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Research paper

Inhibition of the NLRP3 inflammasome by OLT1177 induces functional protection and myelin preservation after spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) leads to irreversible functional deficits due to the disruption of axons and the death of neurons and glial cells. The inflammatory response that occurs in the injured spinal cord results in tissue degeneration; thus, targeting inflammation after acute SCI is expected to ameliorate histopathological evidence indicative of damage and, consequently, reduce functional disabilities. Interleukin 1 beta (IL-1β) and interleukin 18 (IL-18) are pro-inflammatory cytokines members of the IL-1 family that initiate and propagate inflammation. Here, we report that protein levels of IL-1β and IL-18 were increased in spinal cord parenchyma after SCI, but with different expression profiles. Whereas levels of IL-1β were rapidly increased reaching peak levels at 12 h after the injury, levels of IL-18 did not increase until 7 days after the injury. Since activation of the NLRP3 inflammasome is required for the processing and release of IL-1β and IL-18, we intraperitoneally administered OLT1177, a selective inhibitor of the NLRP3 inflammasome, to reduce the contribution of these cytokines to SCI. At a dose of 200 mg/kg, OLT1177 protected against neurological deficits and histological evidence of damage. OLT1177 also reduced the levels of IL-1β in the spinal cord after contusion injury and diminished the accumulation of neutrophils and macrophages at later time points. These data suggest that targeting the NLRP3 inflammasome with OLT1177 could be a novel therapeutic strategy to arrest neuroinflammation and reduce functional impairments after acute SCI in humans.

1. Introduction

Acute inflammation is the host's rapid physiological response aimed at combating infection and clear cell debris (Libby, 2008). This response is tightly regulated in order to prevent acute or chronic inflammation that leads to tissue damage, as occurs after spinal cord injury (SCI) (David et al., 2012; Fleming et al., 2006; Francos-Quijorna et al., 2017). Among the multiple mediators that participate in the initiation of the inflammatory response, members of the interleukin 1 (IL-1) family, particularly IL-β and IL-18, play key roles (Dinarello, 2011).

IL-1β and IL-18 are the main regulators of the innate and adaptive immune responses (Dinarello, 2019, 2011). IL-1β signals through its specific receptor IL-1R1 and a promiscuous co-receptor IL-1R3 (Dinarello, 2019). IL-18 binds to its receptor IL-1R5 (also known as IL-18Rα) and its specific co-receptor IL-1R7 (IL-18Rβ). At physiological conditions, IL-1β and IL-18 are present at very low levels in the central nervous system (CNS). However, after an insult, high expression is observed in microglia, infiltrating macrophages, and also neurons (de Rivero Vaccari et al., 2008; Gustin et al., 2015).

IL-1β and IL-18 are first synthesized as inactive precursors that require processing, via the intracellular enzyme caspase-1, to generate active cytokines (Franchi et al., 2009). Inflammasomes are intracellular macromolecular complexes that provide the upstream activation of caspase-1 (Franchi et al., 2009; Martinon et al., 2002). Inflammasomes are composed of three elements: a pathogen recognition receptor (PRR); an adaptor protein known as ASC (apoptosis-associated speck-like

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protein containing a caspase recruitment domain); and the procaspase-1 protein (Broz and Dixit, 2016). ASC adaptor protein harbors the pyrin domain (PYD) from the PRR subunit and the caspase recruitment domain (CARD) from procaspase-1 protein allowing the assemble of the three elements of the inflammasome (Broz and Dixit, 2016). To date, six different inflammasomes have been identified, each being dictated by its unique PRR. They are the nucleotide-binding oligomerization domain (NOD) leucine-rich repeat (LRR)-containing protein-1 family members NLRP1, NLRP2, NLRP3, and NLRC4, AIM2 (absent in melanoma 2), and pyrin (Broz and Dixit, 2016; Minkiewicz et al., 2013). Among the different inflammasomes, the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome is the best characterized.

NLRP3 senses a wide variety of stimuli including bacterial toxins, RNA viruses, and extracellular ATP (Muñoz-Planillo et al., 2013; Yang et al., 2019). After stimulation, NLRP3 oligomerizes resulting in the formation of the catalytically active caspase-1, which initiates the processing and release of IL-1β and IL-18. Active caspase-1 also induces pyroptosis, which is a lytic form of cell death by forming pores in the plasma membrane (Broz and Dixit, 2016; Yang et al., 2019). This classic way of maturation and release of IL-1β and IL-18 is known as canonical pathway. However, occasionally both cytokines can mature following a non-canonical pathway in response to LPS from Gram-negative bacteria. This non-canonical pathways involves caspase-11 in mice and caspase 4 and/or caspase 5 in humans (Yang et al., 2019).

The NLRP3 inflammasome was observed at protein level after traumatic spinal cord injury (Grace et al., 2016; Jiang et al., 2017, 2016; Zendedel et al., 2016) and traumatic brain injury (TBI) (Chen et al., 2019). Specifically, elevated NLRP3 expression is found in microglia, but not astrocytes, in the mouse brain after stimulation with different classical inflammasome activators (Gustin et al., 2015). However, NLRP3 has also been detected in neurons from the injured brain (Liu et al., 2013). A recent study reported that administration of MCC950 (also known as CP-456773 or CRID3), a potent and selective NLRP3 inhibitor, enhances functional outcomes after spinal cord compression injury (Jiao et al., 2020) highlighting that NLRP3 could be a therapeutic target for treating acute SCI. However, MCC950 caused liver toxicity in phase II clinical trials for rheumatoid arthritis (Mullard, 2019) and it is now being developed as a less toxic analogue with extended half-life and higher maximum concentration (Chen et al., 2021).

OLT1177 (rINN:dapansutrile) is a β-sulfonyl nitrile synthetic compound of 133.17 KDa that selectively inhibits the NLRP3 inflammasome (Marchetti et al., 2018a). This compound has been used to reduce disease severity in mouse models of joint inflammation (Marchetti et al., 2018b), myocardial ischemia reperfusion injury (Toldo et al., 2019), multiple sclerosis (Sánchez-Fernández et al., 2019), and Alzheimer's disease (Lonnemann et al., 2020).

In the present work, we assessed whether OLT1177 exerts therapeutics effects in spinal cord contusion injury in mice. We demonstrate that treatment with this NLRP3 inhibitor reduced inflammation and improved functional outcomes and myelin preservation after acute SCI. Since OLT1177 crosses the brain blood barrier, it demonstrated to be safe in human toxicology studies (Marchetti et al., 2018a), and effective in a phase IIa clinical trials for gout flares (Klück et al., 2020) and phase Ib for heart failure (Wohlford et al., 2020), the results of the present work suggest that OLT1177 could be a potential candidate to treat acute SCI in humans.

2. Materials and methods

2.1. Ethics declarations

All the experiments were approved by the Ethics Committee on Animal and Human Experimentation from the Universitat Autonoma de Barcelona (CEEAH: 4057) and followed the European Communities Council Directive 2010/63/EU. All procedures were carried out following approved guidelines.

2.2. Spinal cord injury

Spinal cord injury procedure was performed according to described protocols (Amo-Aparicio et al., 2018). Adult (8–10 weeks old) female mice were anesthetized by intramuscular injection with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). After skin and muscle incision, laminectomy at 11th thoracic vertebrae was performed and the exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation). A force of 60 kdynes and a tissue displacement of 450–550 μm were applied.

2.3. OLT1177 treatment

Dapansutrile (lab code: OLT1177; Olatec Therapeutics LLC) diluted in sterile saline solution was injected intraperitoneally, starting 1 h after lesion, for seven days. Two different administration protocols were used: (i) 60 mg/kg twice a day and (ii) 200 mg/kg once a day, based on previous studies (Marchetti et al., 2018a; Sánchez-Fernández et al., 2019). Control mice were treated with sterile saline solution using the same administration protocol.

2.4. Protein detection

Mice received an intraperitoneal injection of sodium pentobarbital (Dolethal). Blood was removed by perfusion with 60 mL of 0.9% NaCl in distilled water and a 6 mm piece of spinal cord, centered into the injury site, was taken from each mouse. Samples were frozen in liquid nitrogen and homogenized in RIPA buffer (Thermo Fisher) supplemented with protease inhibitor (Roche) using a TissueRuptor (Qiagen) and an Ultrasonic Homogenizer (Biologics Inc.). Protein concentration was quantified using BCA Protein Assay Kit (Thermo Fisher) according to manufacturer's instructions.

To measure levels of IL-1β and IL-18 in the naïve condition and from 1 h to 28 days after injury, samples were quantified by Luminex (bead-based multiplex assay). Samples were diluted to 2 μg/μL and processed using a ProcartaPlex Panel (ThermoFisher) on MAGPIX Luminex reader (Millipore) as previously described (Amo-Aparicio et al., 2018). Final values were normalized by the protein concentration of each sample. 4 mice per time-point were used.

For immunoblotting studies, 60 μg of protein per sample were resolved on a Mini-PROTEAN TGX 4–20% gel (Bio-Rad) and transferred to a 0.1 μM nitrocellulose membrane (GE Healthcare). Membranes were blocked in 5% dried milk in PBS-T 0.5% for 1 h at room temperature. Samples were incubated with primary antibody for mouse NLRP3 (1:1000, AdipoGen), caspase-1 (1:200, Santa Cruz Biotechnology), ASC (1:1000, Cell Signaling), and IL-1β (1:500, Santa Cruz Biotechnology). Peroxidase-conjugated secondary antibodies and chemiluminescence were used to detect the protein concentration. Conjugated antibody against β-actin (1:1000, Santa Cruz Biotechnology) was used to normalize protein concentrations. 5 mice per group were used.

For ELISA, DuoSet kit for IL-1β (R&D Systems) was used following the manufacturer's protocol. This ELISA kit mostly detects active IL-1β since it recognizes only 5% of the precursor IL-1β protein. 96-well plates (ThermoFisher Scientific) were coated with capture antibody. Samples were incubated for 2 h at room temperature. Detection antibodies and streptavidin-HRP were added. TMB ELISA (eBioscience) was used as substrate solution. Results were measured in a microplate reader (Bio-Tek). Final values were normalized by the protein concentration of each sample. 5 mice per time-point were used.

2.5. Functional assessment

Locomotor recovery after SCI was evaluated at 1, 3, 5, 7, 10, 14, 21, and 28 days post-injury (dpi) in an open-field using the nine-point Basso Mouse Scale (BMS) (Basso et al., 2006). BMS evaluation was performed by two researchers who were blinded to the experimental groups.

Consensus score between two researchers was taken. 15 mice were used for the saline group. 12 and 8 mice were used for the groups treated with the 60 mg/kg and 200 mg/kg doses of OLT1177, respectively.

2.6. Histology

At 28 dpi, mice were perfused with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer. A piece of 6 mm of spinal cord, centered into the lesion site, was removed from each mouse and cryoprotected with 30% sucrose in 0.1 M phosphate buffer saline (PBS) at 4 °C. Samples were cut in ten series of 15 μm thick sections in a cryostat and picked up on glass slides. Three series were obtained to assess myelin preservation, neuronal sparing, and GFAP immunoreactivity. Analysis was done at 150 μm intervals, from 1800 μm rostral to 1800 μm caudal to the injury epicenter.

For myelin quantification, sections were placed in a 1 mg/mL Luxol Fast Blue (LFB) (Sigma-Aldrich) solution in 95% ethanol and 0.05% acetic acid and left overnight at 37 °C. Sections were then washed in 95% ethanol and distilled water before clearing with 0.5 mg/ml of Li_2CO_3 in distilled water for 1.5 min. After several washes, sections were dehydrated and mounted in DPX mounting media (Sigma-Aldrich). After fixation, the epicenter of the contusion was localized by determining the tissue section with the lowest LFB stained area. NIH ImageJ software was used to perform the quantifications. 15 mice were used for the saline group. 12 and 8 mice were used for the groups treated with the 60 mg/kg and 200 mg/kg doses of OLT1177, respectively.

For neuron and astrocyte assessment, sections were blocked with 5% Normal Donkey Serum (Millipore) for 1 h at room temperature. Samples for neuron quantification were also treated with Endogenous Biotin Blocking Kit (Invitrogen) following manufacture's protocol. Sections were then incubated with antibodies against mouse NeuN (1:200, biotinylated, Sigma-Aldrich) and GFAP (1:200, rabbit, Millipore) overnight at 4 °C. Streptavidin conjugated to Alexa fluor 594 (1:200, Invitrogen) and donkey anti-rabbit secondary antibody conjugated to Alexa fluor 488 (1:200, Invitrogen) were applied for 1 h at room temperature. Finally, slides were coverslipped in Mowiol mounting media (Sigma-Aldrich). Neuronal survival was assessed by manually counting the number of $NeuN^+$ cells in the ventral horns. Astrocyte immunoreactivity was calculated at the whole section by measuring the integrated density using NIH ImageJ software. 6 and 7 mice were used for the saline and OLT1177 (200 mg/kg) groups, respectively.

2.7. Flow cytometry

Flow cytometry from spinal cord was performed according to described protocol (Amo-Aparicio et al., 2018). At 1, 3, and 5 days after injury, animals received an intraperitoneal injection of sodium pentobarbital (Dolethal). Blood was removed by perfusion with 60 mL of 0.9% NaCl and a piece of 6 mm of the spinal cord, centered into the injury site, was taken from each mouse. Cell suspensions of the spinal cord were obtained by enzymatic disaggregation using a mixture of collagenase (Sigma-Aldrich) and DNase (Roche) and mechanic disaggregation followed by passage through 70-μm cell strainers (Fisher). Cells were labeled with the following antibodies: CD45-PerCP (eBioscience), CD11b-PE-Cy7 (eBioscience), and F4/80-PE (eBioscience). 1:200 dilutions were used for each antibody. Proper isotypes for each antibody were used. After cell staining, cells were fixed with 1% paraformaldehyde (Sigma-Aldrich) in 0.1 M of phosphate buffer and analyzed using the FACSCanto flow cytometer (BD Bioscience). Cell gating and quantification were performed using FlowJo® software. Microglia cells were defined as $CD45^{low}$ and $CD11b^+$, whereas macrophages were defined as $CD45^{high}$, $CD11b^+$, and $F4/80^+$. Granulocytes, mainly neutrophils, were defined as $CD45^{high}$, $CD11b^+$, and $F4/80^-$ (Amo-Aparicio et al., 2018; Francos-Quijorna et al., 2016). 4 mice per group were used.

2.8. Statistics

All analyses were conducted through GraphPad Prism v8. The Kolmogorov–Smirnov test was used to test normality. Cytokine levels obtained from Luminex were analyzed using one-way ANOVA with Dunnett's post hoc correction. Functional follow-ups for BMS score were analyzed using two-way ANOVA with repeated measures (RM) with Bonferroni's post hoc correction. Functional proportions from BMS were compared by chi-square test. Myelin sparing was analyzed using one-way ANOVA with Holm-Sidak's post hoc correction. Neuron and astrocyte sparing were analyzed using multiple t-test comparisons with the same post hoc correction. Protein levels obtained by western blot and ELISA were analyzed using t-test. Numbers of myeloid cells from flow cytometry were analyzed using t-test. Results from all the experiments were expressed as mean ± SEM and differences were considered significant at $p < 0.05$.

3. Results

3.1. Protein levels of IL-1β and IL-18 increase after SCI

Since damage to the spinal cord leads to the immediate increase in the production of pro-inflammatory cytokines (de Rivero Vaccari et al., 2008; Francos-Quijorna et al., 2016; Gustin et al., 2015), we first evaluated the protein dynamics of IL-1β and IL-18 in the injury environment from 1 h to 28 days after contusion surgery using Luminex technology.

The data revealed that the protein levels of IL-1β and IL-18 were very low in the spinal cord at physiological conditions, being in some samples below the detection limits (1.49 pg/mg protein and 9.88 pg/mg protein for IL-1β and IL-18, respectively). However, levels of both cytokines changed significantly after contusion injury. Levels of IL-1β in the spinal cord parenchyma peaked at 12 h post-lesion and remained at significantly high levels up to 24 h post-injury (Fig. 1a). Protein levels of IL-18 in the injured spinal cord were increased at later time points after SCI. Indeed, levels of IL-18 did not show any increase until day 3 post-lesion, reaching maximal levels at day 7 and remaining at significant greater levels up to day 21 (Fig. 1b).

3.2. OLT1177 improves locomotor recovery and tissue preservation after SCI

Since the data reported above indicate that inflammasome activation occurs in the spinal cord after lesion (Fig. 1a,b), we tested the contribution of NLRP3 to SCI pathology. For this purpose, we first assessed the effect of OLT1177, a specific NLRP3 inhibitor safe in humans (Klück et al., 2020; Marchetti et al., 2018a; Wohlford et al., 2020), on functional outcomes after SCI. The experiments revealed that mice treated with 60 mg/kg of OLT1177 twice a day for 7 days improved functional recovery, although statistical differences against saline group were not observed (Fig. 2a). Mice treated with 200 mg/kg of OLT1177 once a day for 7 days showed a significant enhancement in the functional performance in comparison with mice treated with saline. Differences in the BMS score between OLT1177 (200 mg/kg) and saline groups were statistically significant from day 10 to day 28 after SCI (Fig. 2a).

At the end of the follow-up, the 73% of the mice treated with saline showed hindlimb plantar placement, meaning that they were able to place the full feet on the ground, but only the 6% showed occasional stepping (Fig. 2b). In the case of the animals treated with the 60 mg/kg dose of OLT1177, all of them showed hindlimb plantar placement and 50% of them also showed hindlimb plantar stepping, being occasional (42%) or frequent (8%). Finally, all mice treated with 200 mg/kg of OLT1177 showed hindlimb plantar placement and 75% of them were able to perform occasional (37.5%) or frequent (37.5%) hindlimb plantar stepping (Fig. 2b). Chi-square test verified that functional proportions were significantly determined by the group.

We then evaluated the effects of OLT1177 on myelin sparing in the

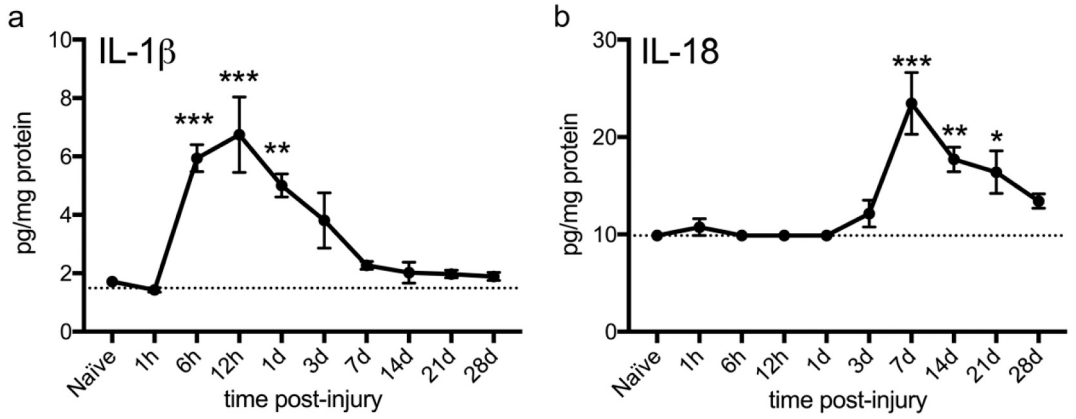

Fig. 1. Levels of IL-1β (a) and IL-18 (b) in the spinal cord at different time points after injury. Spinal cords without injury were used as naïve condition. Dashed line marks the detection limit of each cytokine based on Luminex assay (1.49 pg/mg protein for IL-1β and 9.88 pg/mg protein for IL-18). Data are represented as mean ± SEM. N = 4 per time point. One-way ANOVA with Dunnett's post hoc correction was used to analyze significant differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Naïve.

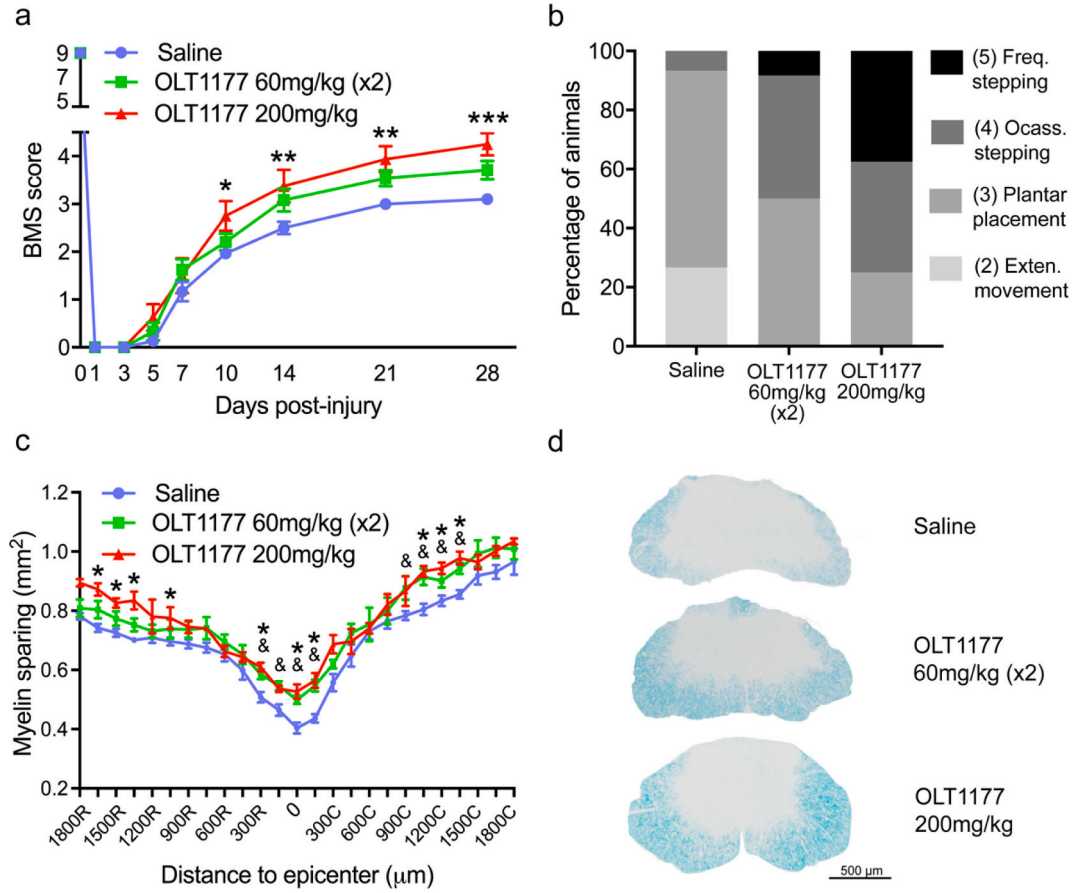

Fig. 2. Effects of OLT1177 in the functional recovery and tissue preservation after SCI. (a) BMS score showing the locomotor progression of mice receiving 60 mg/kg twice a day (green) or 200 mg/kg once a day (red) of OLT1177 for 7 days after SCI. Control mice were treated with saline (blue). (b) Functional distribution of different groups at 28dpi. Darker colors represent more skilled functional performances. BMS values of each type of movement are indicated in parenthesis. (c) Myelin sparing in the lesion area of mice treated with 60 mg/kg twice a day (green) or 200 mg/kg once a day (red) of OLT1177 for 7 days after SCI. Control mice were treated with saline (blue). (d) Representative micrographs of each group showing the myelinated are at the injury epicenter. Data are represented as mean ± SEM. N = 15 for Saline, N = 12 for 60 mg/kg dose, and N = 8 for 200 mg/kg dose of OLT1177. Two-way RM-ANOVA with Bonferroni's post hoc correction was used to analyze differences between groups in (a). Chi-square test to compare proportions in (b). One-way ANOVA with Holm-Sidak's post hoc correction was to analyze significant differences between groups in (c). & $p < 0.05$ for OLT1177 60 mg/kg (x2) vs Saline; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for OLT1177 200 mg/kg vs Saline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spinal cord at day 28 post-injury. Spinal cord cross sections stained for LFB revealed that both 60 mg/kg and 200 mg/kg doses of OLT1177 protected against myelin loss. Differences were evident from 300 μm rostral to 150 μm caudal sections from the lesion core (Fig. 2c,d). However, the 200 mg/kg dose, but not 60 mg/kg dose of OLT1177, was also able to promote significant protections against myelin loss at more distant regions from the injury epicenter (Fig. 2c,d).

Since the 200 mg/kg dose of OLT1177 resulted in greater efficacy than the 60 mg/kg dose, we then evaluated whether this high dose of OLT1177 minimize neuronal loss and astrocytes reactivity.

We observed that OLT1177 increased number of $NeuN^+$ cells at ventral horns at rostral and caudal regions from the injury epicenter. Significant differences against saline-treated mice were found at sections located at 750 μm rostral and 600 μm caudal from the lesion core (Fig. 3a,b). No significant differences in the GFAP signal were found after OLT1177 treatment, however an increase tendency of astrocyte immunoreactivity was observed at the lesion epicenter and adjacent sections (Fig. 3c,d).

These results demonstrate that OLT1177 protects against the neurological deficits, myelin degeneration, and neuronal loss consequences of SCI when used after damage in mice.

3.3. OLT1177 reduces the inflammatory response after SCI

After demonstrating that OLT1177 has therapeutic effects in our SCI model, we next assessed the effects of this compound on the neuroinflammatory response. For this purpose, we measured the levels of the proteins implicated in the processing of IL-1β at 24 h after SCI. We observed that the levels of NLRP3 and ASC were maintained in all the samples (Fig. 4a,b). However, levels of cleaved caspase-1 were significantly reduced in those animals treated with 200 mg/kg of OLT1177 (Fig. 4a,b). This reduction in the levels of cleaved caspase-1 correlated with a reduction in the levels of the active form of IL-1β by OLT1177 (Fig. 4c) while the levels of the inactive form of this cytokine remained unaltered (Fig. 4a,b). These data demonstrate the ability of OLT1177 to inhibit NLRP3 activation in the contused spinal cord.

We also assessed whether OLT1177 affected the number of microglia, macrophages, and neutrophils in the spinal cord at 1, 3, and 5 days after injury. We observed that OLT1177 did not reduce to the number of any of these myeloid cell populations at day 1 after SCI, after one single injection of 200 mg/kg (Fig. 5a). However, the number of neutrophils in the spinal cord were reduced ~50% in mice treated with OLT1177 at day 3 after injury (Fig. 5b,d). Finally, at day 5 after injury, the number of

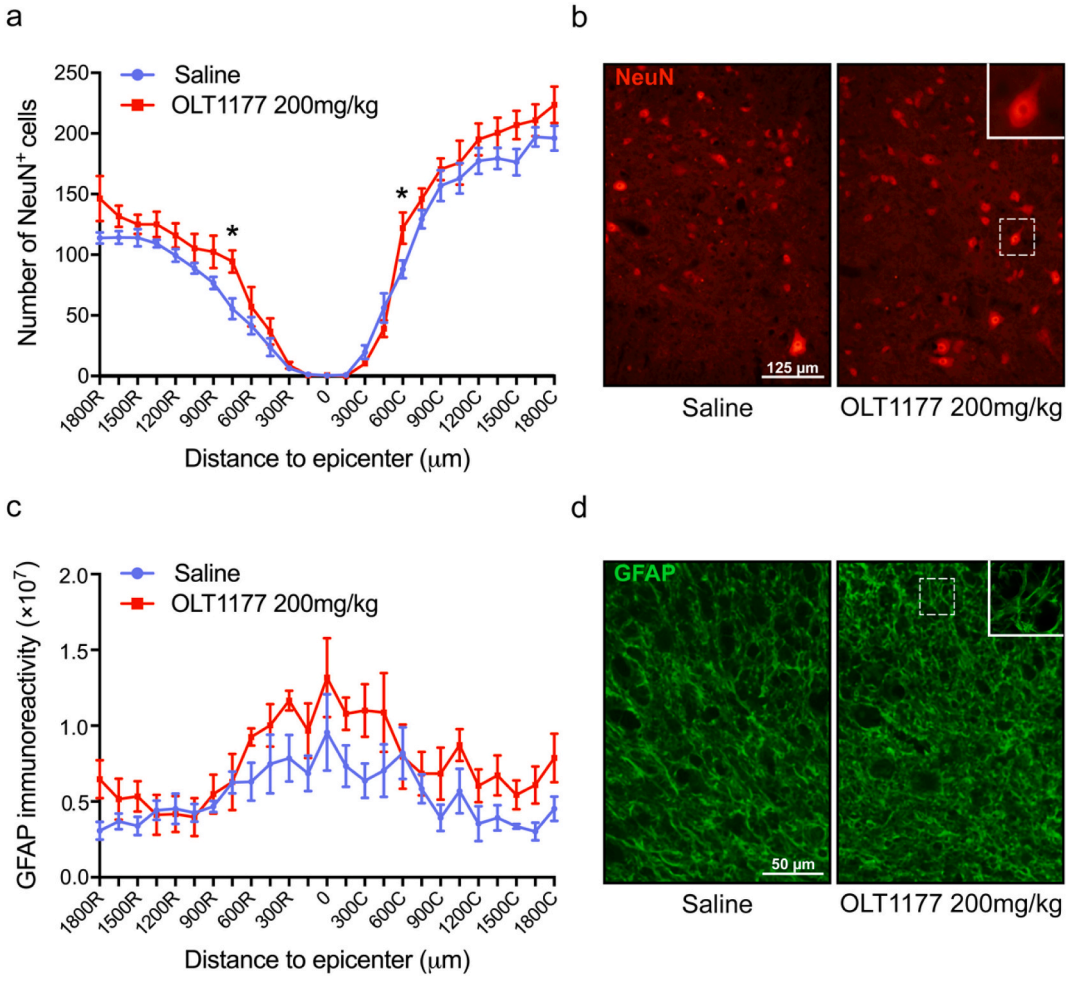

Fig. 3. Assessment of neurons and astrocytes after treatment with OLT1177. (a) Quantification of NeuN-positive cells in the ventral horns at various rostral and caudal distances from the injury epicenter in mice treated with saline (blue) or OLT1177 at 200 mg/kg (red). (b) Representative micrographs showing spinal cord ventral neurons (750 μm rostral from epicenter) in mice administered with saline or OLT1177 (200 mg/kg). The area outlined in the box is shown in higher magnification in the inset. (c) Rostro-caudal quantification of GFAP immunoreactivity in the spinal cord of mice treated with saline (blue) of OLT1177 at 200 mg/kg (red). (d) Representative micrographs showing GFAP immunostaining at the epicenter in mice treated with saline or OLT1177 (200 mg/kg). The area outlined in the box is shown in higher magnification in the inset. Data are represented as mean ± SEM. N = 6 for Saline and N = 7 for OLT1177 (200 mg/kg). Multiple t-test comparisons with Holm-Sidak's post hoc were used to analyze significant differences between groups. * $p < 0.05$ vs Saline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

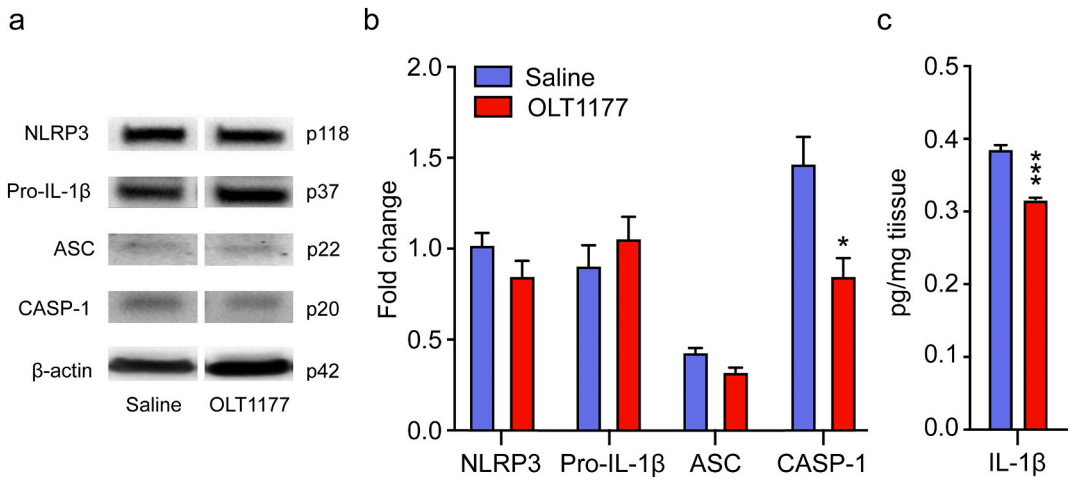

Fig. 4. Effect of OLT1177 on NLRP3 activation in the spinal cord at 24 h post-injury. (a) Representative immunoblotting showing NLRP3, pro-IL-1β, ASC, and caspase-1. Molecular weight (KDa) of each protein is marked on the left side. β-acting was used as loading control. (b) Graphs showing the quantification of NLRP3, pro-IL-1β, ASC, and caspase-1 in mice treated with saline or OLT1177 (200 mg/kg) after SCI. (c) ELISA levels of active IL-1β in the injured spinal cord after treatment with OLT1177 or vehicle. Data are represented as mean ± SEM. N = 5 for each group. Multiple t-test comparisons with Holm-Sidak's post hoc were used to analyze significant differences between groups. * $p < 0{,}01$ and ***$p < 0{,}0001$ vs Saline.

macrophages were reduced ~50% in mice treated with OLT1177, further demonstrating the effects of this compound on minimizing inflammation after SCI (Fig. 5c,e). OLT1177 also seemed to reduce the number microglia and neutrophils at 5 days after injury, but these differences were not significant.

Together these results demonstrate that OLT1177 ameliorates inflammation after SCI and leads to beneficial effects on functional and histopathological outcomes after SCI.

4. Discussion

Traumatic SCI leads to the loss of motor, sensory, and autonomic functions below the lesion site (Burns et al., 2012). Despite SCI is one of the main causes of death and disability (Ahuja et al., 2017), there is no effective treatment available (Hutson and Di Giovanni, 2019). Neurological deficits after SCI are due to the immediate neural damage produced by the injury itself but also to a secondary phase of tissue degeneration that occurs over several weeks after initial trauma (Muldoon et al., 2013). Various events are involved in triggering secondary damage after SCI, such as ischemia, excitotoxicity, necrosis, apoptosis, and inflammation among others (Oyinbo, 2011). The inflammatory response elicited after neurotrauma is thought to be one of the main contributors to secondary tissue damage (David et al., 2012). Therefore, suppressing inflammation could be a clinically beneficial approach to minimize functional impairments after acute SCI.

Today, there is a wide variety of drugs including biologics with anti-inflammatory properties. However, the use of anti-inflammatory reagents can be associated with unwanted side effects, including the risk of opportunistic infections (Dinarello et al., 2012). For instance, reactivation of *Mycobacterium tuberculosis* in patients receiving anti-TNF therapies can be 25 times higher than in untreated individuals (Solovic et al., 2010). Indeed, methylprednisolone for acute SCI, which was a standard of care for years following the publication of NASCIS II, was discontinued due to the lack of efficacy in neurological recovery and the adverse side effects such as increased risk of opportunistic infections (Hurlbert, 2009; Liu et al., 2019). Due to the nature of opportunistic infections, they become a major concern in many anti-inflammatory therapies. What distinguishes IL-1-based therapies from other agents is the lack of opportunistic infections. Although IL-1 blocking biologics increase the risk of routine bacterial infection, these are easily treated with antibiotics and opportunistic infections are rare with IL-1 blockers (Fleischmann et al., 2003).

IL-1β affects homeostatic functions such as body temperature, sleep, and cell metabolism among others (Dinarello, 2011; Dinarello et al., 2012). This cytokine is a regulator of inflammation, even in CNS conditions (Garlanda et al., 2013). Here, we found that the expression of IL-1β increased rapidly after SCI, reaching the highest levels during the first 24 h. The contribution of IL-1β to secondary damage after SCI is controversial. An early study using knockout mice for this cytokine revealed that the lack of IL-1β attenuated inflammation and protected against functional deficits and tissue damage (Boato et al., 2013). Nevertheless, a more recent publication did not report any protection on functional or histopathological outcomes after SCI in knockout mice for *Il1b* even though they showed reduced neuroinflammation (Bastien et al., 2015). Blockade of IL-1β can also be obtained by the administration of interleukin 1 receptor antagonist (IL-1RA, anakinra) (Dinarello et al., 2012). The administration of IL-1RA after SCI resulted into reduced infiltration of neutrophils (Yates et al., 2021) and tissue damage (Hasturk et al., 2015; Li et al., 2018). However, effects of IL-1RA in locomotor performance are unknown.

IL-18 deficiency resulted in reduced microglia activation after influenza A virus infection (Mori et al., 2001), possibly due to less immune cells infiltration. In contrast to IL-1β, we found that the protein levels of IL-18 were not increased at early stages after SCI but rather at later time points. This delayed expression pattern of IL-18 was also observed after spinal cord compression in rats (Jiang et al., 2017). In our mouse model of SCI, the peak of expression of IL-18 was found at 7 days post-lesion, coinciding with the maximal accumulation of microglial cells in the spinal cord (Francos-Quijorna et al., 2017). Indeed, a recent work reveals that the main cells expressing IL-18 in the injured spinal cord in humans are $TMEM119^{+}$ (Zrzavy et al., 2021). These findings suggest that microglial cells are likely to be the main source of IL-18. Previous reports revealed that IL-18 contributes to the pathogenesis of experimental autoimmune encephalomyelitis (Schif-Zuck et al., 2014) and constriction injury of the sciatic nerve (Pilat et al., 2016) suggesting that this cytokine may be also involved in the SCI physiopathology. In this line, a recent study revealed that the microRNA MiR-92b-5p increased the levels of IL-18 binding protein (IL-18BP), a high-affinity soluble IL-18 inhibitor, and ameliorated tissue damage and neurological deficits after SCI (Lin et al., 2019). Improved neurological recovery was also obtained with the administration of IL-18BP following TBI (Yatsiv et al., 2002). In fact, clinical studies suggest that increased levels of serum IL-18 are associated with the exacerbation of long-term outcomes in brain injured patients (Ciaramella et al., 2013).

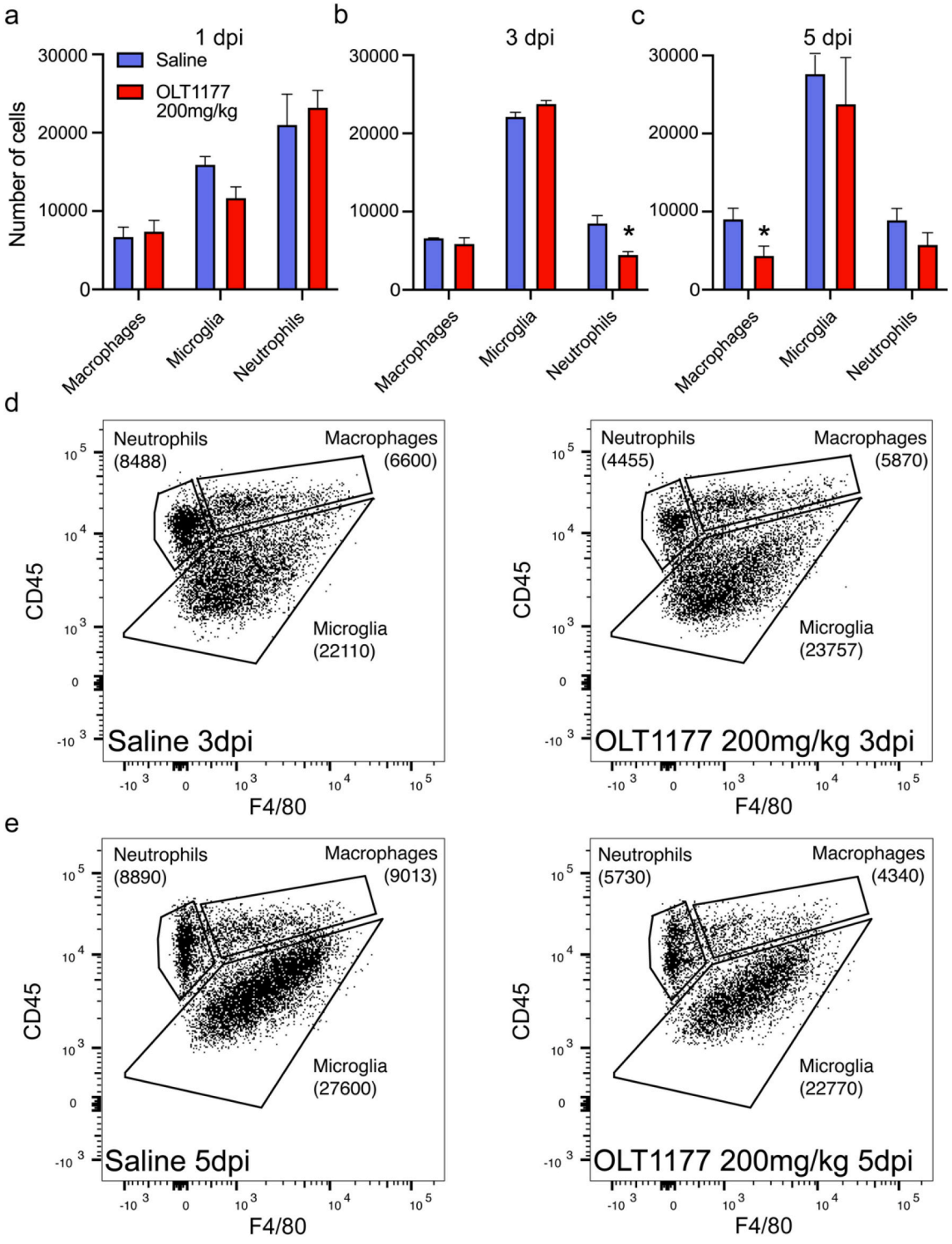

Fig. 5. Effects of OLT1177 in myeloid cell accumulation in the spinal cord after contusion injury. (a, b, c) Number of macrophages, microglia, and neutrophils in the spinal cord of mice treated with daily administration of OLT1177 (200 mg/kg) (red) or saline (blue) at 1 (a), 3 (b), and 5 (c) days after lesion. (d, e) Representative density plots of flow cytometry analysis showing microglia, macrophages, and neutrophils in the spinal cord at 3 (d) and 5 (e) days post-injury. Cell numbers are included in parenthesis. Note the marked reduction in the number of neutrophils and macrophages in the OLT1177 group in comparison with Saline. Data are represented as mean ± SEM. N = 4 for each group. Multiple t-test comparisons with Holm-Sidak's post hoc was to analyze significant differences between groups. * $p < 0.05$ vs Saline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As previously explained, both IL-18 and IL-1β are synthesized as inactive cytoplasmic precursors that require proteolytically processing to be active. This processing is performed by caspase-1, which is activated by inflammasomes, such as NLRP3 (Dinarello et al., 2012). Assembly of NLRP3 inflammasome requires two steps: priming and activation (Broz and Dixit, 2016; Yang et al., 2019). The priming step (signal 1) is provided by inflammatory stimuli such as TLR4 agonist that induce the NF-κB-mediated *Nlrp3* and *Il1b* gene expression. The expression of IL-18 as an inactive precursor is, in contrast, constitutive in the CNS (Alboni et al., 2010). The activation step (signal 2) starts with

the recognition of inflammatory ligands by NLRP3 sensor protein. This results in interaction with ASC via PYD and the recruitment of procaspase-1 via CARD forming an NLRP3-ASC-procaspase-1 complex. Proximity-induced auto-processing results in the formation of the catalytically active caspase-1, which initiates downstream responses, including the release of IL-1β and IL-18 (Broz and Dixit, 2016; Yang et al., 2019). In the context of nervous system, the NLRP3 inflammasome has been found to be activated in microglia and neurons in animal models of traumatic damage (Grace et al., 2016; Jiang et al., 2017, 2016; Zendedel et al., 2016).

Prevention of inflammasome activation exerts greater anti-inflammatory actions than blocking IL-18 or IL-1β separately since it minimized the signaling of both cytokines (Dinarello, 2019). During the last years, attention to NLRP3 inflammasome inhibitors has increased significantly (Yang et al., 2019). Here we demonstrated that the administration of the NLRP3 inhibitor OLT1177 improved neurological recovery and myelin preservation after SCI in female mice, especially when administered daily at the highest dose. At 200 mg/kg, OLT1177 reduced neuronal loss after SCI but it did not attenuate astrogliosis, at least by assessing GFAP intensity. These results support previous publications showing that inhibition of NLRP3 reduced neuronal apoptosis after SCI (Jiao et al., 2020) and did not alter GFAP expression in a mouse model of multiple sclerosis (Hou et al., 2020). Although all the experiments on this paper have been performed in female mice, no sex differences in lesion size or locomotor recovery have been found in this SCI contusion model in mice or rats (McFarlane et al., 2020; Walker et al., 2019).

In line with functional and histological outcomes, OLT1177 also attenuated the inflammatory response at the injury milieu. We found that OLT1177 reduced the processing of caspase 1 and, consequently, the levels of IL-1β, which it is indicative of reduced inflammasome activity. We speculate that longer exposition to OLT1177 after SCI, when levels of IL-18 are increased, would also reduce the processing of IL-18 since NLRP3 is critical for IL-18 activation. We also revealed that OLT1177 exerted a marked reduction in neutrophil and macrophages counts in the lesioned spinal. This correlates with previous publications showing that the inhibition of IL-1β signaling by gene deletion of *Il1r1* or *MyD88*, the adaptor downstream IL-1R1, also limited neutrophils accumulation in the lesioned spinal cord (Pineau et al., 2010). However, in that case, no changes in macrophages infiltration were observed (Pineau et al., 2010). Reduced infiltration of neutrophils after SCI was also described by blocking the actions of IL-1β through IL-1RA (Yates et al., 2021).

The effects of OLT1177 in SCI are in agreement with a recent work showing locomotor improvement in motor function after the deletion of the inflammasome adaptor protein ASC (Shiraishi et al., 2021). Functional recovery after SCI was also obtained with MCC950, another NLRP3 inhibitor (Jiao et al., 2020). These works support the therapeutic potential of NLRP3 inhibition for the treatment of acute neurotrauma. MCC950 was shown to mediate liver toxicity in phase II clinical trials for rheumatoid arthritis and its clinical development was discontinued (Mangan et al., 2018). Contrarywise, OLT1177 has demonstrated to be safe in its preclinical toxicology studies in humans (Marchetti et al., 2018a), in phase IIa clinical trials for gout flares (Klück et al., 2020) and phase Ib clinical trials for heart failure (Wohlford et al., 2020). Moreover, OLT1177 crosses the blood-brain barrier and it has proved efficacy even when given orally (Lonnemann et al., 2020; Marchetti et al., 2018a, 2018b; Sánchez-Fernández et al., 2019).

Overall, our data demonstrates that administration of OLT1177 exerts potent beneficial effects in a mice model of SCI. This study suggests that the inhibition of the NLRP3 inflammasome may constitute a valuable therapeutic avenue for the treatment of neurological conditions and present OLT1177 as a promising candidate for clinical development in SCI.

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Authors' contributions

JAA, DSK, CAD, and RLV designed the study; JAA performed most of the experiments; JGG, MP, and IFQ performed complementary experiments; JAA and RLV collected and analyzed the data; JAA, DSK, CAD, and RLV drafted the manuscript. All the authors have seen and approved the final version of the manuscript.

Declaration of Competing Interest

C.A.D. serves as Chairman of Olatec's Scientific Advisory Board, is co-Chief Scientific Officer, and receives compensation. D.B.S. serves as Chairman and Chief Executive Officer of Olatec. The authors declare that this study did not receive funding from Olatec Therapeutics LLC.

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