation of Arg 1 by ARI or HNE stimulation. Our results suggest that AR works
38 as a switch which can regulate microglia by polarizing cells to either the M1 or the
39 M2 phenotype under M1 stimulation based on its states of activity. We suggest that
40 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

43 **Introduction**

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

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

67 One of the well-characterized pathological processes occurring early after SCI is the
68 formation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) [9,
69 30]. RNS and ROS cause lipid oxidation as well as nitrative and oxidative damage to
70 proteins and nucleic acids, leading to neuronal loss by necrosis or apoptosis [30, 54].
71 Microglia/macrophages are the major cells that produce neurotoxic RNS and ROS
72 after SCI [14]. Oxidative stress-induced inflammation is a major contributor to
73 secondary injury after SCI [9]. ROS-mediated activation of redox-sensitive
74 transcription factors, such as nuclear factor-kappa B (NF- κ B) and activator protein
75 (AP)-1, as well as subsequent expression of pro-inflammatory cytokines, chemokines,
76 and growth factors, are characteristics of inflammatory diseases [5, 48]. The
77 pro-inflammatory cytokines TNF- α and IL-1 β , which are up-regulated immediately
78 after SCI combining with the ligands of pattern recognition receptors (PRRs), such as
79 high mobility group box protein (HMBG1) and LPS, can cause
80 microglia/macrophages to enter an overactive state and begin releasing ROS [13, 14,
81 23]. Cytokines and LPS cause oxidative stress to form toxic lipid aldehydes such as
82 4-hydroxynonenal (HNE), which triggers multiple signaling cascades that variably
83 affect cell growth, differentiation, and apoptosis through lipid peroxidation [36].

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

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

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

114 **Materials and Methods**

115 **Animals and SCI model**

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

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

132 **Behavioural assessment**

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

138 **Estimation of lesion area**

139 Spaced serial 10 µm thick sections 100 µm apart were stained with Luxol Fast Blue
140 (LFB, Sigma-Aldrich) [4]. Briefly, the sections were dehydrated and incubated in
141 0.1% LFB dissolved in 95% ethanol at 60°C overnight. Stained sections were cooled
142 at RT and differentiated in 0.05% lithium carbonate and 70% ethanol. Sections were
143 then counterstained with 0.1% Cresyl Violet (Sigma-Aldrich) at 60°C for 10 min and
144 mounted in neural resin . Digital images were acquired using a DP70 digital camera
145 (Olympus) mounted on a BX51 Olympus microscope equipped with a 10× objective.
146 Five random sections were quantified per mouse and three mice of each kind were
147 measured per group. Quantification of LFB stained areas was performed using
148 Photoshop CS3 software.

149 **Cell cultures**

150 **Microglial cell lines culture**

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

163 **Primary cortical neurons cultures**

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

171 15 min. The enzymatic digestion was followed by a brief mechanical trituration using
172 polished Pasteur pipettes and centrifuged at 800 rpm for 5 min. Cells were suspended
173 in Neurobasal A (Gibco) supplemented with B27 (Gibco) and 2 mM L-glutamine. Cell
174 suspension were seeded in poly-L-lysine (25 µg/ml, Sigma) coated 6-well culture
175 plates at a density of 4×10^4 cells/cm² at 37 °C/5% CO₂ for 7-14 d culture. The cell
176 populations consisted of >95% neurons before used, which was determined by
177 immunocytochemical stain with anti Neuron- specific β-tubulin III (data not shown)
178 [46].

179 **Primary astrocytes cultures**

180 Primary astrocytes were cultured from the cerebral cortex of 1-2 d postnatal mouse
181 pups [17]. The cerebral cortices were dissected and trypsinized as indicated above for
182 the primary neurons cultures. Cells were suspended in DMEM medium supplemented
183 with 10% FBS (Gibco), 2 mM glutamine, 50 U/ml Penicillin and 50 µg/ml
184 Streptomycin (Gibco). Cells were plated on PLL coated 75 cm² culture flasks at a
185 density of 1×10^5 cells/cm² and cultured in 5% CO₂ at 37 °C. After 7-10 d incubation,
186 cultures were purified by shaking for 20 h on an orbital shaker (180 rpm) at 37°C,
187 resulting in cultures of 95% GFAP-positive cells. Purified astrocytes were digested by
188 trypsinization, and cells were again plated at the density of 5×10^5 /cm² onto PLL
189 coated 6-well plates and incubated in 5% CO₂ at 37 °C for two days before used.

190 **Oligodendrocyte cultures**

191 Oligodendrocyte precursor cells (OPC) were generated from primary mixed glial
192 cultures above mentioned as described previously [49, 55]. Briefly, after ten days
193 culture in DMEM medium with 10% FBS (Gibco) and supplemented with 2 mM
194 glutamine, 50 µg/ml Streptomycin and 50 U/ml Penicillin. Oligodendrocyte precursor
195 cells were shaking off based on the different adhesion properties of different glial cell
196 types. Cell cultures were shaken first at 200rpm, 37°C for 30min and rinsed with fresh
197 DMEM medium with 10% FBS (Gibco) and supplemented with 2 mM glutamine,
198 50 µg/ml Streptomycin and 50 U/ml Penicillin. After cells were cultured for another
199 14-16 h, cell cultures were shaken at 280 rpm, 37°C for 18-20 h to collect the OPC.
200 Precursor cells were obtained by sequential dislodging procedure. Purified OPC were
201 plated onto PLL coated 6-well plates at the density of 4×10^4 cells/cm² and cultured
202 in Satomedium (DMEM/F12 supplemented with 4 mM Glu, 0.1 mM sodium
203 pyruvate, 0.1% BSA, 50µg/ml transferrin, 5µg/ml Insulin, 30 nM sodium selenate, 10
204 nM Vitamin H and 10 nM hydrocortisone) supplemented with 10 ng/ml PDGF-AA
205 and 10 ng/ml bFGF to promote cell growth. OPC were cultured in above expansion
206 medium for 2-3 d and passaged with DMEM/F12 supplemented with 0.01% EDTA ,
207 0.2 mg/ml DNase and 5 µg/ml insulin. The supernatant was collected and NG2⁺ cells
208 were plated onto PLL coated 6-well plates at the density of 8×10^4 cells/cm² in
209 differentiation medium which contains Satomedium supplemented with 15 nM
210 triiodothyronine (T3), 10 ng/ml ciliary neurotrophin factor (CNTF), and 5 µg/ml
211 N-acetyl-l-cysteine (NAC) for 6 days before used.

212 **Primary microglia cultures**

213 Primary microglia cultures were also obtained from the cerebral hemispheres of
214 newborn mouse brains (P1-2) as previously described [3, 24]. In brief, mixed glial
215 cells were seeded onto PLL coated culture flasks at a density of 1×10^5 cells/cm². Two
216 weeks later, microglia were shaken off at 200 rpm for 30min. Highly enriched
217 microglial suspension were collected and filtered through 41 μ m cell strainer. After
218 centrifugation at 800 rpm for 5 min, cells were resuspended in DMEM medium with
219 10% FBS, and plated onto 6-well plates at a density of 2×10^5 cells/cm². The purity of
220 cells obtained was harvested > 95% based on staining with Iba-1 antibody before
221 used.

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

225 **Immunohistochemistry**

226 Mice were killed by injection of an overdose of 2% sodium pentobarbital. The
227 animals were transcardially perfused with ice-cold saline followed by 4%
228 formaldehyde in 0.1 M phosphate buffer, pH 7.4, for at least 15 min at room
229 temperature (RT). After perfusion, the spinal cords were dissected out and postfixed
230 4-6 h at 4°C in the same solution used for perfusion. Spinal cord tissue was then
231 immersed into 20% sucrose solution in 0.1 M phosphate buffer, pH 7.3, at 4°C for
232 cryoprotection. Tissues were embedded in Tissue Tek (Sakura Finetek, Zoeterwoude,
233 The Netherlands), cut transversely or longitudinally into 10- μ m slices in a cryostat,

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

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

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

249 Table 1. Antibodies for IHC and WB

| Antibodies | Host | Vendor | Dilution | |
|------------|--------|--------|----------|--------|
| | | | 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- κ B | Rabbit | Anbo | N/A | 1:1000 |
| pNF- κ B | 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 |

250

251 **Quantitative Real-time PCR**

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

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

260 electrophoresis. Equal amount of RNA from each group was used for Poly(A) tailing
 261 and quantitative reverse transcription consisted of 4 μ l 5 \times reaction mix, 2 μ l 10 \times
 262 SuperScript enzyme mix, and 200 ng total RNA in a final volume of 20 μ l. Then real
 263 time PCR was performed using the NCodeTM EXPRESS SYBR GreenERTM
 264 microRNA qRT-PCR Kit (Invitrogen) containing 1 μ l of the RT product, 10 μ l
 265 EXPRESS SYBR green qRT-PCR SuperMix, 0.4 μ l microRNA-specific forward
 266 primer (10 μ M), and 0.4 μ l universal qPCR primer (10 μ M) in a final volume of 20 μ l.
 267 PCR cycling began with template denaturation and hot start Taq activation at 95°C for
 268 2 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min performed in a
 269 CFX96 Real-Time PCR System (BioRad). Beta-actin was used as the internal
 270 standard reference and normalized expressions of targeted genes were calculated
 271 using the comparative CT method and fold changes were derived from the equation
 272 $2^{-\Delta\Delta C_t}$ for each gene. All primers were used in this study are list in Table 2.

273 Table 2. Primer sequences for QRT-PCR analysis

| Accession | | | |
|----------------|----------------|-------------------------|------------------------|
| Gene | number | Forward primer (5'-3') | Reverse Primer (5'-3') |
| AR | NM_009658.3 | ACGGCTATGGAACA ACTA | TGTGGCAGTATTCAATCAG |
| Arg 1 | NM_007482.2 | GAACACGGCAGTGGCTTTAAC | TGCTTAGCTCTGTCTGCTTTGC |
| iNOS | NM_010927.2 | CCCTTCAATGGTTGGTACATGG | ACATTGATCTCCGTGACAGCC |
| NF- κ B | 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 | CATGAGCTGGGTCATCTTTTC |

274 **Western blotting**

275 Tissue samples about 1 cm of spinal cord with the injured site in middle were lysed by
276 homogenizing per 100 mg tissues in 1ml RIPA (50 mM Tris-HCl, pH 8.0, 150 mM
277 NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) buffer or the cells in 6-well
278 plates were lysed in 300 μ l RIPA buffer containing 1 mM PMSF. Lysates were
279 centrifuged at 12,000 rpm for 10 min, and the supernatant was collected and added
280 same volume sample buffer. The protein amount was determined using BSA Protein
281 Assay Kit (Pierce). Samples with equal amounts of protein were then separated by
282 10% SDS-PAGE, and electrotransferred onto (300mA for 60min) Polyvinylidene
283 Fluoride (PVDF) Membranes (Millipore, 0.45 μ m) with Transblot Turbo (Bio-Rad,
284 USA). Membranes were incubated with 5% nonfat milk in Tris buffer containing
285 Tween 20 (TBST; 10 m M Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for
286 2h at RT and incubated with the primary antibodies which listed in Table 1 at 4°C
287 overnight. Membranes were then washed three times for 10 min with TBST and
288 probed with relative 2nd antibodies conjugated with horseradish peroxidase (abcam) at
289 RT for 2 h. Membranes were finally washed three times for 10 min with TBST to
290 remove unbound 2nd antibodies and visualized using enhanced chemiluminescence
291 (Thermo Scientific). The density of specific bands was measured using ImageJ
292 densitometry software and normalized against a loading control (β -actin).

293

294 **Statistical analysis**

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

299

300 **Results**

301 **Time-course changes in mRNA and protein levels of AR after** 302 **SCI**

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

312 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 immunofluorescence 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 astrocytes 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 secrete pro-inflammatory
328 cytokines, such as $\text{INF-}\gamma$ and $\text{TNF-}\alpha$, 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 $\text{INF-}\gamma$ + $\text{TNF-}\alpha$ 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),

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

342

343 **AR deficiency promoted locomotor recovery after SCI**

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

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

359

360 **M1/M2 ratio decreased significantly during early stages of** 361 **SCI**

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

376 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 densitometric
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

420 signals in macrophages promote NF- κ B dependent expression of inflammatory
421 cytokines and chemokines [37-40]. AR also regulates LPS-mediated inflammation
422 through the cAMP/PKA/CREB pathway [42]. Thus, we focused on the expression
423 profiles of NF- κ B and CREB after SCI in AR^{+/+} and AR^{-/-} mice. The mRNA of the
424 p65 subunit of NF- κ B increased gradually postinjury, with AR^{+/+} mice showing
425 higher mRNA levels of p65 at 1, 3, and 7 dpi compared with AR^{-/-} mice at same
426 time-point after SCI (Fig. 4a). Additionally, mRNA levels of CREB were higher at 4
427 hpi and 1, 3, and 7 dpi in AR^{-/-} mice (Fig. 4b). At 14 dpi, however, the mRNA level
428 of CREB was lower in AR^{-/-} mice compared to in AR^{+/+} mice (Fig. 4b). In AR^{+/+}
429 mice, the protein level of p65 gradually increased at 1, 3, 7, and 14 dpi (Fig. 4c and e).
430 In AR^{-/-} mice, however, the profile of p65 continued decreasing after SCI (Fig. 4d
431 and e). The other transcription factor studied, CREB, also showed a different
432 expression pattern after SCI in AR^{+/+} mice and AR^{-/-} mice. In AR^{+/+} mice, the peak of
433 CREB expression occurred at 14 dpi, while CREB level was highest at 3 dpi and
434 decreased at 14 dpi in AR^{-/-} mice (Fig. 4d and f). Amounts of pCREB were always
435 higher at same time-point after SCI in AR^{-/-} mice compared with in AR^{+/+} mice (Fig.
436 4g). Consistent with the higher baseline level of iNOS and Arg 1 (Fig. 3e), the levels
437 of NF- κ B, CREB, and pCREB were also higher at baseline in AR^{-/-} mice (Fig. 4c and
438 d). These results showed that there were lower levels of the p65 subunit of NF- κ B and
439 higher CREB and pCREB during early stages after SCI in AR^{-/-} mice compared to
440 those in AR^{+/+} mice.

441

442 **HNE and CREB were upregulated in microglia following AR**
443 **inhibition.**

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

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

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

476 N9 cells that had been treated with a combination of LPS+ARI, LPS+HNE, and
477 LPS+HNE+ARI all showed an increase in Arg1 expression. iNOS levels slightly
478 increased following stimulation with these compounds compared to HNE or ARI
479 stimulation alone, but decreased significantly compared with LPS stimulation (Fig.
480 5c). Additionally, the level of p-CREB was clearly increased. However, the level of
481 p-NF- κ B was clearly decreased following stimulation with a combination of these
482 compounds (Fig. 5c). These results further indicate that HNE and ARI can induce
483 Arg1 expression through the CREB pathway.

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

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

498

499 **Discussion**

500 Inflammation appears to be the most important pathological process during
501 secondary injury after SCI. Residential microglia and hematogenous macrophages,
502 which are the main inflammatory subpopulations, from surrounding tissues arrive at
503 the injury site within hours after the primary injury [14, 34]. The inflammatory
504 response after SCI is thought as a ‘double-edged sword’, with both neuroprotective
505 and neurotoxic properties based on the polarized states of microglia/macrophages [10,
506 23, 44]. In the first few days after injury, iNOS and pro-inflammatory cytokines such

507 as TNF α , IL-1 β , and IL-6 are upregulated by microglia/macrophages in SCI-induced
508 aseptic conditions [27, 32]. These proinflammatory cytokines and other mediators
509 including HMBG1, which are released from dead cells, activating
510 microglia/macrophages through Toll-like receptors (TLRs), result in NF- κ B-mediated
511 production of proinflammatory cytokines and iNOS activation, the hallmarks of M1
512 macrophage and microglia activation during early stages of SCI [13, 23, 44]. The
513 reports of M2 polarization in SCI are controversial. In the contused spinal cord of
514 mouse, most microglia/macrophages are M1 cells, with only a transient and small
515 number showing M2 polarization. The expression of one of the classic M2 markers,
516 Arg1, was transient and returned to normal levels by 14 days post-injury [23]. A
517 recent study reported accumulation of two distinct subsets of macrophages with
518 different kinetics. The Ly6c^{hi}Cx3cr1^{lo} macrophages, which express characteristic M1
519 markers (IL-1 β and TNF- α), are present at 1 dpi and peaked at 3 dpi at the injury site
520 after SCI. Subsequently, Ly6c^{lo}Cx3cr1^{hi} macrophages, which express characteristic
521 M2 surface markers (Dectin 1, IL-4R α and CD206), infiltrated beginning at 3 dpi and
522 peaked at 7 dpi at the injury site after spinal cord contused injury [44]. Our results
523 also showed that both mRNA and protein levels of Arg1 and iNOS are upregulated in
524 early stages after SCI in AR^{+/+} mice. However, mRNA and protein levels of Arg1 and
525 iNOS were expressed with diverging patterns; Arg1 was upregulated, while iNOS was
526 downregulated during early stages after SCI in AR-deficient mice. The M2-type
527 microglia/macrophages were dominant at the injured spinal cord in AR^{-/-} mice,
528 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-1 β , 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 I κ B- α , IKK α/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 μ M) 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- κ B

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 activate the redox-sensitive transcriptional
554 factors NF- κ B 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- κ B 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

573 promotes activation of M2-associated genes with repressing M1 activation [1, 16, 43].
574 CREB induces C/EBP β expression, which specifically regulates M2-associated genes
575 [43]. Both STAT6 and C/EBP β have recently been shown to be essential for Arg1
576 expression in macrophages, but in a stimulus-specific manner due to the selective use
577 of a distinct *cis*-acting element in the *Arg1* promoter [16]. C/EBP β is responsible for
578 Arg1 expression in response to TLR ligands, whereas STAT6 regulates Arg1
579 expression in response to IL-4 and/or IL-13 stimulation [16, 45]. CREB also shows
580 anti-inflammatory cytokine expression in macrophage in response to LPS, which is
581 mediated by the p38 mitogen-activated protein kinase (MAPK) and mitogen- and
582 stress-activated kinase 1 (MSK1) and MSK2 [1]. As it was shown previously, the
583 M1-polarized microenvironment with high pro-inflammatory cytokines (e.g. IL-1 β
584 and IL-6) and TLR ligands (e.g. HMGB1) are dominant, which is preferred for
585 polarizing microglia/macrophages into the M1 type during early stages of SCI [13, 27,
586 32]. In this study, we found that AR deficiency could switch microglia/macrophages
587 polarized to the M2 type rather than the M1 type even at early stages of an
588 M1-polarized microenvironment, such as in injured spinal cord. Thus, it is reasonable
589 that AR deficiency causes accumulation of HNE in microglia, which stimulates
590 CREB to regulate the expression of characteristic M2 genes with the repression of
591 M1-associated genes (Fig. 6 b).

592 AR can be detected in a number of human and animal tissues including various
593 regions of the eyes, testis, liver, placenta, ovary, kidney, erythrocyte, cardiac and
594 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

617 neural and non-neural cells.

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

628

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634

635 *Conflict of interest*

636 The authors declare that they have no conflict of interest.

637

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

661 with statistical analysis in (c) in WT and AR^{-/-} mice. Bar = 500 μm. d, Representative
662 figures showing the amount of iNOS (green)- and F4/80 (red)-positive cells in the
663 sections of injured spinal cord in WT and AR^{-/-} mice. Boxed area in each panel was
664 enlarged at corner of each panel. Bar = 200 μm. e, The representative figures showing
665 that the amount of Arg1 (green)- and Iba1 (red)-positive cells in the sections of
666 injured spinal cord in WT and AR^{-/-} mice. Boxed area in each panel was enlarged at
667 corner of each panel. Bar = 200 μm. iNOS= inducible nitric oxide synthase; Arg
668 1=Arginase 1.

669

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

678

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

683 Time-courses of NF- κ B, CREB, and p-CREB protein levels after SCI in AR^{-/-} mice. e,
684 NF- κ B 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 μ m. b,
690 Time-course changes of density of fluorescence of HNE in BV2 microglial cells under
691 20- μ M Fidarestat stimulation *in vitro*. * p < 0.05. c, analysis of the protein levels of
692 Arg1, iNOS, pCREB, CREB, pNF- κ B, and NF- κ B 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- κ B, and NF- κ B 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- κ B 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

705 genes expression.

706

707

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