Supplementary Information for

A dual-effect approach to the treatment of multiple sclerosis: both immunomodulation and direct remyelination

Yuan Zhang[1,2†], Xing Li[1,2†], Bogoljub Ciric[1], Mark T. Curtis[3], Wan-Jun Chen[4], Abdolmohamad Rostami[1]*, and Guang-Xian Zhang[1]*

[1] Department of Neurology, Thomas Jefferson University, Philadelphia, PA, USA

[2] Key Laboratory of the Ministry of Education for Medicinal Resources and Natural Pharmaceutical Chemistry, College of Life Sciences, Shaanxi Normal University, Xi'an, China

[3] Department of Pathology, Thomas Jefferson University, Philadelphia, PA, USA

[4] Mucosal Immunology Section, OPCB, NIDCR, NIH, Bethesda, MD, USA

[†] Equal contribution

[*] **Corresponding authors:**

Abdolmohamad Rostami, MD, PhD, Email: a.m.rostami@jefferson.edu. Tel: 215-955 8100 Fax: 219-955 1390.

Guang-Xian Zhang, MD, PhD, Email: Guang-xian.Zhang@jefferson.edu. Tel: 215-955 8935. Fax: 215-503 5848.

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Supplementary figure Figure S1 to S4

Materials and Methods

References

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Supplementary figure legend

Figure S1. Effects of UA treatment on different immune cells. Splenocytes of mice treated with UA at 25 mg/kg/d or PBS described in (Figure B) were harvested at day 30 p.i. Cells were stimulated with MOG_{35-55} (25 μg/ml) for 72 h, and analyzed by flow cytometry (A). Culture supernatants were analyzed for cytokine production by ELISA (B). (C) mRNA levels of T-bet, RORγt, GATA3, and Foxp3 from spleens of EAE mice treated with UA or PBS were analyzed by real-time PCR. Quantitative data refer to mean ± SD (n = 5 mice/group). *p < 0.05; **p < 0.01; ***p <0.001, compared to PBS-treated group, one-way ANOVA comparison with Tukey's multiple comparisons test. One representative of 3 independent experiments is shown.

Figure S2. UA inhibits Th1 and Th17 cell differentiation. Naïve $CD4^+$ T cells from C57BL/6 mice were isolated with Microbeads and activated with anti-CD3/anti-CD28 under Th1 (with IL-12 and anti-IL4) and Th17-polarizing condition (TGF-β1, IL-6, IL-1β, anti-IL4 and anti-IFN-γ), in the presence or absence of UA at various concentrations. (A) Intracellular levels of IFN-γ (for Th1) in differentiated $CD4^+$ T cells were determined by flow cytometry. (B) The percentage of Th1 cells in $CD4^+$ T cells was analyzed by intracellular IFN-γ secretion. (C) Intracellular levels of IL-17A (for Th17) in differentiated $CD4^+$ T cells were determined by flow cytometry. (D) Percentage of Th17 cells was analyzed by intracellular staining of IL-17. Dot scatter plot figures presented are representative of three independent experiments. **p < 0.01, ***p < 0.001, compared to control group, one-way ANOVA with Tukey's multiple comparisons test.

Figure S3. Molecular docking of UA binding to PPARγ. (A) Low-energy binding conformations of UA bound to PPARγ generated by virtual ligand docking. The crystal structure of PPARγ (PBD code: 2Q5S) bound to the synthetic ligand's thiazolidinedions (TZDs) was used (https://www.rcsb.org/structure/2Q5S), and depicted in ribbon form. UA depicted as the ball-and-stick

model showing carbon (grey), hydrogen (white), and oxygen (red) atoms. Hydrogen bonds are represented in dotted lines. (B) UA binding pocket residues.

Figure S4. UA induced astroglial CNTF production. (A) Schematic of the astrocyte-specific shRNA targeting lentiviral vector. (B) Clinical scores of UA-(25 mg/kg/d) or PBS-treated EAE mice that were intracerebroventricularly (i.c.v.) injected with LV-GFAPpro-shPPARγ or LV-GFAPpro-shCtrl 3 days before immunization (day -3 p.i.) and at disease onset (day 12 p.i.). (C) Astrocytes infected with LV-GFAPpro-shPPARγ or LV-GFAPpro-shCtrl were purified using anti-ACSA-2 microbeads; GFP^+ astrocytes were then sorted by FACS. PPARγ and CNTF expression from GFP^+ astrocytes was detected by real-time PCR. (D) Double labeling of astrocytes for GFAP and CNTF was performed under indicated treatment: PBS, UA (10 μg/ml), or UA+GW-9662 (PPARγ specific antagonists, 10 nM). Scale bar = 50 μm. Primary mouse astrocytes isolated from 2-3 day-old pups were cultured in glass slide chambers. One of five representative experiments is shown. (E) Quantitative analysis was performed for numbers of $GFAP^+CNTF^+$ cells. Data are shown as mean values ± SD (n = 5-6 each group) and are representative of three experiments. Groups designated by the same letter are not significantly different, while those with different letters are significantly different ($p < 0.05$-0.001), one-way ANOVA with Tukey's multiple comparisons test.

Figure S1

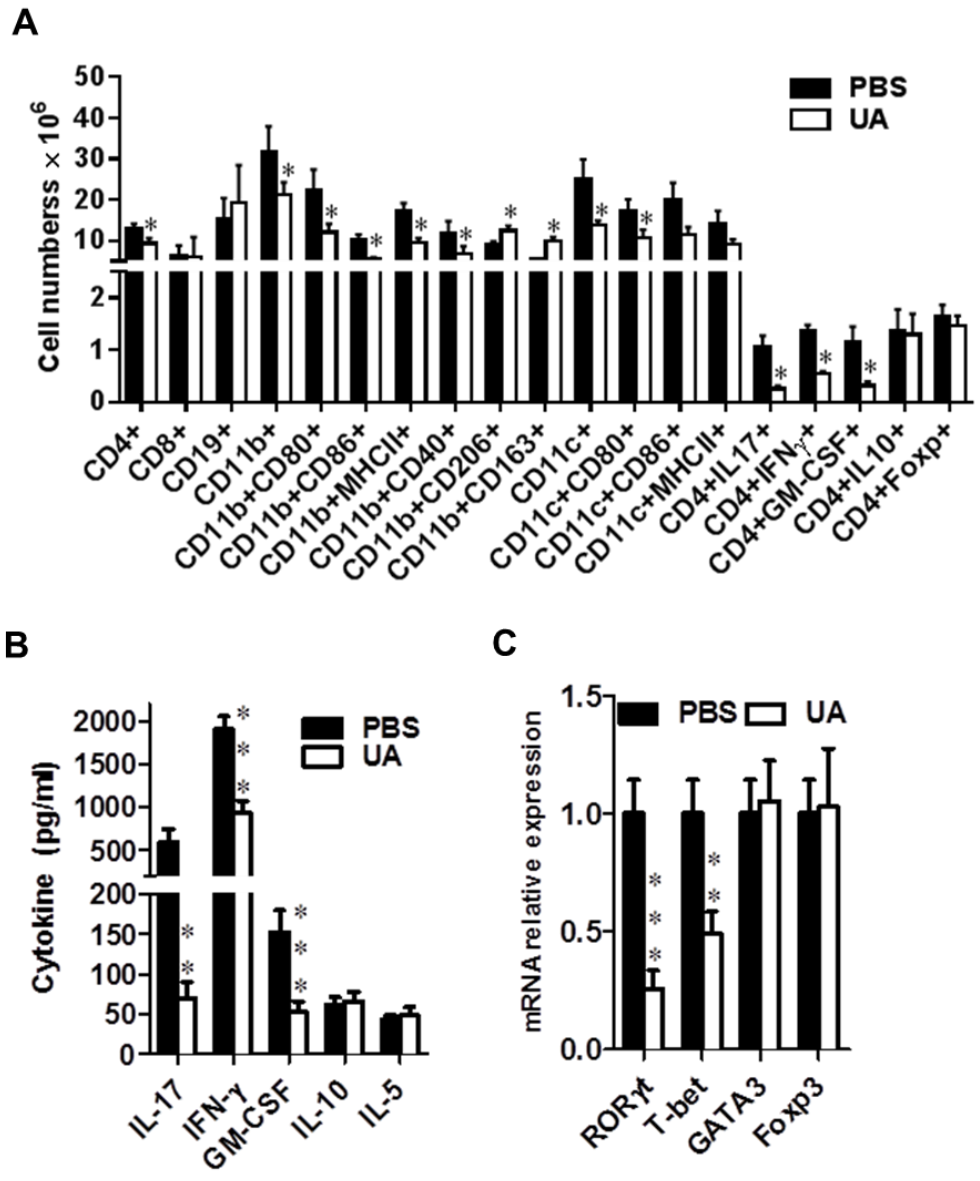
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Figure S2

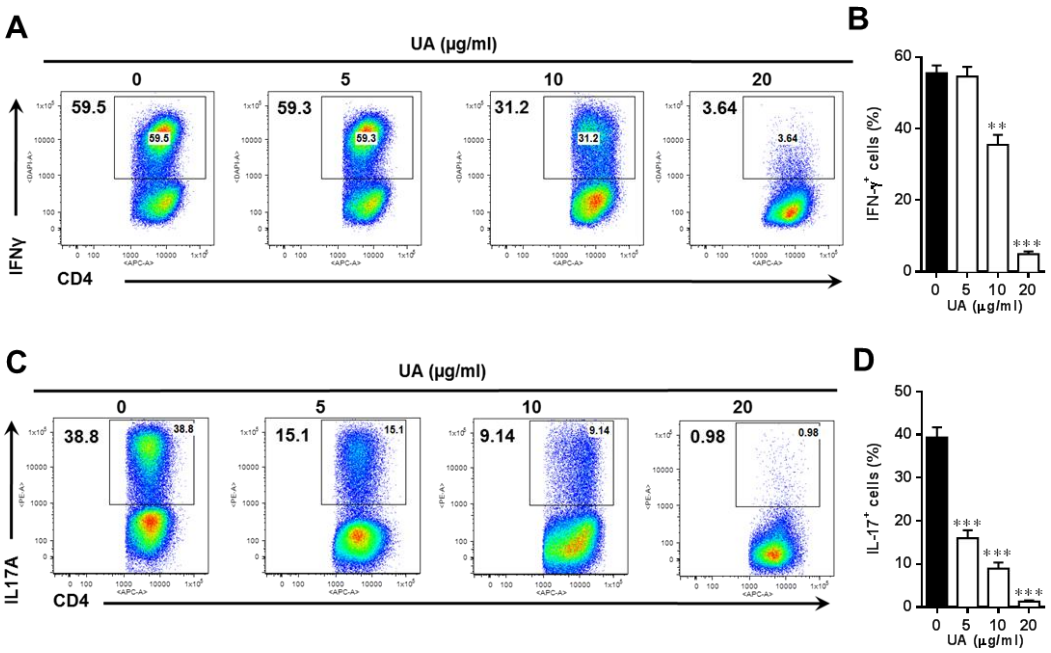


Figure S3

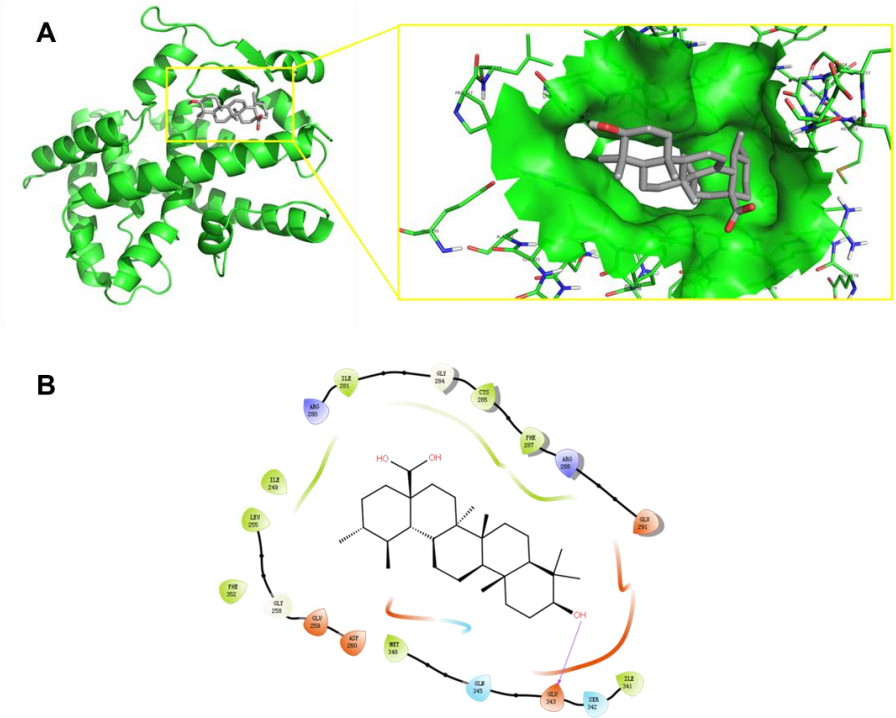
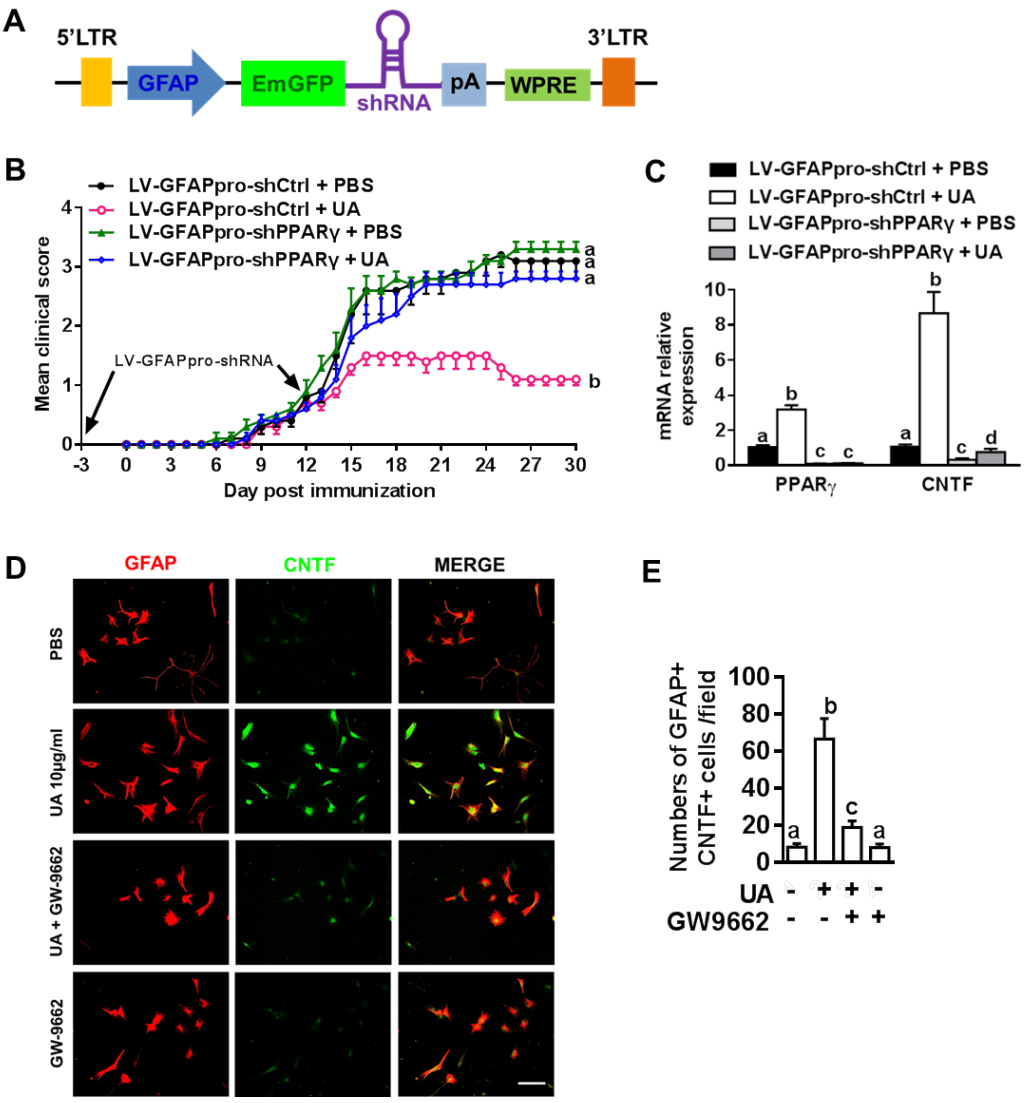


Figure S4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LV-GFAPpro-shPPARγ + UA
Mean clinical score
LV-GFAPpro-shRNA
Day post immunization
C
LV-GFAPpro-shCtrl + PBS
LV-GFAPpro-shCtrl + UA
LV-GFAPpro-shPPARγ + PBS
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mRNA relative expression
PPARγ
CNTF
D
GFAP
CNTF
MERGE
PBS
UA 10μg/ml
UA + GW-9662
GW-9662
E
Numbers of GFAP+ CNTF+ cells /field
UA
GW9662

Materials and Methods

EAE induction and UA treatment

Female C57BL/6J mice (8-10 weeks of age) and PPARγ$^{+/-}$ mice (C57BL/6J background) were purchased from Jackson Laboratory (Bar Harbor, ME). Age-matched PPARγ$^{+/+}$ (wild-type) and PPARγ$^{+/-}$ offspring were used in the experiments. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and were carried out in accordance with the approved institutional guidelines and regulations. Mice were immunized at 2 sites on the back with 200 μg of myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}) (Genescript, Piscataway, NJ) in 200 μl of emulsion containing 50% complete Freund's adjuvant with 5 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Difco, Lawrence, KS). All mice were intraperitoneally (i.p.) injected with 200 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO) in PBS on days 0 and 2 p.i. Clinical EAE was scored daily in a blind manner, according to a 0-5 scale as described previously (1): 0, no clinical signs; 0.5, stiff tail; 1, limp tail; 1.5, limp tail and waddling gait; 2, paralysis of one limb; 2.5, paralysis of one limb and weakness of another limb; 3, complete paralysis of both hind limbs; 4, moribund; and 5, death. EAE mice were randomly enrolled in the following treatment groups: 1) Sham-treated control group: EAE mice were gavaged with PBS; 2) UA-treated group: UA (Sigma-Aldrich) at different doses (5, 12.5, 25, 50 mg/kg/d) were orally gavaged daily at onset (day 11 p.i.), peak (day 18 p.i.), or chronic stage (day 60 p.i.) of EAE.

Histological analysis

Mice were sacrificed at different stages of the disease, before (day 10 or 18 p.i.) or after treatment (day 30 or 120 p.i.), and transcardially perfused with PBS. Lumbar spinal cords or brains were harvested for pathological assessment and spleen was isolated for immunological assessment. CNS tissues were cut into 7 μm sections, fixed with 4% paraformaldehyde, and stained with hematoxylin

and eosin (H&E) for assessment of inflammation, and with Luxol fast blue (LFB) for demyelination. Slides were assessed and scored in a blinded fashion for inflammation (1): 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, abundant perivascular cuffing with extension into the adjacent tissue. For demyelination quantification, total white matter was manually outlined, and area (%) of demyelination was calculated using Image-Pro Plus software.

Preparation of infiltrating MNCs from the CNS

Spleen was mechanically dissociated through a 70 μm cell strainer (Falcon, Tewksbury, MA) and incubated with red blood cell lysis buffer (Miltenyi) ~2 min. Harvested cells were washed with cold PBS before *in vitro* stimulation. To acquire CNS cells, spinal cords and brains were mechanically dissociated and incubated with Liberase (Roche, Nutley, NJ) for 30 min, passed through a 70 μm cell strainer and washed with cold PBS. Cells were then fractionated on a 70/30% Percoll (Sigma-Aldrich) gradient by centrifugation at 2000 rpm for 20 min and MNCs were collected from the interface and washed with PBS.

Cytokine measurement by ELISA

Splenocytes at 1.0×10^6 cells/ml were cultured in triplicates in RPMI 1640 supplemented with 10% FBS in 24-well plates and stimulated with 25 μg/ml MOG_{35-55} for 72 h. Supernatants were collected and assayed for IFN-γ, IL-17, GM-CSF, IL-5 and IL-10 by ELISA Kits (R&D Systems, Minneapolis, MN).

Flow cytometry

For surface-marker staining, cells were incubated with fluorochrome-conjugated Abs to CD4, CD8, CD19, CD11b, CD11c, CD80, CD86, MHC II, CD40, CD206, and CD163 (BD Biosciences, San Jose, CA) at the recommended dilution or isotype control Abs for 30 min on ice. To analyze

MOG-specific Th cells, CNS-infiltrating MNCs were stimulated with 25 μg/ml MOG peptide overnight, followed by stimulation with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of GolgiPlug for 4 h. Cells were surface-stained with mAbs against CD4 and CD8. Cells were then washed, fixed, and permeabilized with Fix & Perm Medium (Invitrogen), and intracellular cytokines were stained with Abs against IL-17, IFN-γ, IL-10, or GM-CSF (BD Biosciences). Foxp3 staining was carried out using a commercial kit, according to the manufacturer's instructions (eBioscience, San Diego, CA). Flow cytometry analysis was performed on FACSAria (BD Biosciences, San Jose, CA) and data were analyzed with FlowJo software (Treestar, Ashland, OR).

Molecular docking

The crystal structure of PPARγ (PBD code: 2Q5S) bound to the synthetic ligand's thiazolidinedions (TZDs) was used (https://www.rcsb.org/structure/2Q5S). Docking was performed by using ICM 3.8.1 modeling software on an Intel i7 4960 processor (MolSoft LLC, San Diego, CA) as described previously (2).

Cuprizone-induced demyelination and UA treatment

For the cuprizone model, 8-week-old male C57BL/6 mice were fed the standard rodent diet containing 0.2% copper chelator cuprizone, which causes CNS demyelination. For induced remyelination experiments, mice were fed cuprizone for 6 weeks to achieve complete demyelination of the corpus callosum, after which cuprizone was withdrawn. UA (25 mg/kg) or PBS was orally gavaged daily for up to 2 weeks to induce remyelination. Mice were anesthetized and perfused with 4% PFA; brains were removed for histopathological, immunohistochemistry, and electron microscopy analyses.

Organotypic slice cultures

Organotypic cultures were prepared and analyzed as described previously (3). Briefly, postnatal day 1-2 mouse pups were decapitated and their brains were placed in ice-cold HBSS. The cerebella and brainstems were cut into 300 μm sagittal slices using a McIlwain tissue chopper (Stoelting Co., Wood Dale, IL). Slices were randomly distributed between conditions and 3-4 slices were placed on each Millicell® -CM organotypic culture insert (Millipore, Billerica, MA). Slices were cultured using an interface method with 1 mL of medium per 35 mm well. For the first 2 days, slices were grown in serum-based medium (50% MEM, 25% HBSS, 25% heat-inactivated horse serum and supplemented with 2 mM Glutamax, 28 mM D-glucose, 100 U/mL penicillin/streptomycin, and 25 mM HEPES) at 37°C and 5% CO_2. Slices were transferred into fresh media every 2-3 days. Slices were left untreated for the first 6 days to allow clearance of debris and myelination to occur. After 6 days of recovery, demyelination was induced by the addition of 0.5 mg/ml lysophosphatidylcholine (LPC, Sigma) into the medium for 16-18 h, after which the slices were transferred back into normal medium ± 10 μg/ml UA. Cultures were maintained for a further 2 or 14 days and then processed for immunohistochemistry or RNA extraction. Culture supernatants were collected before immunostaining to measure concentrations of CNTF using a Mouse CNTF ELISA Kit (LifeSpan, Seattle, WA) following the manufacturer's protocol.

Isolation of primary mouse astrocytes and OPCs

Primary astrocytes and oligodendrocyte progenitor cells (OPCs) were isolated from newborn mouse brain (P3), by dissociation with Neural Tissue Dissociation Kit (Miltenyi Biotech Inc.) and purification with either anti-ASCA-2, or anti-A2B5 microbeads (Mitenyi Biotech Inc.), respectively. Astrocytes were cultured in DMEM/10% FBS cell culture medium, and OPCs in DMEM/F12 supplemented with 2% B27, 2 mM Glutamax, 20 ng/ml bFGF and 20 ng/ml PDGF-AA (Invitrogen). For differentiation assay, proliferation medium for OPCs was replaced by DMEM/F12 with 2% B27, 2 mM Glutamax, 1% N2, and 20 ng/ml T3. Cells were treated with vehicle (control) or UA (1, 5, or 10

μg/ml), and medium was changed every two days. OPCs changed morphology and developed oligodendrocyte markers over 1 to 2 weeks, as determined by immunocytochemistry staining (MBP, mature oligodendrocyte).

Electron microscopy

Mice were deeply anesthetized and perfused with 4% PFA, 1.5% glutaraldehyde and 1 mM $CaCl_2$ in 0.1 M cacodylate buffer. A section of ventral spinal cords was harvested and fixed in the same solution at 4°C for 24 h. Samples were washed, post-fixed with 1% OsO4 in 0.1 M PBS (pH 7.4) for 2 h at room temperature, and subsequently dehydrated in graded ethanol series. Embedding was performed in TAAB resin. Sections, 1.0 μm thick, were cut, stained in toluidine blue (1%), and examined by light microscopy (E800, Nikon) for general histological assessment. Ultrathin sections (60-80 nm) were cut, viewed and photographed with a HT7700 (Hitachi) transmission electron microscope operated at 120 kV. Images were analyzed in Image-Pro for thickness of myelin sheath and g-ratio.

Immunofluorescence

For immunohistochemistry, spinal cord or brain tissues were fixed using 4% paraformaldehyde for 1 day and then cryo-protected using a 30% sucrose solution for 3 days. Fixed tissues were embedded in OCT compound (Tissue-Tek, Sakura Finetek, Japan) for frozen sections and then sectioned coronally at 12 μm thickness. Transverse sections of brain and spinal cord were cut, and immunohistochemistry was performed using different Abs following established procedures. Organotypic slice cultures were fixed in ice-cold 4% paraformaldehyde for 5 min followed by 20% methanol for 3 min. Slices were then washed in PBS for 5 min and transferred to a PBS solution containing 10% bovine serum albumin (BSA), 5% heat inactivated horse serum, and 0.25% Triton X-100, and blocked and permeabilized for 24 h at 4°C. Primary and secondary Ab incubations were

conducted overnight at 4^{o}C, after which the slices were washed twice for 1 h in PBS and then mounted. Immunofluorescence controls were routinely performed with slides in which primary Abs were not included.

For immunocytochemical staining, astrocytes or OPCs maintained in growth or differentiation medium were fixed with 4% paraformaldehyde for 30 min at room temperature and washed twice with PBS. Cells were permeabilized with 0.3% Triton X-100 (in PBS) for 5 min at room temperature and washed twice with PBS. Sections were incubated with 10% goat serum in PBS for 30-60 min; primary Abs were then added and incubated at 4°C overnight. Primary Abs were washed out with PBS 3 times after overnight incubation. Sections were then incubated with species-specific secondary Abs for 60 min at room temperature, followed by washing with PBS 3 times. Immunofluorescence controls were routinely prepared by omitting primary Abs. Nuclei were stained with DAPI. Slides were covered with mounting medium (Vector Laboratories, Burlingame, CA, USA).

Primary Abs used for these studies were specific for: myelin basic protein (MBP, Abcam), neurofilament (NFH, Abcam), glial fibrillary acid protein (GFAP, Abcam), CD45 (Abcam), A2B5 (Millipore), adenomatous polyposis coli/CC1 (Millipore), and MAP2a (Abcam). Appropriate fluorescent secondary Abs were used (Alexa Fluor, Invitrogen).

Image Analysis

Images were captured by fluorescent microscopy (Nikon Eclipse E600; Nikon, Melville, NY) or confocal microscopy (Zeiss LSM 510; Carl Zeiss, Thornwood, NY). Approximately 8-10 images were captured per slice to cover most of the total area of the slice (excluding the edges), thus removing any bias or variations in image acquisition. Five slices were quantified per treatment/control and the experiment was repeated three times using cultures from different mice. Image acquisition settings were kept the same across different treatments. Myelinated axons were quantified by confocal microscopy as described previously (4). Stacks of images of myelin (MBP^{+}) and neurofilament

(NFH^+) immune labeling were obtained at 1 μm intervals in white matter areas at ×40 magnification. Percentage myelinated area was measured by extracting a mask image representing MBP^+NFH^+ colocalization from each layer of a confocal stack and carrying out an 'extended depth of field' projection of these mask images to form a single image representing the total myelinated area throughout the stack, the value of which was obtained using ImageJ (NIH) and ImagePro (Media Cybernetics). Total myelinated area was then divided by the NFH^+ area measured in that field, multiplied by 100. The area of NFH, MBP, or GFAP immunostaining per field was determined using ImagePro software. The numbers of CD45, A2B5, or CC1 positive cells were counted in a blinded fashion either from representative ×20 or ×40 objective images or a series of images derived from Z-stack imaging.

Transient transfection and luciferase reporter assay

Astrocytes were plated at a density of 5×10^4 per well in 24-well plates and allowed to adhere overnight. Cells were then transfected with 32 ng of PPRE X3-TKluc (a kind gift from Bruce Spiegelman, Addgene plasmid no. 1015) (5) and 3.2 ng of renilla luciferase plasmid per well (Promega, Madison, WI) using 0.8 μl of lipofectamine 2000 transfection reagent for each well (Invitrogen, Grand Island, NY). After 24 h of transfection, cells were stimulated with different concentrations of UA or PPAR-specific antagonists for 6 h, and activity of firefly and Renilla luciferase was monitored in cell lysates by Dual-Glo® Luciferase Assay kit (Promega) according to the manufacturer's instructions. Luciferase activity of each sample was normalized to the level of renilla activity. Data are represented as mean fold changes in treated cells as compared to control cells.

Cloning of cAMP response element-binding protein (CREB) promoter and site-directed mutagenesis

Mouse genomic DNA was extracted from mouse tails using DNeasy™ Tissue Kit following the

manufacturer's instructions (Qiagen, Valencia, CA). A 1704-bp (from (−1460 to +244) of murine CREB promoter was amplified from mouse genomic DNA by PCR with specific primers to CREB gene according to a previous study (6). PCR products were gel-purified, digested, and sub-cloned into the pGL4.20 firefly luciferase vector (Promega, Madison, WI). Sequences of cloned promoter regions were confirmed by DNA sequencing. Site-directed mutagenesis was done by using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene; Santa Clara, CA). Two primers in opposite orientation were used to mutate PPRE consensus sequence AGGTCAAAGGACA into ATTTTAAAGGACA.

Construction of lentivirus-delivered CREB-shRNA and in vitro infection

To knock down CREB expression, siRNA target design tool from http://sirna.wi.mit.edu was used to design shRNA targeting CREB. The vectors expressing CREB-shRNA were synthesized in Integrated DNA Technologies (Coralville, Iowa), which consisted of a BamHI site, a 19-nucleotide sense sequence, a loop sequence, a 19-nucleotide antisense sequence, a RNA polymerase site, and an EcoRI site. The forward and reverse strand oligo sequences are as follows (the CREB sense and antisense sequences are siCREB Forward:

5'- *GATCC*gggcagtacattgccatta***CTTCCTGTCAGAtaatggcaatgtactgcccTTTTTG*** -3'; siCREB Reverse:

5'-AATTC**AAAAAgggcagtacattgccattaTCTGACAGGAAGtaatggcaatgtactgccc**G-3'.

(Set 2: 5'- *GATCC*ctgccacaaatcagattaa***CTTCCTGTCAGAttaatctgatttgtggcagTTTTTG*** -3'; 5'-AATTC**AAAAActgccacaaatcagattaaTCTGACAGGAAGttaatctgatttgtggcag**G-3'

Set 3: 5'- *GATCC*gccacaaccagaaagacaa***CTTCCTGTCAGAttgtctttctggttgtggcTTTTTG*** -3'; 5'-AATTC**AAAAAgccacaaccagaaagacaaTCTGACAGGAAGttgtctttctggttgtggc**G-3').

The following complementary oligonucleotide was used as a negative control oligo sequence: Ctrl forward: 5'-GATCC**TTCTCCGAACGTGTCACGT*TTCAAGAGA*ACGTGACACGTTCGGAGA**

ATTTTTG-3'; Ctrl reverse: 5'-AATTC**AAAAATTCTCCGAACGTGTCACGT*TCTCTTGAA*AC**

GTGACACGTTCGGAGAAG-3'. The forward and reverse strand oligo sequences were annealed to create double stranded oligonucleotides, which were then cloned into the pSIH1-H1 vector without GFP. Viruses were produced according to the user's manual (SBI, CA). For cell infection, 5×10^5 IU/ml virus and 8 μg/ml polybrene (Millipore, MA) were incubated with mouse primary astrocytes. After overnight incubation, the medium was replaced by fresh medium, and cultured for future use.

Western Blot Analysis

Cells cultured under different treatments were washed with PBS and lysed by cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1× proteinase inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations of all samples were determined using the Pierce BCA Protein Assay Kit (Thermo, Rockford, IL). Protein samples (equal amount/lane) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane. The transformed membrane was blocked for 2 h followed by incubation with primary antibodies at 4°C overnight. The membrane was washed three times with TBST buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 5 min each and then incubated with 1:200 diluted anti-rabbit or mouse IgG-horseradish peroxidise (HRP) (Thermo Scientific, Rockford, IL) at room temperature for 1 h. The protein band was detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Real-time RT-PCR

Total RNA was extracted from spinal cords using RNeasy® Plus Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was conducted using QuantiTect® Reverse Transcription Kit (Qiagen,). Real-time PCR was performed using the Custom RT² Profiler PCR Array according to the manufacturer's instructions (Qiagen), and detection was performed using the ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City,

CA). All data were normalized to an average of five housekeeping genes Gusb, Hprt, Hsp90ab1, Gapdh and Actb. Qiagen's online web analysis tool was utilized and gene relative expression was calculated by log2 of $-\Delta\Delta Ct$ values from triplicate of PCR. More than two fold changes ($log2 < -1$ or $log2 > 1$) were considered significant between groups.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA). Data are presented as mean ± SD. When comparing multiple groups, data were analyzed by analysis of variance (ANOVA) with Tukey's multiple comparisons test. A significance criterion of $p < 0.05$ was used for all statistical analysis.

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