

Neuronal alarmin IL-1 α evokes astrocyte-mediated protective signals: Effectiveness in chemotherapy-induced neuropathic pain

Lorenzo Di Cesare Mannelli^{a,1}, Laura Micheli^{a,1}, Chiara Cervetto^b, Alessandra Toti^{a,*}, Elena Lucarini^a, Carmen Parisio^a, Manuela Marcoli^b, Carla Ghelardini^a

^a Department of Neuroscience, Psychology, Drug Research and Child Health - Neurofarba - Pharmacology and Toxicology Section, University of Florence, Florence, Italy

^b Department of Pharmacy - DIFAR - Pharmacology and Toxicology Section, University of Genova, Genova, Italy

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ABSTRACT

The distinction between glial painful and protective pathways is unclear and the possibility to finely modulate the system is lacking. Focusing on painful neuropathies, we studied the role of interleukin 1 α (IL-1 α), an alarmin belonging to the larger family of damage-associated molecular patterns endogenously secreted to restore homeostasis.

The treatment of rat primary neurons with increasing doses of the neurotoxic anticancer drug oxaliplatin (0.3–100 μ M, 48 h) induced the release of IL-1 α . The knockdown of the alarmin in neurons leads to their higher mortality when co-cultured with astrocytes. This toxicity was related to increased extracellular ATP and decreased release of transforming growth factor β 1, mostly produced by astrocytes.

In a rat model of neuropathy induced by oxaliplatin, the intrathecal treatment with IL-1 α was able to reduce mechanical and thermal hypersensitivity both after acute injection (100 ng and 300 ng) and continuous infusion (100 and 300 ng/die⁻¹). Ex vivo analysis on spinal purified astrocyte processes (gliosomes) and nerve terminals (synaptosomes) revealed the property of IL-1 α to reduce the endogenous glutamate release induced by oxaliplatin. This protective effect paralleled with an increased number of GFAP-positive cells in the spinal cord, suggesting the ability of IL-1 α to evoke a positive, conservative astrocyte phenotype.

Endogenous IL-1 α induced protective signals in the cross-talk between neurons and astrocytes. Exogenously administered in rats, IL-1 α prevented neuropathic pain in the presence of spinal glutamate decrease and astrocyte activation.

1. Introduction

Chemotherapy treatments can promote the onset of neuropathic diseases (Griffith et al., 2017) causing a damage of the peripheral nervous system and alterations of the central nervous system (CNS) which consequently induce chronic pain hypersensitivity (Branca et al., 2018; Di Cesare Mannelli et al., 2012, 2015b). Oxaliplatin is one of the most neurotoxic anticancer drug inducing a chronic neuropathic syndrome that forces oncologists to dose reduction or therapy discontinuation; cancer survivors experience the persistence of sensory disorders for years after the end of therapy (Kerckhove et al., 2017). No treatment can prevent chemotherapy-induced neuropathy, the best available data support a moderate recommendation regarding duloxetine (Hershman et al., 2014).

The etiopathology of oxaliplatin neurotoxicity involves a direct damage to the peripheral nervous system, evolving in a chronic syndrome that encompasses the spinal cord and pain-related brain areas (Di Cesare Mannelli et al., 2014) with neurons hyperexcitability and glial cells activation (Di Cesare Mannelli et al., 2013a, 2015b). In particular, in several neuropathic syndromes, astrocytes emerged as main players in pain physiopathology as well as in neuroprotective mechanisms (Benarroch, 2010; Milligan and Watkins, 2009). At molecular level, a “cascade” of cytokine production, among which interleukin 1 (IL-1) and tumor necrosis factor α (TNF α) are the main ones, leads to chronic pain promotion (Miller et al., 2009). IL-1 is a prototypic proinflammatory cytokine involved in the immune response to infection or injury and it is the main mediator of inflammatory responses to acute and chronic CNS diseases (Allan et al., 2005; Rothwell, 2003). IL-1 refers to two

* Corresponding author.

E-mail address: alessandra.toti@unifi.it (A. Toti).

¹ These authors contributed equally to this work.

cytokines, IL-1 α and IL-1 β , encoded by two separate genes on chromosome 2 (Bensi et al., 1987; Webb et al., 1986). They share a similar three-dimensional structure with a so-called β -trefoil fold formed by 12 β -strands, even if they have different amino acid sequences with only 26% homology (Graves et al., 1990; Priestle et al., 1989). They are translated as 31- to 33- kDa precursors (pro-IL-1 β and pro-IL-1 α) which are subsequently proteolytically processed to the 17- kDa protein mature active form (Kobayashi et al., 1990). Both cytokines are produced by immune (Dinarello, 1994) and nervous cells such as neurons, microglia, astrocytes and oligodendrocytes (Hanisch, 2002; Shaftef et al., 2008; Vela et al., 2002). Moreover, they both bind IL-1 receptor type I (IL-1RI) (Sims et al., 1988). As autocrine signal, IL-1 dependent IL-1RI stimulation leads to the expression of pro-IL-1 α and pro-IL-1 β (Di Paolo and Shayakhmetov, 2016). Nevertheless, relevant differences mark the fate of their signals. The cleavage of pro-IL-1 β is a well established mechanism mediated by the inflammasome complex through the activation of caspase-1 (Franchi et al., 2009); on the contrary pro-IL-1 α is not a substrate for caspase-1 (Lamkanfi and Dixit, 2014; Martinon et al., 2009; van de Veerdonk et al., 2011) leaving its maturation processing and trafficking largely unclear (Brough and Denes, 2015) even if it is believed to be cleaved to a mature form by calcium-dependent proteases of the calpain family (Luheshi et al., 2009).

Unlike pro-IL-1 β , pro-IL-1 α is able to bind IL-1RI due to the similar conformation of the mature form (mIL-1 α) (Dinarello, 2011; Kim et al., 2013); alternatively, the pro-IL-1 α can act as transcription factor via nuclear translocation, independently from IL-1RI binding (Gabay et al., 2010; Weber et al., 2010) due to the presence of an N-terminal nuclear localization signal that directs this cytokine to the nucleus (Werman et al., 2004; Wessendorf et al., 1993). The mature IL-1 α is a dual-function cytokine since it is able to exert its actions through mechanisms that are dependent or independent from IL-1RI binding by signaling both as a secreted and as a membrane-bound cytokine, whereas IL-1 β exists only as released protein (Di Paolo and Shayakhmetov, 2016; Kurt-Jones et al., 1985). IL-1 α is an alarmin (damage-associated molecular pattern; DAMP) released in response to injury or tissue necrosis (England et al., 2014), but it is constitutively expressed at the steady state in various cell types of healthy tissues, while IL-1 β is absent in cells and its expression is induced by growth factors signaling and proinflammatory or stress-associated stimuli (Di Paolo and Shayakhmetov, 2016). Further, IL-1 α , but not IL-1 β , induces brain cells to generate the LG3 neuroprotective protein fragment of the extracellular matrix component perlecan, a prominent component of the blood-brain barrier (Saini et al., 2011).

The dual profile of IL-1 α seems to conceal also protective functions that slowly emerge clarifying the role of this alarmin. A neuroprotective effect of IL-1 α (at low sub-pathological doses) has been described both in vitro, on cortical neurons subjected to oxygen-glucose deprivation, and in vivo, when systematically administered in mice who underwent a transient stroke (Salmeron et al., 2019). Systemically administered in rats, IL-1 α , was \sim 3000 folds less able to exert nociceptive effects compared to IL-1 β (Ferreira et al., 1988). Moreover, Mika et al. highlighted that intrathecal administration of IL-1 α (in a concentration range of 50–500 ng) in rat exposed to chronic constriction injury of the sciatic nerve reduced allodynia and hyperalgesia (Mika et al., 2008). The mechanisms of the homeostatic properties of IL-1 α as well as the therapeutic potential of the modulation of its signaling remain largely undiscovered.

The present work aimed to study the neuroprotective and pain modulatory properties of IL-1 α in a model of neuropathy induced by the anticancer drug oxaliplatin. In cell cultures, the role of IL-1 α in the crosstalk between neurons and astrocytes was investigated; in rats, pain threshold, glial activation profile and cell hyperexcitability mediators were evaluated after intrathecal IL-1 α administration.

2. Materials and methods

2.1. Cell culture preparation

Primary cultures of astrocytes were obtained according to the method described by McCarthy and de Vellis (McCarthy and de Vellis, 1980). Briefly, the cerebral cortex of new-born Sprague-Dawley rats (Envigo, Varese, Italy) post natal day 1–3 was dissociated in Hanks balanced salt solution containing 0.5% trypsin/0.2% EDTA and 1% DNase (Merck, Milan, Italy) for 30 min at 37 °C. The suspension was mechanically homogenized and filtered with 70 μ m filters. Cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose, (Merck, Milan, Italy) supplemented with 20% FBS (Life Technologies, Milan, Italy), 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin (Merck, Milan, Italy). Confluent primary glial cultures were used to isolate astrocytes, removing microglia and oligodendrocytes by shaking. After 21 days of culture, astrocytes were plated according to experimental requirements. GFAP-positive cells were 95–98%.

Cortical neurons were isolated from rat embryos (embryonic days 14–16; Sprague-Dawley rats; Envigo, Varese, Italy). The cortex was dissociated in Hanks balanced salt solution containing 0.5% trypsin/0.2% EDTA and 1% DNase for 10 min at 37 °C under mild stirring. The suspension was mechanically homogenized and filtered with 100 μ m filters. Cells were plated in Neurobasal Medium supplemented with 2% B27 Supplement (Life Technologies, Milan, Italy), 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin (Merck, Milan, Italy) to allow the selective growth of neurons. After 5 days of culture, neurons were plated according to experimental requirements.

Both astrocyte and neuron cultures were released from the culture plates by treatment with 0.5% trypsin in PBS containing 0.03% EDTA for 1 min and allowed to seed for at least 24 h before being used for the experiments. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

2.2. Neuron astrocyte co-culture

Neurons and astrocytes were separately cultured for 5 and 21 days, respectively. Then astrocytes were plated in transwells (0.45 μ m pore size; BD Biosciences, Durham, NC, USA) while neurons were plated in multiwells as described below. The day after plating, inserts containing astrocytes were placed on the wells containing primary neurons and, after 24 h, treated with oxaliplatin (Sequoia Research Products, Pangbourne, UK). In this model, although neurons and astrocytes face each other, they are out of contact, and the effect of soluble factors released from activated astrocytes on neurons can be studied, allowing separate analysis of neuronal and glial populations.

2.3. Cell plating protocol

For cell viability assay, in co-culture experiments, primary neurons were plated in 24-well plates (2×10^5 cells/well) while primary astrocytes were plated in appropriate transwells (8×10^4 cells/well).

For the dosage of IL-1 α , TGF β 1 and extracellular ATP, neurons or astrocytes in mono-culture were plated in 6-well plates (5×10^5 cells/well). In co-culture experiments neurons were plated in 6-well plates (7×10^5 cells/well) while astrocytes were plated in appropriate transwells (4.5×10^5 cells/well).

2.4. Cell viability assay

Cell viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Merck, Milan, Italy) as an index of mitochondrial compartment functionality. Cells were treated for 48 h with oxaliplatin (0.3–100 μ M). 1 mg/mL MTT was added to each well and incubated for 2 h at 37 °C. After washing, formazan

crystals were dissolved in 800 μ L dimethyl sulfoxide. The absorbance was measured at 580 nm. Experiments were performed in quadruplicate on at least three different cell batches.

2.5. IL-1 α release assay

Cells were treated for 48 h with oxaliplatin (0.3–100 μ M). After treatment, supernatants were collected and the amount of IL-1 α released in culture medium was evaluated by a rat IL-1 α ELISA kit (biorbyt, USA) according to manufacturer's instruction. Normalization was performed on total protein content.

2.6. IL-1 α knockdown

IL-1 α expression was downregulated in neurons by the treatment with a specific siRNA. siRNA oligonucleotides were obtained from IDT (Italy):

5'-GGUCAAUUGGCAGAACUGUAUUCUUCU-3'.

Transfection with siRNA (5 pmol/1 \times 10⁵ cells; transfection reagent Saint Red, Synvolux, The Netherlands) was carried in neurons for 72 h. Control cells were transfected with negative control siRNA (scrambled sequence) (IDT, Italy).

2.7. TGF β 1 dosage

Cells were treated with oxaliplatin 1 μ M for 48 h. After treatment, the culture medium was collected and used for determining the amount of TGF β 1 by ELISA kit (BioLegend, Inc., USA). Culture medium samples were activated by acidification and processed according to the manufacturer's protocol. The absorbance was measured at 450 nm. Declared assay sensibility is 1 pg/mL. Normalization was performed on total protein content.

2.8. Extracellular ATP quantification

24 h after plating in complete growth medium, cells were starved and stimulated with oxaliplatin (0.3–100 μ M) for 48 h. After treatment, the medium was harvested and 50 μ L were processed following the manufacturer's procedure (ATPlite – Luminescence ATP Detection Assay System, PerkinElmer Italia, Milan, Italy). The extracellular ATP was quantified by luminescence using a VICTOR microplate reader (PerkinElmer, Milan, Italy). Each experiment was performed three times, in triplicate. Normalization was performed on total protein content.

2.9. Animals

For all the experiments described below, male Sprague–Dawley rats (Envigo, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure were used. Animals were housed in Ce.S.A.L. (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 \times 41 cm²); animals were fed with standard laboratory diet and tap water ad libitum, kept at 23 \pm 1 $^{\circ}$ C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278–01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 498/2017-PR) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of

animals used.

2.10. Intrathecal catheterization

Rats were anesthetized with 2% isoflurane and the intrathecal catheter was surgically implanted according to (Yaksh and Rudy, 1976). Rats were shaved on the back of the neck and placed in the stereotaxic frame with the head securely held between ear bars. The skin over the nap of the neck was cleaned with ethyl alcohol and incised for 1 cm. The muscle on either side of the external occipital crest was detached and retracted to expose about 3–4 mm² of the atlanto-occipital membrane. The membrane was incised by a needle, which led to the escape of cerebrospinal fluid. The caudal edge of the cut was lifted and about 7.0 cm of 28 G polyurethane catheter (0.36 mm outer diameter; 0.18 mm inner diameter; Alzet, USA) was gently inserted into the intrathecal space in the midline, dorsal to the spinal cord until the lumbar enlargement. The exit end of the catheter was connected to 4.0 cm polyurethane (0.84 mm outer diameter; 0.36 mm inner diameter) and was taken out through the skin, flushed with saline solution, sealed and securely fixed on the back of the head with a silk suture. The proper placement of the catheter was verified with intrathecal injection of 2% lidocaine (15 μ L), which temporarily paralyzed the hind limbs if the catheter was intact and exactly placed (Micheli et al., 2015b). For the chronic treatments, osmotic pumps (Alzet, USA) were used. Pumps were attached with the exit end of the catheter and subcutaneously fixed on the back of the head. The incision site in the skin was sutured with polyamide suture and animals were allowed to recover for 24 h before the study began.

Only animals free from motor impairments induced by the surgical operation for the catheter implantation were used for the behavioral tests. The evaluation of motor dysfunctions were investigated using the Rota rod test (Micheli et al., 2015a). As already mentioned, the animals displaying motor disabilities (approximately 10%) were excluded from the behavioral measurements.

2.11. Oxaliplatin – induced neuropathic pain model

Male Sprague–Dawley rats were treated with 2.4 mg kg⁻¹ oxaliplatin (Sequoia Research Products, Pangbourne, UK), administered intraperitoneally (i.p.) for 10 days (Resta et al., 2018). Oxaliplatin was dissolved in a 5% glucose-water solution. Control animals received an equivalent volume of 5% glucose - water solution i.p. (vehicle).

2.12. IL-1 α treatments

IL-1 α (mature form) was purchased from R&D System (cod: 500-RL-005; Minneapolis, USA) and dissolved in sterile saline solution. Acute measurements were performed after the single i.t. administration of IL-1 α 100 ng/10 μ L and 300 ng/10 μ L (according to Mika et al., 2008) in oxaliplatin treated rats when neuropathy was well established (after 10 oxaliplatin injections over two weeks). Behavioral tests were carried out 15 min, 30 min, 45 min, 1 h, 3 h, 6 h, 24 h and 48 h after injection. Vehicle + vehicle and oxaliplatin + vehicle-treated animals received the i.t. injection of 10 μ L sterile saline solution.

For the repeated infusion, IL-1 α concentrations were adjusted to release respectively 100 ng/die and 300 ng/die i.t. from a mini-osmotic pump ALZET 1002 (USA) with a flow rate of 0.25 mL/h for 10 days. Final solutions were filtered by a 0.22 μ m pore size Hydrophilic PVDF membrane (Millipore, Italy). Mini-pump were attached to the described polyurethane catheters and filled with compounds or vehicle solutions, respectively. All mini-osmotic pumps were incubated in sterile saline solution overnight at 37 $^{\circ}$ C before the implantations (day 0). Oxaliplatin treatment started 1 day after the surgery for the catheter implantation and was performed daily until the end of the experiment (day 10). Behavioral tests were performed daily. Vehicle + vehicle and oxaliplatin + vehicle-treated animals received an equal i.t. infusion of sterile saline solution.

2.13. Paw pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy) according to the method described by (Bird et al., 2016; Leighton et al., 1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical mechanical probe. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. An arbitrary cut-off value of 100 g was adopted. The data were collected by an observer who was blinded to the protocol.

2.14. Cold plate test

Thermal allodynia was assessed using the Cold plate test. The animals were placed in a stainless steel box (12 cm × 20 cm × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 °C ± 1 °C. Pain-related behaviour (licking of the hind paw) was observed, and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s (Baptista-de-Souza et al., 2014).

2.15. Immunofluorescence

Rats were sacrificed, the L4/L5 segments of the spinal cord were exposed from the lumbovertebral column via laminectomy and identified by tracing the dorsal roots from their respective DRG. Formalin-fixed cryostat sections (7 µm) were washed three times with phosphate-buffered saline (PBS 1×), 0.3% Triton X-100 for 5 min and then were incubated, at room temperature, for 1 h in blocking solution (PBS, 0.3% Triton X-100, 5% albumin bovine serum; PBST). The sections were subsequently incubated with anti-GFAP (G3893, Merck, 1:500, mouse) diluted in PBST overnight at 4 °C. The following day, slides were washed three times in PBS 1× for 5 min and then sections were incubated in goat anti-mouse IgG secondary antibody labeled with Alexa Fluor 568 (1:500; Invitrogen, Carlsbad, USA) in PBST at room temperature for 2 h, in the dark. After three washes with PBS 1× for 10 min, slices were mounted using Fluoromount™ (Life Technologies-Thermo scientific, Rockford, IL, USA) as a mounting medium. Negative control sections (no exposure to the primary antisera) were processed concurrently with the other sections for all immunohistochemical studies. Images were acquired using a motorized Leica DM6000 B microscope equipped with a DFC350FX camera (Leica, Mannheim, Germany). GFAP-positive cells were quantified by means of the automatic thresholding and segmentation features of ImageJ.

2.16. Preparation of purified nerve terminals and astrocyte processes

Purified nerve terminals (synaptosomes) and astrocyte processes (gliosomes) were prepared from the spinal cord of oxaliplatin-treated (and vehicle-treated) rats on day 15 of treatment, as previously reported (Amaroli et al., 2018; Bruzzone et al., 2010; Cervetto et al., 2015). Briefly, the spinal cord was rapidly removed and placed in ice-cold medium, then homogenized in 0.32 mM sucrose with Tris-HCl, pH 7.4, using a glass-Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 4 °C, 1000g) to remove nuclei and debris; the supernatant was stratified on a discontinuous Percoll gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33500 g (5 min). The layer containing gliosomes (between 2% and 6% (v/v) Percoll) and the layer containing synaptosomes (between 10% and 20% Percoll) were collected and washed by centrifugation; gliosomes and synaptosomes were then suspended in HEPES medium (mM: NaCl 128, KCl 2.4, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.0, and HEPES 10 with glucose 10, pH 7.4).

2.17. Glutamate release from superfused synaptosomes and gliosomes

The release of endogenous glutamate was studied from synaptosomes or gliosomes as previously described (Amaroli et al., 2018; Arena et al., 2020; Cervetto et al., 2015; Polini et al., 2020). Briefly, synaptosomes or gliosomes were transferred to parallel superfusion chambers at 37 °C and superfused (0.5 mL/min) with standard medium. After 33 min superfusion, superfusate fractions were collected (3-min samples) till the end of the experiment; after 38 min superfusion, synaptosomes were exposed (120 s) to 2'-3'-O-(benzoylbenzoyl) ATP (BzATP; Merck, Milan, Italy); the effect of IL-1α (1.0 ng/mL) was evaluated by adding the drug 8 min before BzATP. The amount of endogenous glutamate released in the fractions collected was measured by high-performance liquid chromatography, as previously described (Cervetto et al., 2012; Polini et al., 2020). The analytical method involved automatic precolumn derivatization (Waters Alliance; Milford, MA) with o-phthalaldehyde, followed by separation on a C18 reverse-phase chromatography column (Chrompack International, 10 cm × 4.6 mm, 3 µm) and fluorimetric detection. Homoserine was used as an internal standard. The detection limit was 100 fmol/µL. Protein determinations were carried out according to Bradford (Bradford, 1976). The amount of endogenous glutamate released in the fractions was expressed as pmol/mg protein. The mean endogenous glutamate release in B1 and B2 fractions was taken as the 100% control value for each chamber. The BzATP-evoked release (overflow) was calculated by subtracting the basal efflux from the total glutamate released in the fractions collected during and after stimulation, and was evaluated as percent variations with respect to the control value for the chamber. Drugs were dissolved in distilled water or in physiological medium.

2.18. Statistical analysis

Results were expressed as mean ± S.E.M. and analysis of variance (ANOVA) was performed. A Bonferroni significant difference procedure was used as post hoc comparison. All assessments were made by researchers blinded to cell treatments. Data were analysed using the "Origin 8.1" software (OriginLab, Northampton, USA).

3. Results

3.1. Oxaliplatin treatment induces the release of IL-1α in mono- and co-cultures of astrocytes and neurons

Oxaliplatin induces neurons and astrocytes mortality both in mono- and co-culture (Di Cesare Mannelli et al., 2015c). As previously reported, 1 µM oxaliplatin leads to 40% reduction of neurons viability in mono- and co-culture, while astrocytes are more resistant showing a significant mortality only over 10 µM. Given these premises, IL-1α was measured in the medium of neuron/astrocytes co-culture after 48 h of incubation with increasing concentrations of oxaliplatin (0.3–100 µM). Cytokine release increased concentration-dependently starting from 1 µM oxaliplatin (23.2 ± 2.3 pg/mL in comparison to 15.1 ± 1.8 pg/mL of controls, indicated as a dashed line) (Fig. 1a). The 1 µM oxaliplatin treatment of mono-cultures revealed a higher cytokine release from neurons (25.1 ± 2.2 pg/mL) compared to astroglia (17.4 ± 2.1 pg/mL) (Fig. 1b). Moreover, the amount of IL-1α released in co-culture medium cannot be read as the sum of mono-culture, since the use of transwells implies an increased volume of medium, which decreases the concentration of IL-1α (Fig. 1b).

3.2. Effect of neuronal IL-1α knockdown on neuron viability, ATP release and TGFβ1 expression

In order to evaluate the role of this cytokine in neuronal damage response, a knockdown of IL-1α was selectively performed in neurons using specific small interfering RNA (85% decrease of IL-1α expression

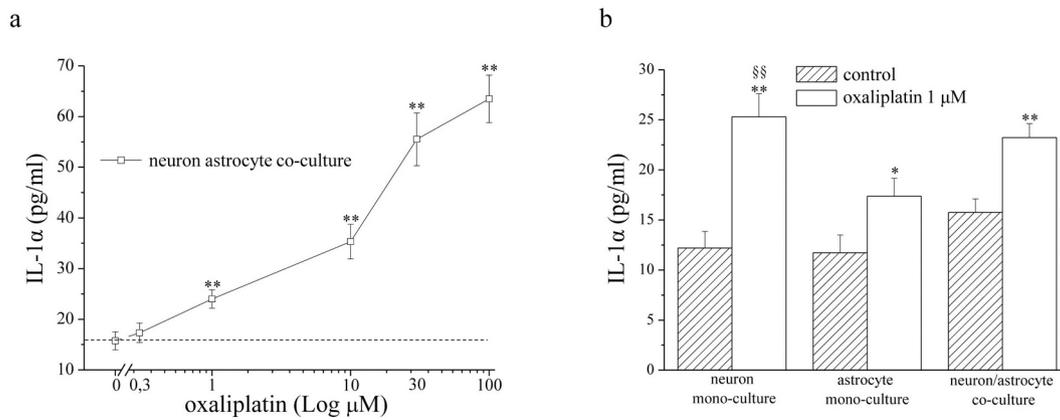


Fig. 1. IL-1 α release induced by oxaliplatin treatment in neurons and/or astrocytes. a) Dosage of IL-1 α in culture medium of neurons co-cultured with astrocytes in the presence of oxaliplatin (0.3–100 μ M) (immunoenzymatic method). Control co-culture was indicated as a dashed line. Values are expressed in pg/mL as mean \pm S.E.M. of 4 experiments. b) Comparison of IL-1 α release in neuron and astrocyte mono- and co-culture treated with 1 μ M oxaliplatin. Values are expressed in pg/mL as mean \pm S.E.M. of 4 experiments. All experiments are normalized to total protein content. One way ANOVA was performed followed by a Bonferroni's significant difference procedure. * $P < 0.05$ and ** $P < 0.01$ vs respective controls; §§ $P < 0.01$ vs oxaliplatin treated astrocytes in mono-culture.

after 72 h incubation vs scrambled treatment, evaluated by western blot analysis; data not shown). The co-culture of IL-1 α silenced neurons with astrocytes allowed to observe a comparable reduction of cytokine concentration in the medium of control and oxaliplatin-treated (1 μ M, 48 h) cells (Fig. 2a). The increased release of IL-1 α in scrambled co-culture under oxaliplatin treatment was not significant, maybe because scrambled cells are treated with Lipofectimine and scrambled siRNA which can interfere with normal cell function. The measurement of neuron viability in co-culture treated with increasing concentration of oxaliplatin (0.3–100 μ M, 48 h; Fig. 2b) revealed that the knockdown of IL-1 α made cells more prone to neurotoxicity (e.g. 1 μ M oxaliplatin increased cell mortality by about 50% in IL-1 α siRNA vs scrambled). The presence of an excitotoxic environment was confirmed by the increased release of extracellular ATP in IL-1 α siRNA co-culture medium compared to scrambled co-culture medium under increasing concentration of oxaliplatin (0.3–100 μ M, 48 h; Fig. 2c) in a concentration dependent manner. Conversely, the silencing of neuronal IL-1 α was correlated to low levels of the protective cytokine TGF β 1 (Fig. 2f). In co-culture, increasing concentrations of oxaliplatin (0.3–100 μ M, 48 h) evoked a progressively increased release of TGF β 1 in the medium, and the increment was significantly lower in the IL-1 α siRNA neuron/astrocyte co-culture compared to scrambled co-culture. In mono-culture, TGF β 1 was significantly higher in neurons medium when treated for 48 h with 1 μ M oxaliplatin (3.4 ± 0.3 pg/mL compared to control neuron mono-culture value 0.3 ± 0.5 pg/mL; Fig. 2d), while 1 μ M oxaliplatin did not increase TGF β 1 in astrocytes (Fig. 2e) and the basal level of the factor was higher in glial cells compared to neurons (14.5 ± 1.2 pg/mL vs neuron value 0.3 ± 0.5 pg/mL). Moreover the TGF β 1 release in astrocytes mono-culture was decreased of almost 30% by the presence for 48 h of 80 μ M fluorocitrate (5.2 ± 2.5 pg/mL vs control astrocytes mono-culture value 14.5 ± 1.2 pg/mL, Fig. 2e), a specific astrocyte metabolism inhibitor (Xu et al., 2016).

3.3. Pain relieving effect of IL-1 α in acute and chronic condition after i.t. injection

In order to analyse the central effect of IL-1 α in vivo, acute and chronic intrathecal infusions of the cytokine were performed in neuropathic rats through a surgically implanted catheter. Neuropathy was induced by repeated administration of oxaliplatin (2.4 mg kg $^{-1}$ i.p., 10 injections) according to previously published protocol (Micheli et al., 2015b). The Paw pressure test was performed to assess the response to a noxious mechanical stimulus (hyperalgesia-like phenomenon). On day 14, the weight tolerated on posterior paw by oxaliplatin-treated rats was

significantly reduced with respect to the control group (42.8 ± 1.3 g vs 66.6 ± 2.1 g, respectively). On the same day, the acute administration of IL-1 α (100 ng i.t. and 300 ng i.t.) significantly increased the weight tolerated on posterior paw starting 15 min after injection up to 6 h (Fig. 3a).

In Fig. 3b and c the effects of a continuous i.t. infusion of IL-1 α are shown. To allow a constant delivery of IL-1 α over days, mini-osmotic pumps connected to the catheters were used. The pumps were filled to ensure a daily infusion of 100 or 300 ng of IL-1 α . The pain threshold was measured as response to a noxious mechanical stimulus (Paw pressure test) and to a non-noxious cold stimulus (allodynia-like phenomenon, Cold plate test) every day starting from the first day of IL-1 α infusion that matched with the first day of oxaliplatin injection (day 1). Oxaliplatin significantly reduced the weight tolerated by the animals on posterior paws and induced cold hypersensitivity starting on day 2, these effects increased up to day 10. Continuous infusion of both dosages of IL-1 α protected the animal from the development of oxaliplatin-induced neuropathic pain. IL-1 α efficacy remained stable over time counteracting oxaliplatin-dependent hypersensitivity until the end of the experiment (day 10) (Fig. 3b–3c).

3.4. GFAP is increased by oxaliplatin and IL-1 α treatment in spinal cord

Immunofluorescence on spinal cord tissues obtained by oxaliplatin-treated rats continuously infused with IL-1 α (100 and 300 ng i.t.) has been performed collecting tissues on day 10. Astrocytes were studied using GFAP as a cell-selective antigen. As shown in Fig. 4, oxaliplatin increased the number of GFAP-positive cells indicating astrocyte reactivity. IL-1 α 300 ng die $^{-1}$ significantly increases GFAP positive cells compared to both oxaliplatin treatment alone and in combination with IL-1 α 100 ng die $^{-1}$ (Fig. 4a–4b).

3.5. IL-1 α reduces the P2X7 mediated release of glutamate spinal cord astrocyte processes and nerve terminals

In order to analyse the effect of IL-1 α on the neuronal and astrocytic release of glutamate from the spinal cord, purified nerve terminals (synaptosomes) and astrocyte processes (gliosomes) were acutely prepared ex vivo from the spinal cord of control and oxaliplatin-treated rats. Synaptosomes have been proved to represent a purified preparation of the nerve terminals, containing neurotransmitter-loaded vesicles and competent for neurotransmitter secretion, negligibly contaminated by non-neuronal particles (Cervetto et al., 2016; Venturini et al., 2019), while gliosomes represent a purified preparation of the astrocyte

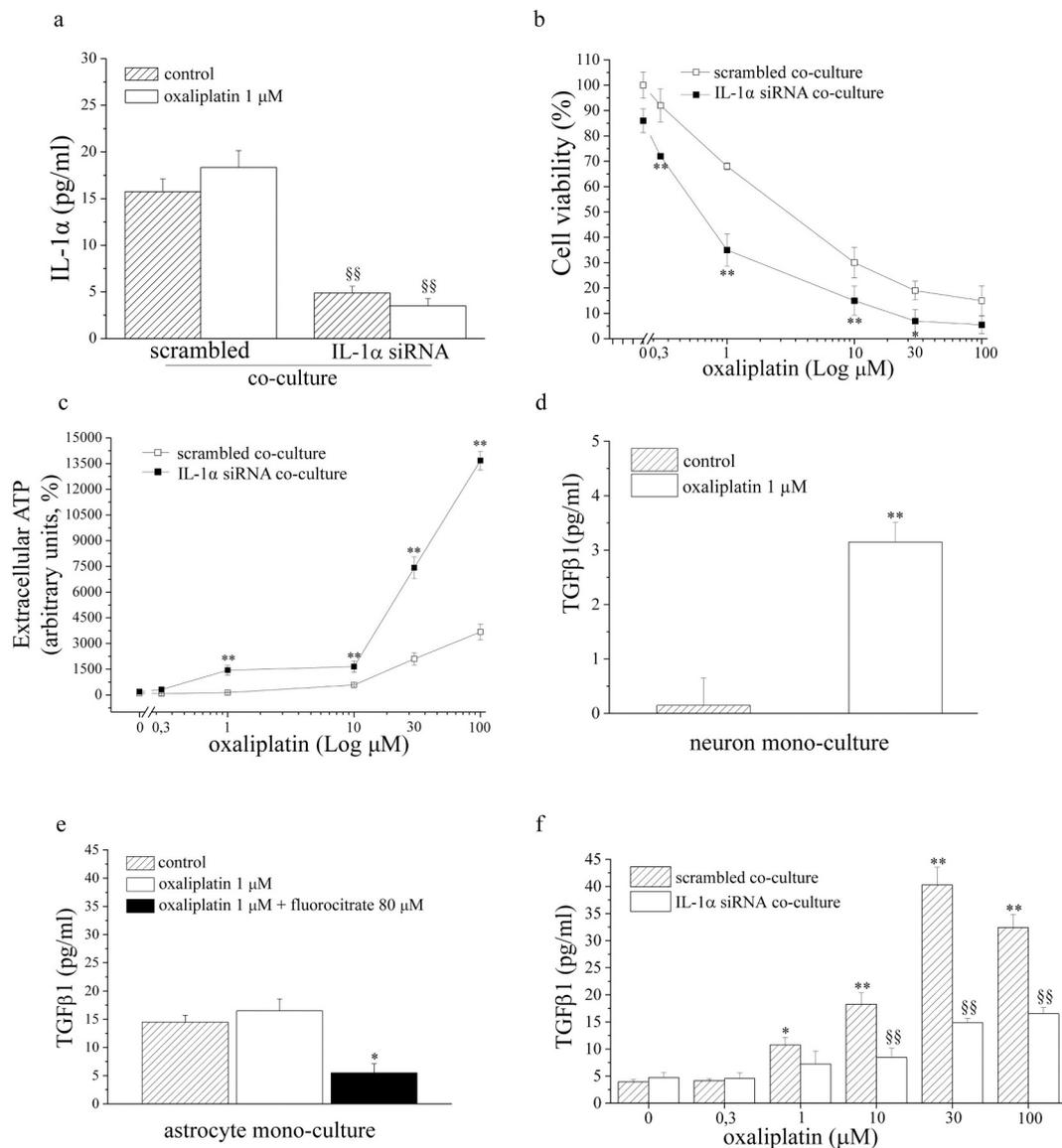


Fig. 2. Effects of IL-1 α -knockdown in neurons in mono-culture and in co-culture with astrocytes. a) Comparison of IL-1 α release in control (scrambled) neuron/astrocyte co-culture and IL-1 α -knockdown neuron (IL-1 α siRNA)/astrocyte co-culture treated with 1 μ M oxaliplatin for 48 h. An immunoenzymatic method was used to measure IL-1 α in culture medium and values are expressed in pg/mL as mean \pm S.E.M. of 4 experiments. Normalization was performed on total protein amount. b) Cell viability of IL-1 α siRNA neuron/astrocyte co-culture compared to scrambled co-culture after 48 h oxaliplatin treatment). Cell viability was measured by MTT assay and values are expressed as a percentage of the control mono-culture without oxaliplatin as mean \pm S.E.M. of 4 experiments. c) Extracellular ATP concentration in the culture medium of scrambled neuron/astrocytes co-culture and IL-1 α siRNA neuron/astrocyte treated with oxaliplatin for 48 h; extracellular ATP was measured by a bioluminescence detection kit. Results are expressed as % of arbitrary units, normalized on total protein amount and values are the mean \pm S.E.M. of 3 experiments. d) Concentration of the anti-inflammatory cytokine TGF β 1 measured in the culture medium of neurons after 48 h incubation with oxaliplatin 1 μ M. Normalization was performed on total protein amount. e) Concentration of TGF β 1 measured in the culture medium of astrocytes after 48 h incubation with oxaliplatin 1 μ M. 80 μ M fluorocitrate was used as specific astrocyte metabolism inhibitor. f) Concentration of TGF β 1 measured in the culture medium of scrambled neuron/astrocytes co-culture and IL-1 α siRNA neuron/astrocyte co-culture treated with increasing concentrations of oxaliplatin for 48 h. Results are expressed as pg/mL of TGF β 1 and values are expressed as the mean \pm S.E.M. of 4 experiments. Normalization was performed on total protein amount. One way ANOVA was performed followed by a Bonferroni's significant difference procedure. * P < 0.05 and ** P < 0.01 vs control; §§ P < 0.01 vs scrambled.

processes, containing gliotransmitter-loaded vesicles and competent for gliotransmitter secretion, with negligible neuronal contamination (Cervetto et al., 2016; Venturini et al., 2019).

The basal endogenous glutamate outflow in the first two fractions collected from superfused purified rat spinal cord gliosomes amounted to 66.5 ± 15.6 pmol/mg protein/3 min ($n = 4$) in oxaliplatin-treated rats, and to 84.2 ± 33.7 pmol/mg protein/3 min ($n = 4$) in vehicle-treated rats. Notably, the P2X7 agonist BzATP (100 μ M) did not evoke glutamate overflow from the gliosomes of vehicle-treated rats, while evoking a glutamate release response from the gliosomes obtained from

the oxaliplatin-treated rats. IL-1 α inhibited the release of glutamate from the gliosomes obtained from oxaliplatin-treated animals (Fig. 5a).

The basal endogenous glutamate outflow in the first two fractions collected from superfused purified rat spinal cord synaptosomes amounted to 69.5 ± 16.5 pmol/mg protein/3 min ($n = 4$) in oxaliplatin-treated rats, and to 82.4 ± 15.4 pmol/mg protein/3 min ($n = 4$) in vehicle-treated rats. The BzATP (100 μ M)-evoked glutamate overflow was higher from synaptosomes obtained from the oxaliplatin-treated with respect to vehicle-treated rats; IL-1 α (1 ng/mL) inhibited the BzATP-evoked glutamate overflow both in oxaliplatin-treated, and in

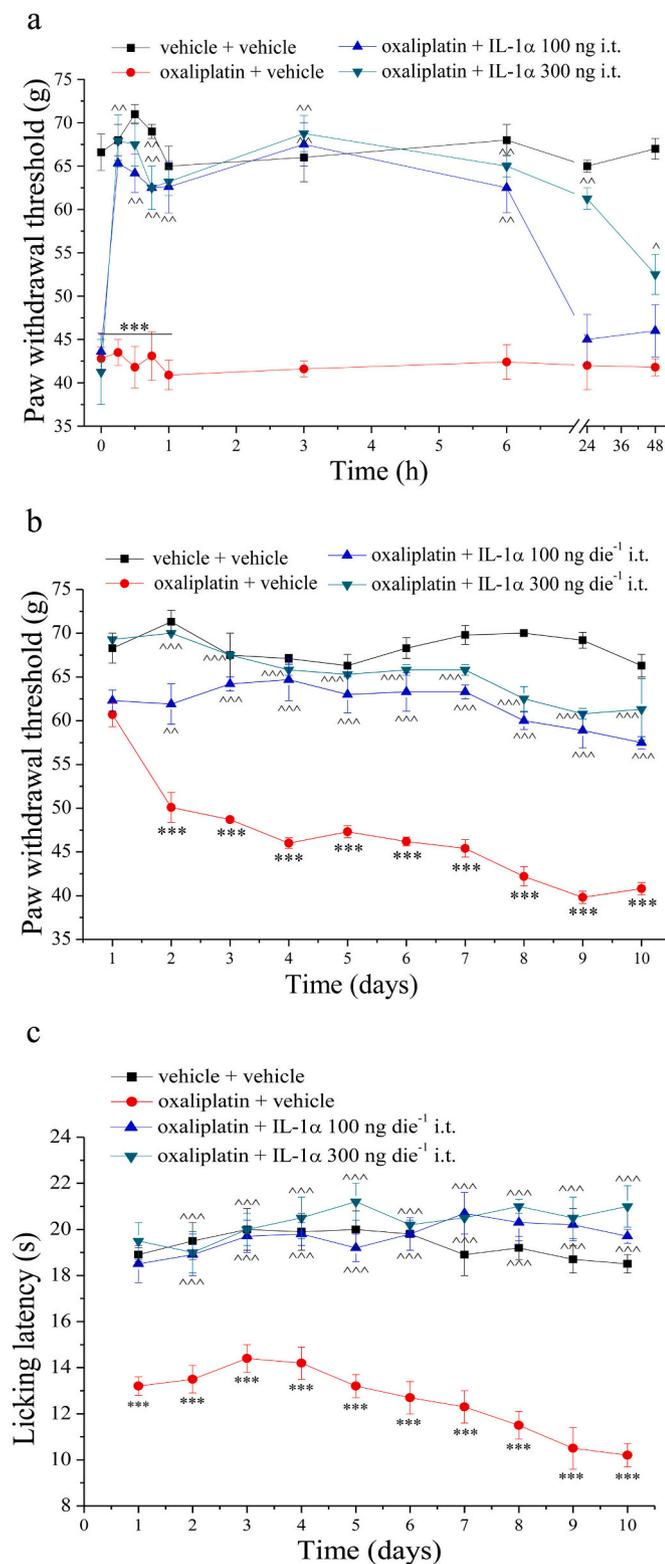


Fig. 3. Pain relieving effect of intrathecal IL-1 α infusion in oxaliplatin-treated rats. **a**) Pain modulation due to an acute i.t. injection of IL-1 α (100 ng and 300 ng) after 10 days of oxaliplatin treatment (2.4 mg kg⁻¹ i.p.) measured by Paw pressure test. Data are mean \pm SEM of 3 independent experiments. *** P < 0.001 vs vehicle + vehicle group, \hat{P} < 0.05 and $\hat{\hat{P}}$ < 0.01 vs oxaliplatin + vehicle group. **b-c**) Effect of a continuous i.t. infusion of IL-1 α (100 and 300 ng/die⁻¹) in oxaliplatin-treated rats (2.4 mg kg⁻¹ i.p.), pain threshold was measured every day by Paw pressure test (**b**) and Cold plate test (**c**). Data are mean \pm SEM of 3 independent experiments. *** P < 0.001 vs vehicle + vehicle group, \hat{P} < 0.01 and $\hat{\hat{P}}$ < 0.001 vs oxaliplatin + vehicle group.

vehicle-treated rats (Fig. 5b). IL-1 α per se did not significantly affect the release from the astrocyte processes or the nerve terminals, in control or in oxaliplatin-treated animals.

The findings are consistent with the ability of IL-1 α i.t. to induce a long-lasting relief of oxaliplatin-evoked neuropathic pain by a mechanism involving a decrease of the spinal glutamate release.

4. Discussion

The present work offers a new mechanism of protection against neuropathy triggered by IL-1 α . In vitro, released by neurons, the alarmin stimulates TGF β signaling in astrocytes promoting neuroprotection; in vivo, in a rat model of oxaliplatin-induced neuropathy, it reduces hyperalgesia, allodynia and spinal glutamate concentration in the presence of activated spinal astrocytes.

The glial compartment plays important homeostatic functions like neuroprotection, myelin formation, mechanical and trophic support (Carozzi et al., 2015; Molina-Gonzalez and Miron, 2019; Sofroniew and Vinters, 2010); astrocytes represent the most abundant glia cells in CNS (Sheridan and Murphy, 2013). In physiological conditions, they maintain redox potential, produce trophic factors, regulate neurotransmitters and ion concentrations, remove toxins and debris from the cerebrospinal fluid (Asanuma et al., 2019; Sidoryk-Wegrzynowicz et al., 2011). In nociceptive conditions (physiological pain), astrocytes contribute to mitigate pain reducing neurons' excitability. On the contrary, chronic pain (pathologic, mainly of neuropathic origin) triggers a central sensitization including the maladaptive response of astrocytes that shift in an active, pain amplifying phenotype (Liddelow and Barres, 2017). Nevertheless, reactive astrocytes continue to play a neuroprotective role through several mechanisms: reducing inflammation, regulating blood brain barrier permeability, forming a physical barrier to prevent immune cell infiltration (Cavallo et al., 2020; Chen et al., 2015; Wanner et al., 2013). Reactive astrocytes become a crossroads of signals able to enhance pain but also to promote CNS recovery and repair, a variety of effects related also to the astonishing morphological, molecular and functional heterogeneity of this cell population (Clarke et al., 2021).

In order to study the role of IL-1 α in the cross-talk between neurons and glial cells in neuropathic condition, a rat neuron/astrocyte co-culture have been performed. As previously reported by our group, the treatment of co-culture with increasing, clinically relevant, concentrations of oxaliplatin simulates in vitro a neurotoxic damage highlighting the stronger resistance of astrocytes compared to neurons (Di Cesare Mannelli et al., 2015c). In the present study, oxaliplatin evoked a significant release of IL-1 α in the medium of neuron/astrocytes co-cultures boosting the question of a detrimental or protective role of this cytokine compared to the other family member IL-1 β . IL-1 α has become recognized as a critical early mediator of sterile inflammatory responses that occur after an injury or tissue necrosis (England et al., 2014), it can be processed and released during cell death by inducing neutrophil recruitment. Like IL-1 β , IL-1 α binds IL-1RI (Garlanda et al., 2013) leading to the recruitment of a second receptor chain called IL-1R accessory protein (IL-1RAcP) (Greenfeder et al., 1995) promoting the activation of several intracellular adaptor molecules (myeloid differentiation factor 88, MYD88; IL-1R-associated kinases, IRAK; TNF receptor-associated factor 6, TRAF6) as well as signaling via NF κ B, p38, c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs) and mitogen-activated protein kinases (MAPKs) (Dunne and O'Neill, 2003). Differently to IL-1 β , IL-1 α is also constitutively expressed in cells at steady state (Di Paolo and Shayakhmetov, 2016) and it can act with mechanisms independent from IL-1R binding (Di Paolo and Shayakhmetov, 2016; Kurt-Jones et al., 1985). To note, pro-IL-1 α (also expressed after the autocrine stimulation of IL-1RI, (Di Paolo and Shayakhmetov, 2016)), unlike pro-IL-1 β , acts as a transcription factor via nuclear translocation (Gabay et al., 2010; Weber et al., 2010). In response to a necrotic damage, IL-1 α works as an 'alarmin' suggesting the activation of a signaling which mainly exerts repairing properties in damaged

a

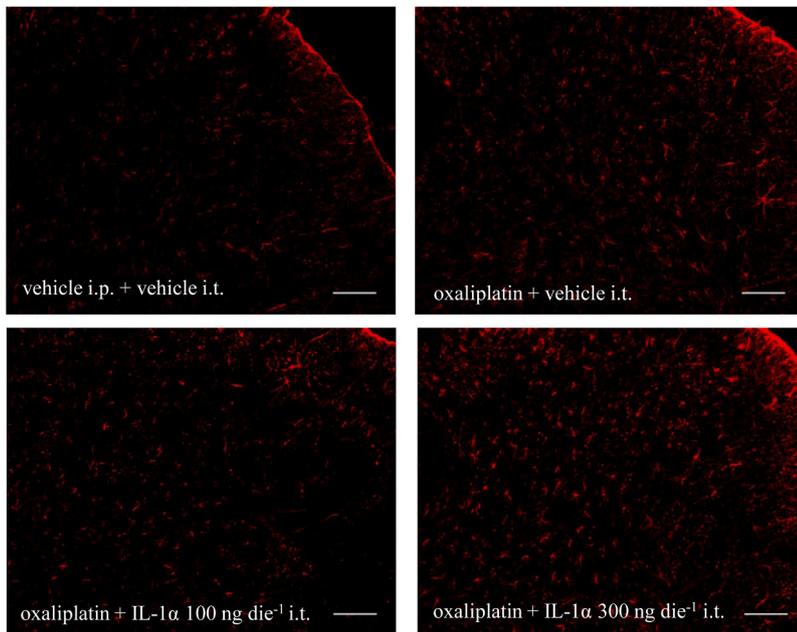


Fig. 4. Astrocyte analysis in the dorsal horn of the lumbar spinal cord. a) Immunofluorescence was performed on spinal cord sections on day 10 of oxaliplatin treatment (2.4 mg kg^{-1} i.p.) and IL-1 α continuous infusion (100 and 300 ng/die^{-1}). Results obtained are a mean of 5 animals per condition, 6 slides for each spinal cord. For each slide 3 randomly acquired images were analysed. Images are representative of three independent experiments. b) Quantification of spinal cord cells positive for GFAP expression per mm^2 . Data are mean \pm SEM of 3 independent experiments. ** $P < 0.01$ vs vehicle-treated rats; $\S P < 0.05$ vs oxaliplatin-treated rats.

b

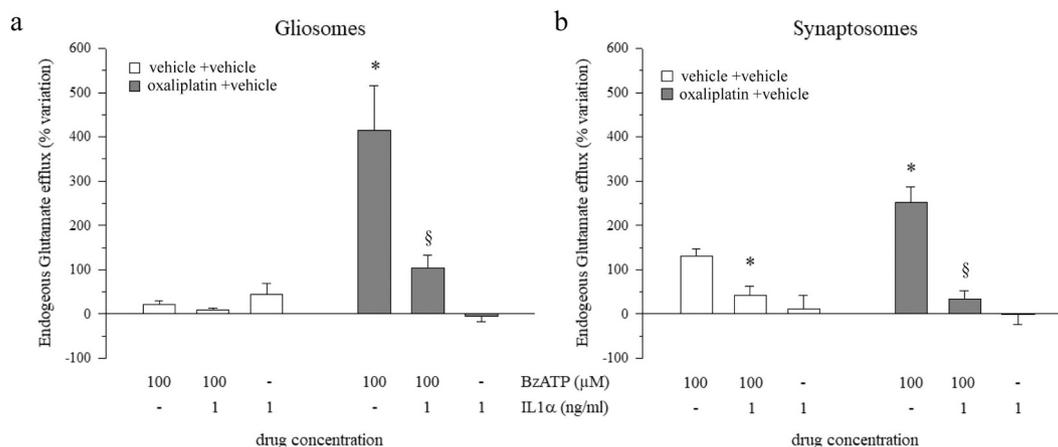
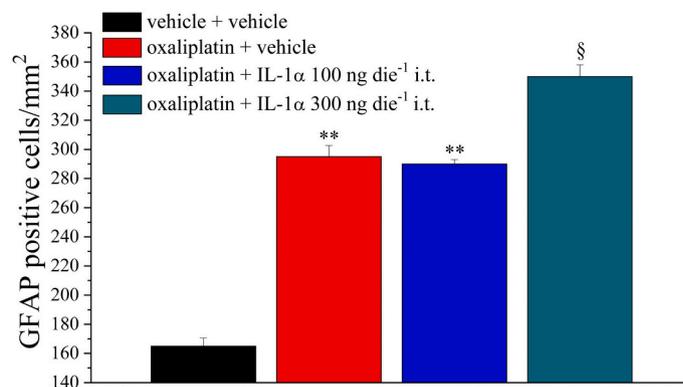


Fig. 5. Glutamate release of spinal cord astrocyte processes and nerve terminals. Effect of the P2X7 receptor agonist BzATP on endogenous glutamate release from (a) purified astrocyte processes (gliosomes) and (b) nerve terminals (synaptosomes) obtained from vehicle-treated (white bar) and oxaliplatin-treated (gray bar) rats. Bars represent glutamate overflow in the presence of the drugs. BzATP was added for 120 s during superfusion; IL-1 α was added 8 min before the agonist. Data are mean \pm SEM of 3–4 independent experiments. * $P < 0.05$ vs BzATP alone in vehicle-treated rats; $\S P < 0.05$ vs BzATP alone in oxaliplatin-treated rats.

tissues. Furthermore, few groups have also highlighted a new protective role of IL-1 α in particular conditions (Mika et al., 2008; Salmeron et al., 2019). Based on these findings we studied the possible protective role of IL-1 α in our model of neuropathy. We showed that IL-1 α was mainly produced by neurons, the cell population most affected by oxaliplatin treatment. The silencing of IL-1 α in neurons significantly reduced neurons viability when co-cultured with astrocytes under increasing concentrations of oxaliplatin. This result revealed the necessary presence of IL-1 α to better survive in neurotoxic damage suggesting that the alarmin can be a player in the cross-talk between neuron and astrocyte to stimulate the conservative role of the latter. Accordingly, the system IL-1 α -knockdown neuron/astrocyte co-culture, showed higher quantity of extracellular ATP released in the medium under oxaliplatin treatment in comparison to scrambled neuron/astrocyte co-culture. ATP is a key mediator of nervous excitability (Fields and Stevens, 2000), acting via P2X purinergic receptors, and it is involved in pain hypersensitivity associated with neuropathic pain and peripheral inflammation (Bernier et al., 2018). ATP released from damaged tissue directly modulates glial functioning, and glia in turn release several cytokines, chemokines, and neurotrophic factors that have relevant effects on neurons (Burnstock, 2006; Hamilton and McMahon, 2000). Instead, the protective transforming growth factor β (TGF β) increased in neuron mono-culture treated with oxaliplatin, but its basal level is higher in astrocytes mono-culture compared to neurons mono-culture, as a recognized glial conservative mechanism (Cekanaviciute et al., 2014). The oxaliplatin-dependent release of TGF β 1 was also observed in neuron/astrocyte co-culture, interestingly this effect was strongly reduced by using IL-1 α silenced neurons supporting the hypothesis that neuroprotective signaling evoked by IL-1 α involves TGF β 1 release. The neurorestorative role of TGF β superfamily is clear, including anti-inflammatory, -apoptotic, -excitotoxic actions as well as the promotion of scar formation, angiogenesis, and neuroregeneration (Dobolyi et al., 2012). TGF β 1 not only regulates growth, differentiation, extracellular matrix formation, neuronal survival and injury repair (Massagué et al., 2000; Zhu et al., 2001), but it also inhibits the release of pro-inflammatory cytokines, oxygen free radicals and nitric oxide (Letterio and Roberts, 1997; Rubio-Perez and Morillas-Ruiz, 2012). In hippocampus, TGF β 1 is able to influence glutamate-evoked currents by acting on ionotropic glutamate receptor expression (Bae et al., 2011). After a damage, astrocytes can upregulate TGF β 1 signaling to limit immune cell recruitment and activate neuroprotective mechanisms (Cekanaviciute et al., 2014) also in neuropathic conditions (Chen et al., 2013; Echeverry et al., 2009). Finally, the intrathecal infusion of TGF β 1 in rats reduce neuropathic pain (Echeverry et al., 2009). Starting from these in vitro findings, we tested the pain modulatory properties of IL-1 α in vivo in rats subjected to oxaliplatin repeated treatments until the development of neuropathy. A single intrathecal infusion of the recombinant cytokine evoked a significant dose-dependent analgesic effect up to complete regression of hypersensitivity. Impressively, efficacy lasted for more than 48 h; in the same model the clinically used compounds like pregabalin and duloxetine were partially effective for a maximum of 45 min (Di Cesare Mannelli et al., 2017). These results agree with the pain relieving effect of IL-1 α shown by Mika and colleagues (Mika et al., 2008) in another model of neuropathic pain (nerve compression by loose ligation of the sciatic nerve). Moreover, the present findings show that the continuous infusion i.t. of IL-1 α alongside the neurotoxic drug treatment protocol fully prevented the development of painful hypersensitivity. This evidence suggests the pivotal role that IL-1 α could play in the complex mechanism of pain generation, highlighting the therapeutic potential of its signaling. The effectiveness throughout several days of treatment allows also to exclude the development of tolerance phenomenon. Interestingly, the described controlled pain condition matched with spinal astrocyte activation. Oxaliplatin per se induces a spinal and supraspinal increase of GFAP-positive cells (Di Cesare Mannelli et al., 2013b), the blockade of astrocyte response by the cell-specific inhibitor fluorocitrate is enough to prevent neuropathic pain

but also inhibiting all astrocyte-mediated neuroprotective signals. IL-1 α did not reduce oxaliplatin-dependent astrocyte density increase, on the contrary significantly increased the number of GFAP-positive cells in the dorsal horns. The increase of GFAP positive cells can be induced by glutamate release through the TGF β 1 pathway activation, as revealed by the work of Romão and colleagues (Romão et al., 2008).

As shown by ex vivo analysis on spinal astrocyte processes and nerve terminals the relief of pain paralleled with a decrease of glutamate levels. As previously shown, oxaliplatin evokes an enhancement of glutamate levels and compounds able to reduce the concentration of the excitotoxic neurotransmitter, like P2X7 antagonists or pannexin-1 inhibitors (Di Cesare Mannelli et al., 2015a) that are potent pain killers. Interestingly, the increased extracellular ATP release observed in vitro justifies the P2X7 activation studied ex vivo by using the ATP analog BzATP. Notably, oxaliplatin causes a stronger increase in the BzATP evoked release of glutamate from astrocytes compared to neurons. In this context the ability of IL-1 α to reduce glutamate efflux, more powerful on reactive astrocytes than on nerve terminals with a evident modulation in the glutamate levels in the neuro-glia crossroads, totally agree with its anti-hypersensitivity profile.

The hypothesis is that IL-1 α may boost astrocytes by a smart modulation, reducing pain and maintaining the neuroconservative properties, as demonstrated in cell cultures.

5. Conclusions

These findings show the efficacy of IL-1 α against oxaliplatin-induced neuropathic pain, an adverse effect that dramatically impairs the life of cancer patients. Moreover, IL-1 α emerges as a neuroprotective signal able to preserve neurons by exploiting astrocyte response. The investigation of the mechanisms by which the alarmin influences astrocyte phenotype will allow to add an important element to the kaleidoscopic plasticity of astrocytes.

Declarations of interest

None.

CRediT authorship contribution statement

Lorenzo Di Cesare Mannelli: Conceptualization, Supervision, Writing – review & editing. **Laura Micheli:** Investigation, Data curation, Writing – original draft. **Chiara Cervetto:** Investigation, Writing – review & editing. **Alessandra Toti:** Investigation, Data curation, Writing – original draft, Writing – review & editing. **Elena Lucarini:** Validation, Data curation. **Carmen Parisio:** Writing – review & editing. **Manuela Marcoli:** Supervision, Writing – review & editing. **Carla Ghelardini:** Conceptualization, Supervision.

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