Inhibition of the RNA Regulator HuR mitigates spinal cord injury by potently suppressing

post-injury neuroinflammation

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Abstract

Background

Neuroinflammation plays a significant role in promoting secondary tissue injury after spinal cord trauma. Within minutes after spinal cord injury (SCI), microglia and astrocytes become activated and produce inflammatory mediators such as TNF-α, IL-6, iNOS and COX-2 which induce tissue injury through cytotoxicity, vascular hyperpermeability, and secondary ischemia. The inflammatory cascade is amplified by chemokines such as CCL2 and CXCL1 that promote recruitment of peripheral inflammatory cells into the injured spinal cord. HuR is a key post-transcriptional RNA regulator that controls glial expression of many pro-inflammatory factors by binding to adenylate- and uridylate-rich elements in 3' untranslated regions of the mRNA. SRI-42127 is a small molecule inhibitor that blocks HuR nucleocytoplasmic translocation, a process critical for its regulatory function. The goal of this study was to assess the potential of SRI-42127 for suppressing neuroinflammation after SCI and improving functional outcome.

Methods

Adult female mice underwent a contusion injury at the T10 level. SRI-42127 or vehicle was administered intraperitoneally starting 1 h after injury and up to 5 days. Locomotor function was assessed by open field testing, balance beam and rotarod. Immunohistochemistry was used to assess lesion size, neuronal loss, myelin sparing, microglial activation and HuR localization. Molecular analyses of spinal cord and peripheral tissues for expression of inflammatory mediators included qPCR, immunohistochemistry, ELISA, or western blot. Post-SCI pain was assessed by the mouse grimace scale.

Results

SRI-42127 significantly attenuated loss of locomotor function and post-SCI pain. Histologic correlates to these beneficial effects included reduced lesion size, neuronal loss, and an increase in myelin sparing. There was reduced microglial activation at the epicenter with concomitant

attenuation of HuR nucleocytoplasmic translocation. Molecular analysis revealed a striking

reduction of pro-inflammatory mediators at the epicenter including IL-6, MMP-12, IL-1β, TNF-α,

iNOS, COX-2, and chemokines CCL2, CXCL1, and CXCL2. Suppression of inflammatory

responses extended peripherally including serum, liver, and spleen.

Conclusion

Targeting HuR after SCI is a viable therapeutic approach for suppressing neuroinflammatory

responses after tissue injury and improving functional outcome.

KEYWORDS

SRI-42127, Spinal cord injury, HuR translocation, neuroinflammation, RNA regulation

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Background

Traumatic spinal cord injury (SCI) is a debilitating neurological injury affecting more the 250,000 individuals per year globally. The injury often results in in permanent disability and shortened lifespan. Additionally, there are life-changing socioeconomic consequences for both the individual and their caregivers, particularly younger people [1, 2]. Treatment in the acute phase of injury is mainly supportive with limited treatment options for neuroprotection to improve recovery and reduce long term disability.

The physical trauma of SCI triggers a robust inflammatory response initiated within minutes by resident microglia and astrocytes in the vicinity of tissue injury. This activation leads to production of pro-inflammatory mediators including IL-1β, IL-6, TNF-α, MMPs, COX-2 and nitric oxide (NO) via iNOS that exert direct toxic effects on neurons, oligodendroglia, and endothelial cells, often leading to apoptosis and peaking within the initial 24 h post injury [3, 4]. These mediators increase vascular permeability by disrupting the blood-spinal cord barrier (BSCB), leading to edema, secondary ischemia, and hemorrhage [5, 6]. Disruption of the BSCB, in combination with glial secretion of chemokines such as CCL2 and CXCL1, further intensifies the inflammatory cascade by facilitating recruitment and infiltration of peripheral neutrophils and monocytes [3].

An integral regulatory component to this inflammatory cascade, beginning with activated microglia and astrocytes, is at the posttranscriptional level, where AU-rich elements (ARE) in the 3' untranslated region (UTR) of pro-inflammatory mRNAs govern their expression through modulation of mRNA stability and translational efficiency [4]. HuR, a major cellular RNA-binding protein (RBP) in glial cells, binds to these AREs and positively regulates the stability and translational efficiency leading to increased expression [4, 7-10]. Primarily located in the nucleus, HuR translocates to the cytoplasm upon activation where it transports and stabilizes bound

mRNAs and facilitates their localization to polysomes for translation. In microglia and/or

astrocytes, HuR translocation is triggered in different inflammatory conditions, including SCI [9,

11], amyotrophic lateral sclerosis [8], lipopolysaccharide (LPS) stimulation [10, 12], hypoxia [10],

and glioblastoma [7]. Knockdown or deletion of HuR in microglia or astrocytes significantly

diminishes the expression of many key inflammatory mediators, including pro-inflammatory

cytokines, chemokines, and iNOS, associated with secondary tissue injury in SCI [7-9]. Our group

has recently developed a small molecule, SRI-42127, that blocks HuR dimerization, a process

critical for cytoplasmic shuttling and RNA binding [13-15]. SRI-42127 inhibits translocation of HuR

in microglial cells and astrocytes after activation with LPS and suppresses the production of pro-

inflammatory mediators [10]. In our prior work, SRI-42127 potently attenuates development of

neuropathic pain in a peripheral nerve injury model, a process that is driven by neuroinflammatory

responses in the spinal cord [16, 17]. With this background, we hypothesized here that inhibition

of HuR with SRI-42127 would suppress neuroinflammatory responses after acute SCI, reduce

secondary tissue injury, and mitigate loss of motor function.

Methods

Animals and contusion model of spinal cord injury

All animal procedures were approved by the UAB Institutional Animal Care and Use Committee

and were carried out in accordance with relevant guidelines and regulations of the National

Research Council Guide for the Care and Use of Laboratory Animals. Female C57/Bl6 mice

between 10–14 weeks of age were anesthetized under isoflurane and a laminectomy was

performed to remove the dorsal aspect of the T10 vertebrae. The animal was transferred to a

spinal stereotaxic frame and clamps were attached to the T9 and T11 vertebral spines to secure

the vertebral column. Using a 0.8-mm diameter tip, a single impact contusion of 50 kdyn was

delivered using the Infinite Horizon spinal cord injury device (Precision Systems &

Instrumentation, Lexington, KY). Sham control mice received a laminectomy only. Post-

operatively, animals received manual bladder evacuation twice daily to prevent urinary tract

infections. One dose of subcutaneous buprenorphine was administered prior to injury and every

12 h for 3 d post injury. For molecular studies, SRI-42127 (10 mg/kg) and vehicle were prepared

as previously described [16] and administered intraperitoneally every 2 h beginning 1 h after injury

for 4 doses. The cohort for behavioral studies received 4 doses daily, starting 1 h after injury, for

5 days.

RNA isolation and qPCR

The thoracic spinal column was harvested, and 2 mm sections were cut from the epicenter of

injury and adjacent rostral, caudal spinal column. Spinal cord was removed from these sections

and RNA was isolated using TRIzol reagent as per manufacturer's protocol (Thermo Fisher

Scientific). RNA was precipitated using isopropanol (Sigma) and glycogen (Thermo Fisher

Scientific) and concentrations were measured by Nanodrop (Thermo Scientific, Waltham, MA).

Complementary DNA was reverse transcribed from 2 μg of total RNA using a reverse transcription

kit (Thermo Fisher Scientific). Quantitative Real Time PCR (qRT-PCR) was conducted using the

ViiA7-Real-Time PCR system and primers and probes from Applied Biosystems as previously

described [10].

Western blot and ELISA

For western blotting, whole cells lysates were prepared using T-PER (Thermofisher) and

quantification was done with a BCA protein assay kit (Thermofisher). Fifty µg of protein per sample

were electrophoresed in 4%–20% mini-PROTEAN gel (Biorad) and transferred to a nitrocellulose

membrane. Blots were immunostained with a COX-2 antibody (Santa Cruz Biotechnology, USA

Inc) was used at a dilution of 1:1000. Each sample was analyzed in triplicate and quantified using

Image lab software (Bio-Rad Laboratories, USA Inc.). For ELISA of peripheral serum samples,

cytokines (IL-1β, IL-6, IL-10, TNF-α, CCL2, CXCL1) were measured using U-plex biomarker

Group 1 (mouse) Assays (K15069M, MSD). Analyses were done using a QuickPlex SQ 120

instrument (MSD) and DISCOVERY WORKBENCH® 4.0 software (MSD).

Immunohistochemistry

Mice were euthanized 8 h or 3 weeks and perfused with an ice-cold phosphate buffer saline (PBS)

solution for 2 min followed by 4% paraformaldehyde (PFA) solution for 5 min [9]. The entire spinal

column was then extracted and post-fixed in PFA for 24 h before being decalcified with 8%

hydrochloric acid and 10% ethanol in PBS. The spinal column was incubated in a PBS solution

with 10% sucrose for cryoprotection then transferred to a 30% sucrose in PBS solution for 48 h.

Two mm samples of the spinal column from epicenter and adjacent rostral (+ 2 mm) and caudal

tissues (- 2 mm) were preserved and cryo-molded in OCT. A cryostat was used to cut samples

into 30 µm serial sections cut transversely.

Sections were washed with PBS and blocked in 10% goat serum for 1 h. Samples were incubated

overnight with primary antibodies (GFAP, 1:500, Z0334, Dako, Carpinteria, CA), (Iba1, 1:500,

FUJIFILM Wako Chemicals U.S.A. Corporation), (NeuN, 1:400, ABN78, Sigma-Aldrich, USA

Ltd.), (HuR, 1:500, sc-5261, Santa Cruz Biotechnology, USA Inc). The following day, slide

sections were washed and incubated with Alexa fluorophore-conjugated secondary antibodies

(Goat anti mouse, A28175, Thermo Fisher Scientific and Goat anti rabbit Cy3, 111-165-144,

Jackson Immuno Research) for 2 h, mounted with antifade DAPI + mountant (ThermoFisher

Scientific P36966). For myelin staining, sections were incubated with FluoroMyelin Green for 20

minutes (1:300, Invitrogen).

Quantitative assessment of HuR localization was done using our previously published method

[10]. Fiji software was utilized for image analysis, and a minimal threshold was established in

order to quantify the mean fluorescence intensity (FI). FI was quantified and normalized with the

DAPI FI for the same region and expressed as arbitrary units (AU). Total lesion area was

determined based on the ratio of GFAP-stained area (which is concentrated on the border of the

injured region) to total spinal cord area. The total area of GFAP and myelin staining was measured

using the freehand selection tool and threshold functions in Fiji. Three biological replicates were

used for the Fiji analysis.

Analysis of microglial morphology

During the early stages of activation, microglia can become hyper-ramified with increase branch

complexity and soma enlargement [18, 19]. Fiji software (https://imagej.net/) was used to convert

all photomicrographs to binary and skeletonized images using AnalyzeSkeleton plugin

(https://imagej.net/plugins/analyze-skeleton/). To analyze the number of branches, the total

branch length, and the endpoints per microglia brightness and contrast are changed by updating

the Fiji lookup table, so pixel values are unchanged. This is followed by a despeckle step to

remove salt-and-pepper noise. Photomicrographs then converted to binary using threshold tool

followed by skeletonizing the image using the toolbar Skeletonize and AnalyzeSkeleton (2D/3D)

to collect data on the branch length. Branch length per cell was calculated by dividing summed

branch length by the number of microglia somas in the corresponding photomicrographs. Data

was obtained from 5007 microglial cells from vehicle control (n=3) and 4538 microglial cells from

SRI (n=3). The soma area was calculated using the Fiji multipoint area selection tool.

Open field test

Motor function of the hind limbs was scored at 1, 2 and 3 weeks after SCI using the open-field

basso mouse scale (BMS) in 10 min observation periods. BMS is a reliable measure and has

been widely used to evaluate motor functions after SCI in mice [20]. Briefly, each mouse was

separately placed in an open field (60 cm x 120 cm) and patterns of limb movement such as ankle

movement, plantar placing and stepping of paw, paw positions and trunk instability were recorded

over a 10 min time interval by a ceiling camera and analyzed by a computerized tracking system

EthoVision XT sofware (Noldus, USA). Prior to the testing day, baseline measurements were

obtained. Videos were carefully inspected by two reviewers (MAH and HAZ) blinded to the

experimental conditions. Hindlimb joint movement, coordination, and weight support were

evaluated using a rating scale from 0 points (no movement of any kind) to 9 points (normal

locomotion). EthoVision software was used to analyze recorded videos to automatically calculate

total distance traveled, mobility and velocity by each mouse within the arena.

Beam walking

The apparatus consists of a round horizontal beam of 100 cm long and 1.2 cm in diameter and

was elevated 50 cm above the ground. A hollow black escape box was attached to one end of

the beam. One fluorescent lamp (60W) was used to illuminate the beam from the start side. One

week prior to SCI, mice were thoroughly trained to traverse the beam from start point to end point

(black box). Baseline measurements were taken one day before the SCI. On the trial days after

SCI mice were placed at the start point and were allowed to walk for 2 min for 3 trials. The test

was recorded by video camera. A scoring was given to the mice as described elsewhere [21].

Briefly, the rating system employs values such as mouse retention, forward motion, and goal-

achieving on a beam. Mice received a high score for effectively using its hindlimbs to move around

the beam. In case of absence of hindlimb movements, mice received no points. Scoring was done

by an observer blinded to the identity of the mouse using recorded video weeks 1, 2, and 3 after

SCI. A mean value was calculated from the three trials for each mouse.

Rotarod

Motor coordination was assessed in weeks 1, 2, and 3 post SCI on an accelerating rotarod (San

Diego instruments CA, USA). Mice were trained one week before SCI by placing them on the

revolving rotor (4 rpm) and allowing them to acclimate for 2 mins. Baseline measures were

acquired one day prior to the contusion injury. On the day of testing day, the rod accelerated from

4 to 60 rpm over 2 mins and the latency to fall off was measured in 2 trials on each testing day,

allowing them 5 minutes of rest with in between each trial. The average time for the 2 trials was

used for statistical analysis to calculate a single score for each mouse.

Non-evoked Pain testing

Mice were placed into tabletop plexiglas cubicles (12 x 8 x 5.5 cm) with perforated metal floors

and clear walls. High-resolution (1920x1080) cameras (Sony Handycam model HDR-CX100)

were set up in front and behind the cubicles as described elsewhere [22]. After 15 min of

habituation, cameras were turned on to record for 15 mins. Recordings were taken prior to surgery

(baseline, BL) and at days 2 and 9 post SCI. Still images with clear visuals of the face were taken

once in each 2-minute period for each recording (8 images total) by an experimenter blinded to

the group assignment. Images were added to a PowerPoint file, randomized, and scored by two

experimenters blinded to the experimental conditions. Scores for action units were averaged

within each recording session. Action unit scores were averaged to calculate a mouse grimace

score (MGS).

Statistics

All statistical analyses were performed by Graphpad (GraphPad Software Inc.). The mean values

are shown on graphs along with error bars. Error bars indicate the standard deviation and

standard error as indicated. An unpaired Welch's t test was used for HuR localization, qPCR,

western blot, FI, and microglia counting. Two-way ANOVA with Tukey post hoc test was used for

CCR2. Data from all behavioral testing were analyzed using an unpaired Welch's t test. A p-value

less than 0.05 was considered statistically significant.

Results

SRI-42127 treatment improves functional recovery after SCI

Wild-type female mice were subjected to a mid-thoracic contusion injury and then treated with

SRI-42127 every 6 h for 5 days, starting 1 h after injury. To assess and quantitate motor activity

over a longer period of time, we used a computerized tracking system in an open field **(Fig. 1A-**

E). We first assessed locomotor function using the Basso mouse scale (BMS) at weeks 1, 2, and

3 post SCI **(Fig. 1A)**. There was a highly significant increase in BMS scores for SRI-treated mice

versus vehicle beginning at week 1 (4.2 ± 1.2 versus 0.2 ± 0.1; $P < 0.0001$). The separation of

BMS scores persisted and remained significant in weeks 2 and 3 with both groups showing some

recovery by week 3. Heat maps of activity within the field were generated (example shown in **Fig.**

1B) from which distance moved, mobility and velocity were calculated **(Fig. 1C, D, E)**. At week 1,

there was more than a two-fold increase in distance moved (3900 ± 200 versus 1800 ± 100 cm;

$P < 0.0001$), mobility (6.5 ± 0.7 versus 4.0 ± 0.5 percent; $P = 0.0029$), and velocity (6.8 ± 1.0

versus 3.0 ± 0.5 cm/sec; $P < 0.0001$). The differences between SRI- and vehicle-treated groups

remained significant throughout the recovery period for each parameter with both groups showing

improvement by week 3. We next assessed the animals with a novel beam walking test which

quantitates locomotor recovery using a scale of 0 to 7 **(Fig. 1F)** [21]. This test provides a more

comprehensive assessment of beam walking by measuring other functional components besides

latency to reach the other side. In both test groups, pre-injury baseline (BL) scores were 7. At

week 1, there was a significant increase in score in the SRI-treated group compared to vehicle

(3.3 ± 1.2 versus 0.6 ± 0.2; $P < 0.0001$). This difference persisted throughout the three-week

observation period, with both groups showing modest improvement by week 3 (5.0 ± 1.2 versus

2.0 ± 0.5; $P < 0.0001$). Next, we assessed rotarod performance **(Fig. 1G)** and found that SRI-

treated mice had a ~5-fold increase in mean latency to fall at week 1 compared to vehicle control

(6.8 ± 3.1 versus 1.3 ± 1; $P = 0.0012$). This difference remained significant throughout the recovery

period with both groups showing increases in latency by week 3. To exclude the possibility of a

drug effect on motor function, independent of SCI, we performed open field testing, BMS, and

beam walking on sham-injured mice given SRI-42127 and observed no difference compared to

the vehicle group over the 3-week testing period **(Supplementary Fig. 1)**. Since pain is a common

and debilitating sequelae of SCI, we assessed non-evoked pain using the mouse grimace scale

(MGS) **(Fig. 1H)**. At baseline, prior to SCI, the groups had similar MGS scores (0.31 ± 0.02 versus

0.29 ± 0.04). Following SCI, mice treated with SRI-42127 had significantly lower MGS scores

when compared to vehicle-treated mice (day 2: 1.56 ± 0.11 versus 1.02 ± 0.09, p < 0.01; day 9:

0.83 ± 0.08 versus 0.46 ± 0.05, p < 0.01). Taken together, SRI-42127 treatment after SCI

significantly attenuated motor function loss and pain.

SRI-42127 mitigates histopathology after SCI

At 3 weeks post SCI, spinal cords were harvested and tissue sections at the epicenter and

adjacent rostral and caudal levels to assess for histopathological changes. To identify the glial

scar border, we immunostained sections with GFAP **(Fig. 2A)**. Using these borders, a lesion size

was calculated and found to be reduced by 60% in SRI-treated mice versus vehicle (P = 0.003)

(Fig. 2B). Inspection of rostral and caudal levels revealed no evidence of glial scar

(Supplementary Fig. 2A). To assess for neuronal loss, sections were immunostained with NeuN

(Fig. 2C). In SRI-treated mice, there was a 7-fold increase in NeuN positive cells in the epicenter

(**Fig. 2D**; P= 0.003). Overall NeuN fluorescence intensity (FI) was significantly higher in SRI-

treated mice **(Fig. 2E)**. In rostral and caudal levels, there were no differences in NeuN FI in SRI-

and vehicle-treated mice **(Supplementary Fig. 2B)**. To assess white matter sparing at 3 weeks

in SRI-treated mice compared to vehicle controls (**Fig 2F, G**; *P* = 0.003). In rostral and caudal

post SCI, spinal cord sections were stained with fluoromyelin. There was a 4.5-fold increase in FI

levels, there was also a significant 40-50% increase in FI in SRI-treated mice (**Supplementary**

Fig. 2C).

SRI-42127 blocks nucleocytoplasmic translocation of HuR in microglia and attenuates microglial activation

A hallmark of HuR activation is its translocation from nucleus to the cytoplasm where it stabilizes and promotes the translational efficiency of pro-inflammatory mediators in microglial cells [4, 10]. Here we assessed HuR localization by immunohistochemistry in Iba1+ cells at 8 h post SCI. At the epicenter, there was prominent HuR translocation in vehicle-treated mice, as indicated by a merged signal with cytoplasmic Iba1 (**Fig. 3A**). This shift was not seen in uninjured rostral or caudal levels (**Supplementary Fig. 3**). In SRI-treated mice, this merged signal was not observed (**Fig. 3B**). A nuclear/cytoplasmic ratio of HuR FI was calculated and found to be more than 2-fold higher in SRI-treated mice (P = 0.008) consistent with nuclear retention of HuR (**Fig. 3C**). To assess microglial activation, we measured Iba1 FI for individual cells in the epicenter and found an overall reduction consistent with reduced activation (**Fig. 3D**). This was underscored by a reduction in total Iba1 FI at the epicenter in SRI-treated mice (**Fig. 3E**) since the overall Iba1+ cell count was unchanged (**Fig. 3F**). Iba1 FI in rostral and caudal segments showed no differences between SRI-treated and vehicle control mice (**Fig. 3E**). Interestingly, we did not see translocation of HuR in astrocytes in vehicle-treated mice at this early timepoint but did at 24 h (**Supplementary Fig. 4A versus 4B**). In summary, these findings indicate prominent HuR cytoplasmic translocation in microglia (and astrocytes) after SCI which is suppressed with SRI-42127 treatment.

SRI-42127 attenuates activation-associated microglia morphological changes

As another measure of activation, we assessed morphological changes of microglia at the epicenter 8 h after SCI. Using Fiji software, a skeleton analysis of microglia was done as previously described [23] (**Fig. 4A**). We observed a decrease in branch length (P = 0.009),

number of branches ($P < 0.0001$), and number of end points per microglia ($P < 0.0001$) in drug-

treated mice, consistent with reduced early-stage activation [24]. In addition to hyper-

ramification, we measured soma size as an indicator of reactive microglia and found a significant

reduction in SRI-treated mice (**Fig. 4B**; $P < 0.0001$). Taken together with results in Fig. 3, SRI-

42127 attenuates microglial activation after SCI.

SRI-42127 suppresses induction of proinflammatory mediators at the epicenter of SCI

At 8 h post-SCI, spinal cords were harvested and divided into 3 segments: injury epicenter, rostral

and caudal. RNA expression of proinflammatory mediators was assessed by qPCR. At the

epicenter, there was a large induction of *IL-6*, *IL-1β*, *TNF-α*, *CCL2*, *MMP12*, *CXCL1*, *CXCL2*, and

iNOS in the vehicle control compared to rostral and caudal segments (**Fig. 5A**). *COX2 and TGF-*

β1 mRNAs were not induced. The induction was most pronounced with *IL-6* (34-fold) and *CXCl2*

(39-fold) while the others ranged from 5- to 15-fold. SRI-42127 treatment suppressed the

expression of all pro-inflammatory mediators, most potently for *IL-6* (~13-fold) and *IL-1β* (~9-fold).

Other inflammatory mediators were suppressed by 3- to 7-fold. Interestingly, there was SRI-

induced suppression of some mRNAs at non-injured spinal cord segments, including *IL-1β*, *CCL2*,

MMP-12, and *CXCL1*. Based on our prior work showing that HuR positively regulates *CCR2* in

microglia [8], we assessed this mRNA after injury and found a nearly 3-fold attenuation at the

epicenter. SRI-42127 suppressed *CCR2* mRNA at the uninjured levels as well. The fold-change

was even larger as there was significantly higher expression of *CCR2* in non-injured levels for the

vehicle control. Since COX-2 is heavily regulated by HuR at the translational efficiency level in

addition to mRNA stability [25, 26], we assessed protein levels by western blot (**Fig. 5B**). We

found a 2-fold increase in COX-2 protein levels in vehicle-treated mice compared to uninjured

levels and this was attenuated by ~4-fold in SRI-treated animals.

To determine the impact of SRI-42127 on inflammatory cytokine induction at a later timepoint, we

assessed spinal cords after five days of SRI-42127 or vehicle treatment (**Supplementary Fig. 5**).

There was a sustained suppression of pro-inflammatory mediators, most potently for *MMP-12*

(~90-fold) and *CXCl2* (~30-fold). Others were suppressed by 3- to 9-fold. *TGF-β1*, on the other

hand, was greater in vehicle-treated mice by ~3-fold. No difference was seen with *IL-1β* while

CXCL1, *iNOS*, and *COX-2* trended toward suppression but did not reach significance due to

higher variability of mRNA levels. Taken together, treatment with SRI-42127 in the acute phase

of SCI broadly suppressed the induction of pro-inflammatory mediators at the level of injury.

SRI-42127 attenuates peripheral inflammatory responses after SCI

We next examined the effects of SRI-42127 on peripheral inflammatory responses at 8 h post

SCI (**Fig. 6**). We first looked at circulating levels of key inflammatory mediators and found a large

increase in IL-6 (5.8 vs. 6719 pg/ml) and CXCL1 (32.7 vs 3268) in vehicle-treated mice after SCI

compared to sham-injured mice (**Fig. 6A**). CCL2 also increased by 3-fold (77.2 vs 226 pg/ml).

With SRI-42127 treatment there was a significant attenuation of these inflammatory mediators:

IL-6 (~5-fold; $P < 0.0001$), CXCL1 (~3.5-fold; $P < 0.0001$) and CCL2 (~2.5-fold; $P = 0.003$). While

there was some increase in IL-10, TNF-α, and IL1-β in vehicle-treated mice after SCI compared

to sham-injured mice, SRI-42127 did not suppress this increase. To gain insight into potential

sources for these cytokines, we looked at liver and spleen, two organs that drive the influx of

inflammatory macrophages (**Fig. 6B**) [27, 28]. In liver tissue, we observed a significant induction

of *CXCL1* (~16-fold) and *IL-6* (9-fold) mRNA which was suppressed by ~16-fold and 11-fold,

respectively, with SRI-42127 treatment. For splenic tissue, there was an increase in *CCL2* (~16-

fold) and *IL-6* (~10-fold) mRNA expression which was suppressed by ~16-fold and ~8-fold,

respectively, with SRI-42127 treatment. For cytokines that were not elevated in serum, including

IL-1β, TNF-α, and IL-10, there was only minimal induction of TNF-α in liver compared to sham

injury **(Supplementary Figure 6)**.

Discussion

A major challenge in therapeutically targeting the inflammatory cascade after SCI is the

heterogeneity and complexity of signaling pathways driven by a broad range of pro-inflammatory

mediators induced after injury [3, 29, 30]. These pathways are numerous, often overlapping, and

orchestrated by many cell types at different stages of acute injury including microglia,

macrophages, neutrophils, and astrocytes [30, 31]. HuR is a major RNA regulator of many pro-

inflammatory mediators and is expressed in these cell types, making it a rational target for

maximizing suppression of multiple components in the inflammatory cascade [3, 4, 7, 30, 32, 33].

In this report, we have shown that inhibiting HuR with the small molecule SRI-42127 potently

suppresses pro-inflammatory responses in the spinal cord and periphery in the acute phase of

SCI, resulting in a significant attenuation of motor function loss and pain. Histologic correlates for

this therapeutic effect include sparing of neurons and whiter matter at the level of injury, reduced

lesion size, and reduced microglial activation.

A deleterious effect of glial HuR in SCI was first reported by our group using a transgenic mouse

model where its selective overexpression in astrocytes accentuated spinal cord edema, neuronal

loss and neuroinflammatory responses after acute injury [9, 11]. In those reports, we described

prominent nucleocytoplasmic translocation of both endogenous (and transgenic) HuR similar to

what we observed in microglia and astrocytes in the current work. In glial cells, HuR is

predominantly nuclear in localization, but upon activation by triggers such as LPS [10], hypoxia

[10], glioblastoma [7, 32], and amyotrophic lateral sclerosis [8], translocates to the cytoplasm to

promote mRNA stabilization and translational efficiency of pro-inflammatory mediators [4, 32].

HuR translocation was prominent in microglia/macrophages and astrocytes at the epicenter early

of injury, but not rostrally or caudally, indicating a specificity of activation to the level of trauma. In

astrocytes, HuR translocation occurred by 24 h post SCI, consistent with our previous reports [9,

11], but not at 8 h, suggesting that microglial HuR is activated earlier on in SCI. In both cell types,

however, SRI-42127 attenuated cytoplasmic HuR which is consistent with its mechanism of

blocking HuR dimerization [10, 13-15].

Inhibition of HuR with SRI-42127 led to a striking suppression of two cytokines, IL-6 and IL-1β, at

the epicenter, both of which are rapidly induced within 1-3 hours after injury [29]. These cytokines

are considered key initiators of the inflammatory cascade and directly produce toxic effects on

neurons, oligodendrocytes, endothelial cells, leading to apoptosis and necrosis [30]. They are

mainly produced by microglia and astrocytes activated upon exposure to damage associated

molecular patterns (DAMPs) produced by the injury [4, 30]. This profound suppression was

consistent with our prior studies indicating that SRI-42127 had the greatest suppressive effect on

IL-1β and IL-6 induction in primary microglia and astrocytes *in cellulo* after LPS stimulation, and

spinal cord microglia *in vivo* after spared nerve injury (SNI) [10, 16]. In the SNI model, SRI-42127

treatment significantly reversed allodynic pain in acute and chronic phases of the injury [16]. IL-6

and its signaling pathway are causally linked to neuropathic pain in SCI [34-36]. In prior reports,

blocking the pathway with a neutralizing antibody to the IL-6 receptor or inhibiting the downstream

transducer, JAK2, abrogated the development of pain and improved functional recovery. Thus,

the early and marked suppression of IL-6 in the epicenter of SRI-treated mice may underlie the

significant attenuation of spontaneous pain observed after SCI.

SRI-42127 suppressed other pro-inflammatory mediators in the initial 8 h post injury that further

enhance the inflammatory cascade and toxic microenvironment via different pathways, including

iNOS (via nitric oxide), COX-2 (via PGE_2), and MMP-12 [3, 29, 30, 37, 38]. Interestingly COX-2

protein but not mRNA was suppressed with SRI-42127 treatment. This may be related to a loss

of translational efficiency rather than mRNA stability (and decrease in mRNA levels) as both

processes represent two distinct posttranscriptional levels of regulation governed by HuR [4]. We

have shown previously that these mediators are regulated by HuR in glial cells in different

inflammatory environments and suppressed either by SRI-42127, MS-444 (another chemical HuR

inhibitor [15]) or with HuR silencing/deletion [7-10, 16, 39]. In addition to direct toxic effects, these

inflammatory mediators also trigger indirect toxicity by promoting vascular permeability, edema,

ischemia, and hemorrhage [1, 30].

In the acute phase of injury, the inflammatory cascade is further amplified by glial and macrophage

production of chemokines, particularly CXCL1, CXCL2 and CCL2 which promote recruitment and

activation of neutrophils, monocyte/macrophages, and other immune cells from the periphery [1,

30]. These chemokines were likewise suppressed by more than 6-fold with SRI-42127 treatment.

In a compressive model of SCI, depletion of CCL2+ microglia significantly attenuated neuropathic

pain [40]. CCL2-depeleted microglia switched from a pro- to anti-inflammatory phenotype and

demonstrated reduced migration and invasion properties. CCL2 also can activate spinal microglia

when injected intrathecally and trigger behavioral patterns consistent with neuropathic pain [41].

Interestingly, our prior work showed that HuR is a major positive regulator of CCR2, the main

receptor for CCL2, in microglia and macrophages [8]. This is consistent with the >60% attenuation

of *CCR2* mRNA levels at the epicenter with SRI-42127 treatment. *CCR2* knockout abrogates

neuropathic pain after nerve injury which may tie into the attenuation of pain observed here [42,

43]. Of note, the fold-suppression of *CCR2* mRNA with drug treatment was even greater at rostral

and caudal levels (~6-fold) due to the higher expression of *CCR2* mRNA in vehicle controls at

those levels. This difference is likely due to injury-related loss of neurons, astrocytes and

oligodendrocytes, all of which express CCR2 [44].

Interestingly, *TGF-β1* was not induced at 8 h post injury or altered with SRI-42127 treatment but

increased by ~5-fold in vehicle-treated mice at 5 days. This timeframe is consistent with a prior

report where TGF-β1 induction occurred more gradually (over days) after SCI [45]. In prior *in vivo*

studies, we did not see suppression of TGF-β1 in brain or spinal cord tissues in SRI-treated mice

[10, 16] and so the increase observed here may relate to enhanced glial scar signaling pathways

due to a larger lesion size. TGF-β1 promotes astrocyte proliferation and glial scar formation and

the 5-day timepoint represents the early stage of that process [46-48].

In SCI (and other forms of CNS trauma), there is a rapid peripheral response in organs such as

liver and spleen that strongly influences the pro-inflammatory microenvironment in the injured

cord by promoting mobilization and recruitment of inflammatory monocyte/macrophages and

neutrophils [27, 28, 49-52]. In liver and spleen there is production and release of chemokines

CXCL1 and CCL2 that drive this recruitment. Consistent with prior reports [50, 52], we observed

a significant increase in circulating CXCL1 at the early timepoint of 8 hours post injury with liver

tissue showing a ~15-fold induction of *CXCL1* mRNA. SRI-42127 potently suppressed both

hepatic mRNA induction and circulating levels of CXCL1. Although early induction of *CCL2* in liver

has been observed with compression spinal cord injury models and later time points [51, 53], we

did not see this at 8 hours in our model. On the other hand, circulating CCL2 levels were increased

and spleen tissue showed a selective ~15-fold induction of *CCL2* mRNA. A prior report identified

spleen as the major source for monocyte-derived macrophages recruited to the injured spinal

cord and pre-SCI splenectomy led to improved recovery [54].The induction of CCL2 after SCI was

attributable to myeloid populations within the spleen that can express this chemokine [55], but it

may serve as a signal for mobilizing and recruiting bone marrow derived monocytes that

eventually infiltrate the injured spinal cord. Thus, the suppression of peripheral CCL2 by SRI-

42127 may further reduce the inflammatory milieu in the injured spinal cord.

IL-6 was induced both in liver and splenic tissues in conjunction with a very large increase in

circulating levels. In liver, IL-6 induction is a component of inflammation that has been observed

in animal models of acute SCI [49]. Selective induction of IL-6 in liver and spleen versus TNF-α

and IL-1β has been observed in other models of immune activation [56, 57] although the

mechanism remains unclear. Our findings do suggest, however, that these organs are major

contributors to circulating IL-6 in early SCI. In humans, elevated serum IL-6 has been detected

in the acute phase of SCI, with some studies correlating levels with clinical severity of injury (see

review [58]). The attenuation of systemic IL-6 by SRI-42127 may also have contributed to the

reduction in pain after SCI as discussed above. Taken together, the suppression of peripheral

inflammatory responses by SRI-42127 underscores the broad reach of HuR in cell types outside

of the CNS [4, 59].

The effectiveness of SRI-42127 in blocking the inflammatory cascade is borne out by the

significantly improved histological and behavioral outcomes. There was attenuation of microglial

activation in the injured spinal cord, and reduced lesion size with sparing of neurons and white

matter. Interestingly, we also observed a significant increase in myelin sparing in rostral and

caudal levels in SRI-treated mice. Loss of myelin in these levels is likely related to Wallerian

degeneration in long white matter tracts both rostral and caudal to injury, reflective of distal axonal

degeneration following separation of axons from their cell body [47, 60].

In our prior work, we found that SRI-42127 penetrates the CNS to therapeutic concentrations

within 10-15 minutes after systemic delivery [13] and rapidly suppresses neuroinflammatory

responses [10]. These are favorable features for clinical translation in acute SCI where "time is

spine" has emerged as a central theme in clinical management [6]. Because SRI-42127 can be

administered systemically, it could be given in the field by emergency personnel shortly after

injury. In our experimental paradigm, SRI-42127 was given 1 hour after SCI for a duration of 5

days.

Neuroinflammatory responses, including activated microglia and macrophages, persist in chronic

phases when their role switches to a restorative one, promoting neural plasticity, angiogenesis

and stem cell proliferation [3, 61, 62]. HuR regulates trophic factors that promote these restorative

functions such as Hif-1α, VEGF, and GM-CSF, so prolonged treatment with SRI-42127 may be

harmful [32, 59, 63-65]. Even IL-6 signaling may promote SCI recovery and neural regeneration

[58]. However, the major deleterious pro-inflammatory cytokines regulated by HuR, approach pre-

injury levels by 7 days, providing a time frame for intervention with HuR inhibitors. Further studies

will be required to delineate the optimal duration for SRI-42127 treatment after SCI.

Abbreviations

Abbreviations

ARE: AU-rich elements

BL: Baseline

BMS: Basso mouse scale

BSCB: Blood-spinal cord barrier

CCL2: C-C Motif chemokine ligand 2

CCR2: C-C chemokine receptor type 2

COX-2: Cyclooxygenase-2

CXCL: chemokine (C-X-C motif) ligand

D: Drug (SRI-42127)

DAMPs: Damage-associated molecular patterns

DAPI: 4',6-diamidino-2-phenylindole

FI: Fluorescence intensity

GFAP: Glial fibrillary acidic protein

HuR: Human antigen R

Iba1 : Ionized calcium binding adaptor molecule 1

IL-6 : Interleukin 6

IL1-β : Interleukin-1 beta

iNOS : Inducible nitric oxide synthase

IP: intraperitoneal

LPS: Lipopolysaccharide

MGS: Mouse grimace scale

MMP: Matrix metalloproteinase

NeuN: Neuronal nuclei

PBS: Phosphate buffer saline

RBP: RNA binding protein

SCI: Spinal cord injury

Sh: Sham

SNI: Spared nerve injury

SRI: SRI-42127

TGF-β1: transforming growth factor beta-1

TNF-α: Tumor necrosis factor alpha

UTR: Untranslated region

V: Vehicle

Ethics approval and consent to participate

The animal study was reviewed and approved by the UAB Institutional Animal Care and Use

Committee and were carried out in accordance with relevant guidelines and regulations of the

National Research Council Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding

author. The data are not publicly available due to privacy or ethical restrictions.

Competing interests

The authors declare no competing interests.

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Authors' contributions

MAH, PHK, and CPC contributed to the conception and design of the study. MAH, RS, RES, AK,

YS, AZH, KAS, and JD performed experiments and acquired data. AG, SAA, LBN, NF contributed

to data analysis and interpretation. MAH and PHK prepared figures and drafted the manuscript.

All authors read and approved the final manuscript.

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Figure Legends

Figure 1: SRI-42127 attenuates motor function loss and pain after SCI. Following SCI, SRI-

42127 (10mg/kg) or vehicle was administered IP at 6 h intervals for 5 days starting 1 h after

injury. **(A)** BMS scores for vehicle control and SRI-treated mice were measured at 1, 2 and 3

weeks after SCI. **(B)** Motor function post SCI was assessed by open field testing. A representative

heat map of activity at 2 weeks post SCI is shown. Different parameters of motor activity were

measured including **(C)** distance moved, **(D)** mobility**,** and **(E)** velocity. **(F)** Beam walking scores

of vehicle and SRI-treated mice were obtained at 1, 2, 3 weeks post SCI. **(G)** Rotarod testing was

performed at 1-, 2- and 3-weeks post SCI and latency to fall was measured. **(H)** Non-evoked pain

was assessed by mouse grimace scores (MGS) which were measured at day 2 and 9 post SCI.

Baseline testing of both test groups, prior to SCI, had similar MGS scores. Evaluations for open

field BMS, beam walking, and non-evoked pain were made by two observers blinded to the status

of the animal. Error bars represent means ± SD of 6 mice/group. P values: * < 0.05, ** < 0.01, ***

< 0.001, **** < 0.0001.

Figure 2: SRI-42127 treatment reduces lesion size and loss of neurons after SCI while

increasing myelin sparing. (A) Representative GFAP-immunostained sections from the

epicenter (3 weeks post SCI) showing reduced lesion size in an SRI-treated mouse compared to

vehicle control. **(B)** Total lesion area was quantified in 3 vehicle and 3 SRI-treated mice. **(C)**

Representative NeuN-immunostained sections from the epicenter showing an increase in NeuN+

cells in the SRI-treated mouse. **(D)** NeuN+ cells were quantified at the epicenter in 3 vehicle and

3 SRI-treated mice at 3 weeks post-SCI. **(E)** NeuN fluorescence intensity (FI) was quantified at

the epicenter. **(F)** Representative fluoromyelin-stained sections from the epicenter showing

increased myelin staining in SRI-treated mouse compared to vehicle control. **(G)** Fluoromyelin

staining at the epicenter was quantified in 3 vehicle and 3 SRI-treated mice. Dashed lines

delineate the boundary of injured spinal cord. Bars represent the mean ± SD of 3 mice/group. P

< **0.01. Scale bars 100 µm.

Figure 3: **SRI-42127 inhibits nucleocytoplasmic translocation of HuR in microglia and**

attenuates microglial activation. Eight hours after SCI, sections from the epicenter were

immunostained with antibodies as shown. **(A)** Cytoplasmic translocation of HuR is observed in

microglia at the epicenter from a vehicle treated mouse as indicated by a merged yellow signal of

HuR and Iba1 (arrow heads). A higher power view of a microglial cell (white arrow) is shown in

the insert. **(B)** SRI-42127 treatment blocked HuR translocation from the nucleus as indicated by

a merged signal of HuR, Iba1 and nuclear DAPI as well as an absence of a merged yellow signal.

A higher power view of a microglial cell (white arrow) is shown in the insert. **(C)** Microglial HuR

localization was quantified in 5 cells from 3 biological replicates as described in the Methods and

expressed as a nuclear/cytoplasmic ratio. **(D)** Quantification of Iba1 fluorescence intensity (FI) in

11-13 cells from 3 biological replicates. **(E)** Total Iba1 FI was quantified at the epicenter, rostral,

and caudal levels. **(F)** Total Iba1+ cell counts at the epicenter were quantified in 3 biological

replicates. P * < .05, P < **0.01, *** < 0.001, **** < .0001. Scale bars 50 µm, 10 µm (inserts).

Figure 4: SRI-42127 attenuates activation-associated changes in microglial morphology

after SCI. (A) Eight hours after SCI, tissue sections from the epicenter were immunostained with

Iba1 antibodies and digitally converted to Skeletonized images using Fiji software. Representative

images are shown. Morphological features were quantified including total microglia branch length,

number of branches per microglial cell**,** and number of branch endpoints per microglial cell. Data

were generated from 5007 microglial cells from vehicle control (n = 3 biological replicates) and

4538 microglial cells from SRI (n=3 biological replicates). **(B)** The soma of Iba1+ microglial cells

were identified as shown in the representative images to the left. Binary images were generated

by adjusting the Fiji threshold level (shown in inserts). Total area was quantified in 11 cells from

3 biological replicates as shown in the graph. Error bars represent SD. P *** < 0.001, **** < .0001.

Figure 5: SRI-42127 attenuates pro-inflammatory cytokine and chemokine mRNA induction

(A) Spinal cords were harvested at 8 h post-SCI and divided into 3 segments: injury epicenter,

rostral and caudal. RNA expression of proinflammatory cytokines and chemokines was assessed

by qPCR in vehicle controls and SRI treated mice. All values were expressed relative to the

vehicle control which was set at 1.0. GAPDH was used as the housekeeping control. P values: *

< 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. Error bars represent means ± SEM of 6 mice/group.

(B) COX-2 western blot of protein lysate from epicenter, rostral and caudal spinal cord levels

obtained 8 h after SCI. A representative blot is shown above. COX-2 levels were quantified by

densitometry from three biological replicates using vinculin as a loading control. All values were

expressed relative to the vehicle controls at rostral and caudal segments which were set at 1.0.

Error bars represent the mean ± SD. P values: * < 0.05.

Figure 6: SRI-42127 suppresses expression of pro-inflammatory mediators peripherally.

(A) ELISA was used to quantify cytokines in plasma samples obtained at 8 h post SCI or sham

injury (Sh). **(B)** At the same time interval, RNA was harvested from liver and spleen and assessed

by qPCR for *CXCL1*, *CCL2*, and *IL-6* mRNA expression. Data represent the mean ± SD of 6

mice/group in plasma and ± SD of 3 mice/group in liver and spleen. P values: ** < 0.01, *** <

0.001, **** < 0.0001.

Supplementary Figure 1: Behavioral assessment in sham mice. Mice were subjected to

sham injury and then given vehicle or SRI-42127 every 6 hours for 5 days. **(A)** Open field BMS

score heatmap, total distance travelled, velocity, and mobility show no difference in motor

activity between vehicle- and SRI-treated mice **(B)** Balance beam test score showed no deficits

in vehicle or SRI-treated mice.

Supplementary Figure 2: Assessment of caudal and rostral sections of spinal cord from

vehicle and SRI-treated mice. At 3 weeks post SCI, rostral and caudal segments of 3 vehicle

and 3 SRI-treated mice were evaluated by immunohistochemistry. **(A)** GFAP staining shows no

evidence of glial scarring. **(B)** NeuN staining shows equal neuronal counts in vehicle and SRI-

treated mice. **(C)** Fluoromyelin staining shows a significant increase in myelin in SRI-treated mice.

P value: ** < 0.01.

Supplementary Figure 3: HuR nucleocytoplasmic translocation was not observed in

microglia in uninjured spinal cord levels. Caudal or rostral segments of vehicle- and SRI-

treated mice were assessed for HuR localization after SCI and showed no evidence of

nucleocytoplasmic translocation.

Supplementary Figure 4: HuR nucleocytoplasmic translocation in astrocytes is delayed

after SCI and blocked by SRI-42127. At 8 and 24 h after SCI, sections from the epicenter of

vehicle and SRI-treated mice were immunostained with HuR and GFAP antibodies as shown. (A)

At 8 h, HuR remained nuclear in location. (B) At 24 h, there is translocation of HuR in a vehicle-

treated mouse as indicated by arrows (upper panels) which is blocked by SRI-42127 treatment

(lower panels)**.** Scale bars, 10 μm, 50 μm.

Supplementary Figure 5: Suppression of pro-inflammatory cytokines persists after 5 days

At 5 days after SCI, the epicenter of injured mice receiving vehicle or SRI-42127 was assessed

for inflammatory mediator expression by qPCR. P values: ** < 0.01, **** < 0.0001. Error bars

represent the mean ± SD of 3 mice/group.

Supplementary Figure 6: Cytokines which had minimal or no mRNA induction in peripheral

tissues. At 8 h after SCI or sham injury, total RNA from liver and spleen was assessed by qPCR

for expression of the inflammatory mediators shown. P value: * < 0.05. Error bars represent the

mean ± SD of 3 mice/group.

Supplementary Figure 7: Unedited western blot of COX-2 and vinculin loading control

from Figure 5B.

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

(A)

Fig 6