



Inhibition of C5aR1 as a promising approach to treat taxane-induced neuropathy

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect of several antitumor agents resulting in progressive and often irreversible damage of peripheral nerves. In addition to their known anticancer effects, taxanes, including paclitaxel, can also induce peripheral neuropathy by activating microglia and astrocytes, which release pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin 1-beta (IL-1 β), and chemokine (C-C motif) ligand 2 (CCL-2). All these events contribute to the maintenance of neuropathic or inflammatory response. Complement component 5a (C5a)/C5a receptor 1 (C5aR1) signaling was very recently shown to play a crucial role in paclitaxel-induced peripheral neuropathy. Our recent findings highlighted that taxanes have the previously unreported property of binding and activating C5aR1, and that C5aR1 inhibition by DF3966A is effective in preventing paclitaxel-induced peripheral neuropathy (PIP) in animal models. Here, we investigated if C5aR1 inhibition maintains efficacy in reducing PIPN in a therapeutic setting. Furthermore, we characterized the role of C5aR1 activation by paclitaxel and the CIPN-associated activation of nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome. Our results clearly show that administration of the C5aR1 inhibitor strongly reduced cold and mechanical allodynia in mice when given both during the onset of PIPN and when neuropathy is well established. C5aR1 activation by paclitaxel was found to be a key event in the induction of inflammatory factors in spinal cord, such as TNF- α , ionized calcium-binding adapter molecule 1 (Iba-1), and glial fibrillary acidic protein (GFAP). In addition, C5aR1 inhibition significantly mitigated paclitaxel-induced inflammation and inflammasome activation by reducing IL-1 β and NLRP3 expression at both sciatic and dorsal root ganglia level, confirming the involvement of inflammasome in PIPN. Moreover, paclitaxel-induced upregulation of C5aR1 was significantly reduced by DF3966A treatment in central nervous system. Lastly, the antinociceptive effect of C5aR1 inhibition was confirmed in an *in vitro* model of sensory neurons in which we focused on receptor channels usually activated upon neuropathy. In conclusion, C5aR1 inhibition is proposed as a therapeutic option with the potential to exert long-term protective effect on PIPN-associated neuropathic pain and inflammation.

Abbreviations: CIPN, Chemotherapy-induced peripheral neuropathy; PIPN, Paclitaxel-induced peripheral neuropathy; DRG, Dorsal root ganglia; PNS, Peripheral nervous system; IL-1 β , Interleukin-1 β ; IL-8, Interleukin-8; TNF- α , Tumor necrosis factor; C-C motif, Chemokine; CCL-2, ligand 2; MCP-1, Monocyte chemoattractant protein 1; KO, Knockout; GPCR, G-protein coupled receptor; C5aR1, Complement component 5a receptor 1; NLR, Nod-like receptor; NLRP3, Nod-like receptor family pyrin domain containing 3; Iba-1, Ionized calcium-binding adapter molecule 1; GFAP, Glial fibrillary acidic protein; CCI, Chronic constriction injury; PTX, Paclitaxel; Veh, Vehicle; NCS-1, Neuronal Calcium Sensor-1.

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1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a common and disabling side effect of antitumor agents, resulting in progressive and often irreversible damage of peripheral nerves [1]. Paclitaxel (Taxol®) is one of the most used taxanes in ovarian, breast, non-small cell lung cancer, and prostate cancer [2]. Although paclitaxel is a potent chemotherapeutic agent that induces tumor cell death through microtubule stabilization [3], it commonly causes axonal sensory neuropathy by triggering inflammation in the spinal cord and dorsal root ganglia (DRG) neurons [4]. PIPN affects up of 97% of all cancer patients treated with paclitaxel, often limiting its therapeutic efficacy [5,6]. Emerging studies suggest that the immune system and immune-mediated neuroinflammation are crucial events in the onset and development of PIPN [7]. Paclitaxel treatment has been also associated with the activation of inflammatory pathways in the peripheral nervous system (PNS) and central nervous system (CNS) inducing overexpression of key mediators of pain sensation including interleukin-1 β (IL-1 β), interleukin-8 (IL-8), and tumor necrosis factor α (TNF- α) [8,9]. Paclitaxel treatment has been also reported to enhance nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome activation, through increase the release of mature IL-1 β [10], and this activation in peripheral nerves is associated with the development of neuropathic pain [11]. NLRP3 is an intracellular pattern recognition receptor (PRR), responsible for promoting the secretion of inflammatory factors, and is involved in innate immunity of the nervous system [12]. Paclitaxel has been also shown to increase monocyte chemoattractant protein 1 (MCP-1) expression in small nociceptive DRG neurons as well as in spinal astrocytes [13] and to induce mutually independent microglial and astrocyte activation in the spinal cord [14]. In addition to the above pathways, other mediators have been identified as involved in PIPN in representative animal models [15–17]. Among them, Neuronal Calcium Sensor-1 (NCS-1) is a protein highly expressed in neurons and involved in functional effects of calcium signaling through the binding to the inositol trisphosphate (InsP₃) receptor. NCS-1 is a critical component of PIPN; in fact, the alterations in Ca²⁺ signaling induced by paclitaxel activate several proteases such as calpain that hydrolyzes NCS-1, thus affecting the subsequent cellular response and triggering the onset of neuronal damage. NCS-1 is considered a potential therapeutic target for prevention of PIPN and this hypothesis is sustained by the observation that lithium, a drug used to treat bipolar disorders, and ibudilast, a phosphodiesterase inhibitor used to treat asthma, were demonstrated able to prevent PIPN in animal models through their binding to NCS-1 [18,19], and lithium alone showed ability to prevent PIPN also in humans [20]. Activation of complement system cascade, part of the innate immune system, has also been associated with PIPN [21]. A recent study reported that paclitaxel-induced mechanical allodynia is reduced in a C3 knockout (KO) murine model, suggesting a pivotal role of complement system in PIPN [22]. C5a, the inflammatory component produced by complement activation, is emerging as a key mediator of inflammatory and neuropathic pain [23] and several findings highlight the importance of the activation of its receptor C5aR1, belonging to the family of G-protein coupled receptors (GPCR), in the onset of painful neuro-inflammation and in the pathophysiology of neuropathic pain [23–26]. Although the pathogenic mechanisms of paclitaxel-induced peripheral neuropathy in the spinal cord and PNS have been extensively studied, a unique molecular mechanism of action accounting for the peripheral neurons sensitization has not been identified thus impairing the research of a specific therapeutic approach for the treatment of PIPN [7]. We recently described that both paclitaxel and docetaxel exhibit specific binding affinity to C5aR1 and that taxanes binding to C5aR1 results in a C5a-independent receptor activation [27]. The results of *in vitro* and *in vivo* experiments confirmed that this event could represent one of the key mechanisms underlying taxanes-induced CIPN. Interestingly, C5aR1 knock-out (KO) mice have been found resistant to paclitaxel-induced neuropathic pain development and

maintenance; similarly, the preventive treatment with a C5aR1 allosteric inhibitor, DF3966A, the R enantiomer of the compound DF3016A, a SMW compound previously described [26,27] as selective and potent C5aR1 inhibitor, was able to switch off the taxane-induced receptor activation. Notably, the compound significantly reduced cold and mechanical allodynia in a mouse model of PIPN. This protective effect was confirmed by RT-PCR for receptor channels TRPV1 and 4 and cytokines expression (IL-1 β and IL-6 and TNF) in spinal cord and paws of the animals, which consequently enhance the transmission of pain. These results were also confirmed by *in vitro* studies (PTX-treated DRG cells) in which C5aR1 inhibition through DF3966A restored neuronal plasticity and downregulated levels of the active form of proteins implicated in neuropathic pain pathway such as cortactin, FAK and STAT3 [27]. The specificity of the mechanism of action was confirmed by *in vitro* data obtained with other known C5aR inhibitors. [27] Our results also show that the activation of C5aR1 mediated by paclitaxel triggers production of peripheral pro-inflammatory mediators including cytokines, which consequently enhance the transmission of pain. Interestingly, pharmacological blockage of C5aR1 activation by DF3966A prevents DRG alterations induced by paclitaxel in rodents, supporting the concept that paclitaxel/C5aR1 axis is a key neuropathological mechanism of taxane-induced neuropathy highlighting the potential of targeting C5aR1 as a new therapeutic approach to treat PIPN [25,26].

Here we investigated the possible therapeutic effects of DF3966A in taxane-induced neuropathy using two different schedules of treatment, starting the administration either during the development of neuropathy or when neuropathy is fully established. In this work we further characterized the molecular mechanisms underlying taxane-induced neuropathy with a focus on the role of paclitaxel-mediated C5aR1 activation on inflammatory spinal and glial activation and on the involvement of inflammasome.

2. Materials and methods

2.1. Animals

All the procedures involving animals were performed on male Balb/C mice (6–8 weeks, Charles River) housed in the animal care facility of the Department of Pharmacy, University of Naples, in a room with controlled temperature (22 \pm 1 $^{\circ}$ C), humidity (60 \pm 10%), and light (12 h per day) with food and water available ad libitum. Animal care was in conformity with International and National law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines [28], and the Basel declaration including the 3R conception). All behavioral tests were performed between 9:00 am and 1:00 pm, and mice were only used once. All efforts were made to minimize animal suffering.

2.2. Drug treatments

Paclitaxel (Selleckchem, USA, cod. S1150) was dissolved in saline according to manufacturer's instruction, to obtain a dose of 4 mg/kg to establish the PIPN model in mice. The protocol used was performed according to Polomano et al., 2001 [29], adapted to mice. Paclitaxel was administrated intraperitoneally for four alternate days (1, 3, 5, and 7).

For the *in vivo* experiments, animals were treated with DF3966A (Dompé farmaceutici S.p.A., L'Aquila, Italy). DF3966A was dissolved in water (30 mg/10 ml), and orally given at a dose of 30 mg/kg every 12 h. The oral dose was selected based on previous pharmacokinetic and efficacy studies [26,27]. Treatment started 4 or 10 days after the first paclitaxel injection, which means during the induction phase of PIPN (4 days) or when the neuropathy is established (10 days), up to day 28. On day 3, 5 and 7 DF3966A was administered 1 h after paclitaxel. Mice were divided into 4 groups:

- Sham: mice receiving the PTX vehicle
- Vehicle: mice receiving PTX and then the Vehicle of DF3966A

- DF3966A (DF4): mice receiving PTX and then DF3966A 4 days after the first paclitaxel injection
- DF3966A (DF10): mice receiving PTX and then DF3966A 10 days after the first paclitaxel injection.

2.3. Mechanical allodynia

To measure changes in sensitivity thresholds to non-noxious mechanical stimulus, mechanical allodynia was measured using the Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Italy). Animals were placed in a chamber with a mesh metal floor covered by a plastic dome that enables the animals to walk freely, but not to jump, in a quiet room 15–30 min before testing. The mechanical stimulus was then delivered in the mid-plantar skin of the hind paw. The DPA automatically records the grams at which the foot is withdrawn. The cut-off for the paw withdrawal was fixed at 5 g (grams). Thus, any mouse that reached the cut-off are excluded from the study [30,31]. Testing was performed 3 h after DF3966A treatment on days 10–14–21 and 26.

2.4. Cold allodynia

Cold sensitivity was measured as the number of foot withdrawal responses after the application of acetone to the dorsal surface of the paw. A drop (50 μ l) of acetone was applied to the dorsal surface of paws with a syringe connected to a thin polyethylene tube. A brisk foot withdrawal response (licking, flinching or lifting) after the spread of acetone over the dorsal surface of the paw was considered as a sign of cold allodynia [32]. Cold responses were measured 3 h after DF3966A treatment on days 10–14–21 and 26.

2.5. Real-time PCR analysis

For this experiment, we used sciatic nerve, DRG, brain and spinal cord samples collected at day 27. Total RNA, isolated from each sample was extracted using TRIzol Reagent (Bio-Rad Laboratories), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μ g total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The PCR conditions were 15 min at 95 °C followed by 40 cycles of two-step PCR denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Each sample contained 20 ng cDNA in 2X QuantiTect SYBRGreen PCR Master Mix and primers pairs, designed from PrimerBank database selecting the validated ones, to amplify c5aR1 (ID: 6680818a1) CCL2 (ID: 6755430a1), GFAP (ID: 30692526a1), Iba-1 (ID: 9506379a1), NLRP3 (ID: 22003870a1), NF- κ B (ID: 6677709a1), TNF- α (ID: 7305585a1), in a final volume of 50 μ l. Mouse primers used are showed in Table 1. The relative amount of each studied mRNA was normalized to β -Actin (ID: 6671509a1), as reference gene, and data were analyzed according to the $2^{-\Delta\Delta CT}$ method [33].

2.6. Data analysis

All data are presented as the mean \pm SEM. Analysis of data was conducted using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The significance of differences between groups *in vivo* behavioral testing experiments was determined on basis of test used, by one or two-way analyses of variance (ANOVA), followed by Tukey post hoc tests for multiple comparisons. The level of significance was set at * $P < 0.05$.

2.7. In vitro studies

To better dissect the underlying mechanisms of paclitaxel-*in vivo* PN, an *in vitro* model of sensory neurons was developed.

Table 1.
Mouse Primers Used for PCR Analysis.

Primers	Primer sequence (5'-3')	Annealing Temperature °C
Actin B	GGTGTATTCCCTCCATCG	61.8
	CCAGTTGGTAACAATGCCATGT	61.1
CCL2	TTAAAAACCTGGATCGGAACCAA	60.1
	GCATTAGCTTCAGATTACGGGT	60.7
C5ar1	ATGGACCCCATAGATAACAGCA	60.4
	AGGGAGTGGAGGAGTCATTCG	60.5
Gfap	CGGAGACGCATCACCTCTG	62.1
	AGGGAGTGGAGGAGTCATTCG	62.7
Iba-1	ATCAACAAGCAATTCCTCGATGA	60.3
	CAGCATTGCTTCAAGGACATA	60.7
NLRP3	ATTACCCGCCGAGAAAGG	61.4
	TCGCAGCAAAGATCCACACAG	62.9
NFkB	AGGCTTCTGGGCCTTATGTG	61.6
	TGCTTCTCTGCCAGGAATAC	61.6
TNF-α	CCCTCACACTCAGATCATCTTCT	60.9
	GCTACGACGTGGGCTACAG	62.1

Specifically, human neuroblastoma SH-SY5Y cells (ECACC, Sigma Aldrich, St. Louis, MO, USA) were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (all materials from Corning, USA) in a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed twice a week, and cells were cultured at about 80% confluence. For the sensory neuron differentiation protocol, 5 \times 10⁴ cells/well were seeded in collagen matrix-coated plates (NC-100 μ g/mL). After 24 h (day 1), the medium was replaced with FBS reduced to 2% (differentiation medium) and supplemented with 10 μ M all-trans retinoic acid (RA, Sigma Aldrich, USA). Then, cells were incubated for 5 days, with daily medium replacement, except for the second day. On day 5 of differentiation, cultures were stimulated with serum-free medium supplemented with human brain-derived neurotrophic factor (BDNF Dompé farmaceutici, S. p.A.). On day 7 of differentiation, neurons were used for the experiments.

2.7.1. Drug treatments

Following the differentiation, cells were treated for 12 h with Paclitaxel (10 nM), C5a (50 nM) and DF3966A (10 mM) and the combination of the three molecules. Paclitaxel stock solution (Sigma-Aldrich; 10 mM) was prepared by dissolving the powder in DMSO and aliquots were stored at -20 °C. C5a stock solution (R&D Systems, Inc. MN, USA; 20 mM) was prepared by dissolving the powder in DMSO 100%; aliquots have been stored at -80 °C. C5aR1 antagonist DF3966A (Dompé farmaceutici S.p.A., L'Aquila, Italy) at stock solution 20 mM in DMSO 100% was prepared. In the final concentration, treatments were diluted in culture media.

2.7.2. Quantitative real-time PCR

For gene expression analysis, the following protocol has been used: total RNA was extracted by Trizol reagent and purified with spin-columns (Direct-zolTM RNA Microprep, Zymo Research, USA) according to the manufacturer's instructions. The total RNA concentration has been determined spectrophotometrically in RNAase-free water, and 1 μ g aliquots of total RNA have been reverse-transcribed into cDNA using ProtoScript First Strand cDNA Synthesis Kit (NEB). RT-PCR has been carried out on ABI 7300HT sequence detection system (ABI), in a total volume of 20 μ l containing EagleTaq Universal Master Mix (Roche), DEPC water, 4 μ l of cDNA and the following Prime Time qPCR Assays for

C5aR1 (hs00704891), TRPV1 (hs00218912m1), TRPV4 (hs01099348m1), TRPA1 (hs00175798m1), Nav1.7 (hs01076699m1), Nav1.9 (hs00204222m1) have been purchased from ThermoFisher (ThermoFisher Scientific; USA). Triplicate samples have been run for each gene. The reference gene GAPDH (hs02786624g1) (ThermoFisher Scientific, USA) was used as an internal control to normalize the expression of target genes. RT-PCR protocol: a pre-heating step for 3 min at 95 °C, 40 cycles at 95 °C for 10 s and 60° for 30 s, and last end-step at 65 °C for 10 s. Relative expression levels have been calculated for each sample after normalization against reference gene, using the $\Delta\Delta Ct$ method for comparing relative fold expression differences.

2.7.3. Data analysis

Data were expressed as mean \pm SEM. Statistical analysis has been performed by the analysis of variance (ANOVA) followed by Tukey's multiple comparisons test analysis using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The level of significance was set at $*P < 0.05$.

3. Results

3.1. C5aR1 inhibition significantly reduces PIPN in vivo

Based on our previous data showing the involvement of C5aR1 in the pathophysiology of PIPN from day 3 until day 14 [27], we investigated the potential therapeutic effects of oral DF3966A administered both

during the development of neuropathy (from day 4) and in the case of overt neuropathic state (from day 10).

As expected, paclitaxel administration induced cold and mechanical allodynia in animals at all time points (day 10, 14, 21, and 26) compared to sham (Fig. 1A, B). In cold allodynia experiments, the number of paw withdrawals of mice receiving paclitaxel + DF3966A from day 4 is significantly reduced from day 14 until day 26, compared to paclitaxel + DF3966A vehicle group, while no effect was observed on day 10 (Fig. 1A). Mice receiving paclitaxel + DF3966A from day 10 showed a significant reduction in the number of paw withdrawals at day 21 and 26 compared to paclitaxel + DF3966A vehicle group; no effect was observed at days 10 and 14 (Fig. 1A). Specifically, the area under the curve (AUC) for the effects of paclitaxel + DF3966A vehicle showed that paclitaxel induced a significant ($p < 0.0001$) increase in the number of paw withdrawals compared to the sham group (Fig. 1C). Animals treated with paclitaxel + DF3966A starting from day 4 and from day 10 showed a significant reduction of number of paw withdrawals compared to paclitaxel + DF3966A vehicle group ($p < 0.0001$) (Fig. 1C). In the DPA test, mice receiving paclitaxel + DF3966A from day 4 showed a significant increase in the paw withdrawal threshold on both paws from day 14 until day 26 compared to paclitaxel + DF3966A vehicle group, while no significant effect was observed at day 10 (Fig. 1B). Mice receiving paclitaxel + DF3966A from day 10 showed a significant increase in the paw withdrawal threshold at day 21 and day 26 compared to the paclitaxel + DF3966A vehicle group; while no effect was observed at day 10 and day 14 (Fig. 1B). The AUC of the groups in which

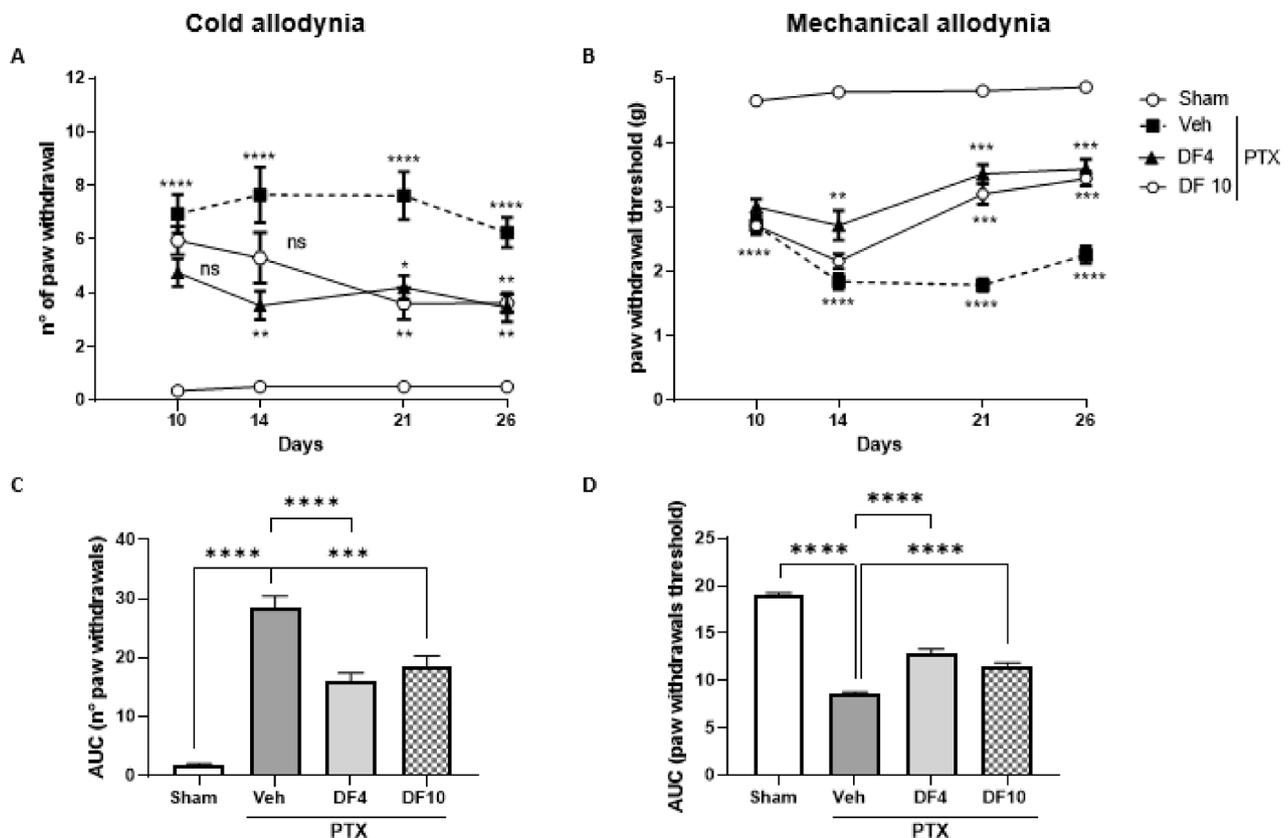


Fig. 1. Time course of paw withdrawal responses in mice displaying paclitaxel (PTX)-induced cold (A) (two-way ANOVA, effect of time \times treatment $F(9,228) = 1.666$; $p = 0.0982$ followed by Tukey's multiple comparisons test) and mechanical (B) (two-way ANOVA, effect of time \times treatment $F(9,228) = 8.842$; $p < 0.0001$ followed by Tukey's multiple comparisons test) allodynia untreated or treated with DF3966A (DF, 30 mg/kg) starting from day 4 (DF4) or 10 (DF10) after PTX. (C) number of paw withdrawal number AUC of the effects of DF3966A on the number of paw withdrawals in the PTX-induced neuropathic pain model. (D) paw withdrawal threshold AUC of the effects of DF3966A on the paw withdrawal threshold (g) in the PTX-induced neuropathic pain model. DF3966A was orally administered every 12 h for 14 days during the induction phase of PIPN. Cold and mechanical allodynia were evaluated from day 10 to day 26. Sham (PTX vehicle, white circle), PTX + Veh (PTX + DF3966A vehicle; black square), PTX + DF4 (PTX + DF3966A starting from day 4 after PTX; black triangle) and PTX + DF10 (PTX + DF3966A starting from day 10 after PTX; white hexagon). **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ vs the respective vehicle group. Data are expressed as mean \pm SEM; $n = 10$ per group.

neuropathic pain was induced by paclitaxel injection was significantly lower than in sham animals ($p < 0.0001$) (Fig. 1D). Notably, treatment with DF3966A starting from day 4 and 10 after paclitaxel significantly increased the AUC of paw withdrawal threshold compared to the paclitaxel- DF3966A vehicle group ($p < 0.0001$).

3.2. C5aR1 inhibition significantly mitigates paclitaxel-induced inflammation and NLRP3 inflammasome activation

We first examined whether DF3966A was able to reduce paclitaxel-induced secretion of pro-inflammatory cytokines. The paclitaxel-induced increase in TNF- α and IL-1 β gene expression was strongly reduced in sciatic nerve of animals receiving paclitaxel + DF3966A both from day 4 and from day 10 (Fig. 2A, B). We also showed that paclitaxel increased mRNA levels of NLRP3 in sciatic nerve, which were significantly reduced by treatment with DF3966A whether administrated from day 4 or from day 10 (Fig. 2C).

In DRG neurons, as in the sciatic nerve, mRNA levels of TNF- α and CCL2 were significantly inhibited by paclitaxel + DF3966A from day 4 but not by paclitaxel + DF3966A from day 10 (Fig. 3A, C), while the increase in IL-1 β induced by paclitaxel was decreased in both DF3966A groups (Fig. 3B), in line with previous findings. In addition, DF3966A administered from day 4 and day 10 significantly mitigated the activation of NLRP3 inflammasome signaling (Fig. 3D).

3.3. C5aR1 inhibition modulates paclitaxel-induced alteration of key inflammatory factors in the spinal cord

In the spinal cord, DF3966A significantly reduced the paclitaxel-induced increase of mRNA levels of TNF- α , a mediator of both spinal microglial activation and hypersensitivity to neuropathic pain (Fig. 4A). GFAP and Iba-1, markers of astroglia and microglial cells respectively, were also induced by paclitaxel and significantly inhibited by DF3966A treatment starting from both day 4 and day 10 (Fig. 4B, C).

3.4. C5aR1 inhibition modulates paclitaxel-induced alteration of C5aR1 expression

In the spinal cord of paclitaxel-treated mice, C5aR1 gene expression was significantly increased compared to sham mice, and this upregulation was completely inhibited by treatment with DF3966A both if administered from day 4 and day 10 after paclitaxel (Fig. 5A).

In the brain, C5aR1 mRNA levels were upregulated in mice treated with paclitaxel but were significantly reduced in DF3966A-treated mice starting from day 4 (Fig. 5B). In addition, NF κ B mRNA levels were increased by paclitaxel and strongly reduced by DF3966A when given from day 4 after paclitaxel (Fig. 5C).

3.5. C5aR1 inhibition modulates PIPN in sensory neurons

To further dissect the underlying mechanisms of C5aR1 inhibition, an *in vitro* model of sensory neurons was developed, stimulated with PTX or C5a, and then treated with DF3966A.

We first examined whether DF3966A was able to reduce C5aR1 also in this model and, remarkably, it was able to significantly counteract the induced expression of this receptor. Interestingly, PTX and C5a showed the same behavior (Fig. 6A). Some members of the transient receptor potential (TRP) family of channels, as the TRPV1 and TRPV4 (vanilloid), TRPA1 (ankyrin) and TRPM8 (melastatin) are expressed on the plasma membrane of primary sensory neurons, where they are activated by physical and chemical stimuli. Recent evidence suggests that TRPV1, TRPV4, and TRPA1 are main contributors of mechanical and thermal hypersensitivity in models of CIPN. In particular, *in vitro* and *in vivo* studies have pointed out the unique role of TRPA1 and oxidative stress in the mechanism responsible for cold and mechanical hyperalgesia in models of CIPN [34–36].

In our experimental conditions, both PTX and C5a significantly increased the expression of these receptors in sensory neurons, while the C5aR1 inhibitor was able to counteract these effects (Fig. 6 B-D).

To investigate whether Nav1.7 and Nav1.9 were involved in PIPN, we measured mRNA expression of these voltage-gated sodium channels in sensory neurons upon treatments (Fig. 6 E-F). Expression of Nav1.7 and Nav1.9 mRNA in sensory neurons was higher upon treatment with PTX and C5a, alone or in combination, compared to control. Notably, DF3966A was able to strongly reduce their expression, most significantly for Nav1.9 (Fig. 6 E-F). The data collected further demonstrate the crucial role of C5aR1 in the neuropathological mechanisms underlying PIPN.

4. Discussion

Taxane-based chemotherapy is associated with frequent and severe CIPN syndrome [37], whose mechanisms are still poorly understood, hampering the development of new therapeutic treatments. Our recent

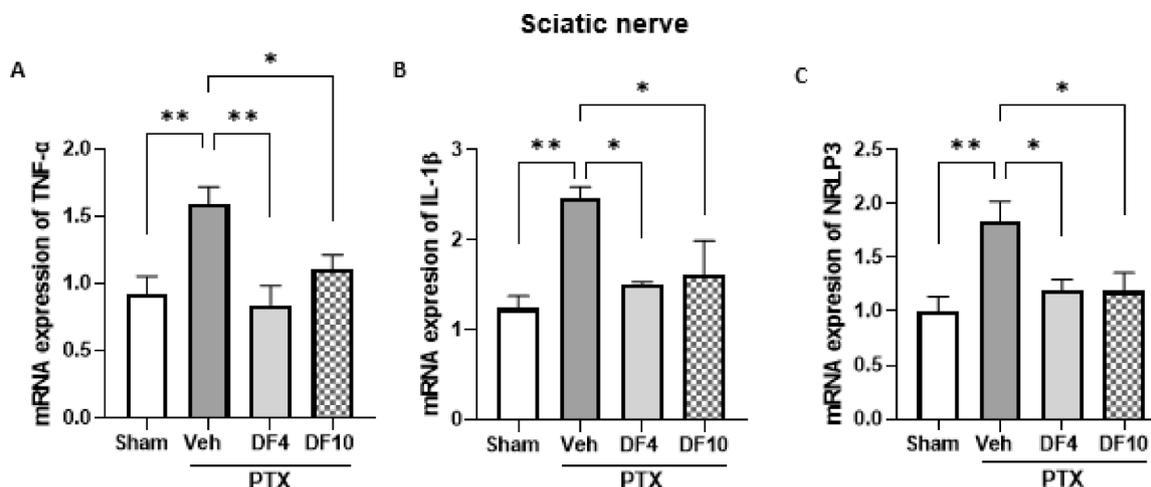


Fig. 2. Real-time PCR analysis of TNF- α (A) (one-way ANOVA, effect of treatment $F(3,36) = 7.237$; $p = 0.0006$ followed by Tukey's multiple comparisons test), IL-1 β (B) (one-way ANOVA, effect of treatment $F(3,36) = 6.134$; $p = 0.0018$ followed by Tukey's multiple comparisons test), and NLRP3 (C) (one-way ANOVA, effect of treatment $F(3,36) = 5.727$; $p = 0.0026$; followed by Tukey's multiple comparisons test) expression levels in sciatic nerve collected at day 27 after the first PTX administration. Sham (PTX vehicle), PTX + Veh (PTX + DF3966A vehicle), PTX + DF4 (PTX + DF3966A starting from day 4 after PTX) and PTX + DF10 (PTX + DF3966A starting from day 10 after PTX). ** $p < 0.01$ and * $p < 0.05$ vs the respective vehicle group. Data are expressed as mean \pm SEM; $n = 10$ per group.

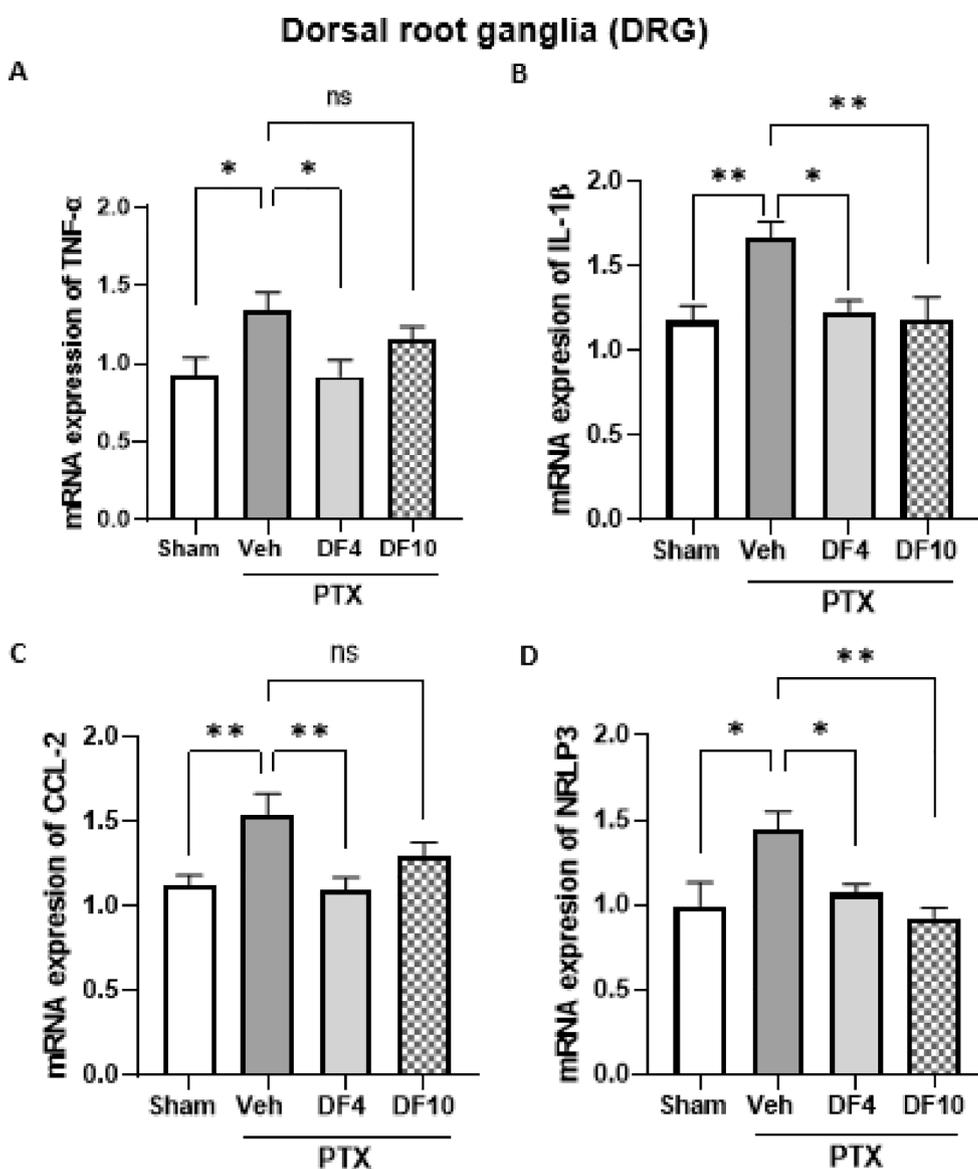


Fig. 3. Real-time PCR analysis of TNF- α (A) (one-way ANOVA, effect of treatment $F(3,36) = 3.946$; $p = 0.0157$ followed by Tukey's multiple comparisons test), IL-1 β (B) (one-way ANOVA, effect of treatment $F(3,36) = 5.874$; $p = 0.0023$ followed by Tukey's multiple comparisons test), CCL2 (C) (one-way ANOVA, effect of treatment $F(3,36) = 5.280$; $p = 0.0040$ followed by Tukey's multiple comparisons test), and NLRP3 (D) (one-way ANOVA, effect of treatment $F(3,36) = 5.708$; $p = 0.0027$; followed by Tukey's multiple comparisons test) expression levels in DRG neurons collected at day 27 after the first PTX administration. Sham (PTX vehicle), PTX + Veh (PTX + DF3966A vehicle), PTX + DF4 (PTX + DF3966A starting from day 4 after PTX) and PTX + DF10 (PTX + DF3966A starting from day 10 after PTX). ** $p < 0.01$ and * $p < 0.05$ vs the respective vehicle group. Data are expressed as mean \pm SEM; $n = 10$ per group.

findings have shown a specific affinity of taxanes for C5aR1, one the complement factor C5a receptor, suggesting that taxane-induced C5aR1 activation may represent a key mechanism in the development of peripheral neuropathy. Evidence of a role for C5aR1 in nociception sensitization was previously reported in several models of inflammatory pain [38], while the importance of C5aR1 as a mediator of neuropathic pain also involved in CIPN has been recently demonstrated [39]. In line with the hypothesis that paclitaxel binding to C5aR1 triggers neurosensitization, our results showed that blocking C5aR1 during the induction phase of PIPN with a C5aR1 inhibitor, specifically DF3966A, prevented the development of neuropathy *in vivo* [27]. Here, we evaluated the potential curative effect of C5aR1 inhibition testing the efficacy of DF3966A when administered to mice during the development phase of paclitaxel-induced neuropathy, and when neuropathy is well established.

DF3966A strongly reduced cold and mechanical allodynia both when it is administered during the development of neuropathy and when the pain was well established, confirming that the inhibition of paclitaxel-induced C5aR1 activation may represent a valuable strategy not only to prevent but also to mitigate and reverse peripheral neuropathy when symptoms are already present. We also analyzed the inflammatory and neuroinflammatory status in the CNS and PNS, as well as the impact of

paclitaxel-activated C5aR1 signal on the immune system, highlighting a new possible correlation between inflammasome and C5aR1 modulation. Inflammasomes are cytosolic multiprotein oligomers involved in the innate immune system and play a role in the activation of inflammation [40]; their activation and assembly promotes maturation and secretion of pro-inflammatory cytokines such as IL-1 β and TNF- α [41,42]. The NLRP3 inflammasome is present primarily in immune and inflammatory cells, including mast cells, neutrophils, macrophages [43–45], and was recently identified in neurons of the sensory system [12]. In a chronic constriction injury (CCI) mouse pain model, it was recently shown that NLRP3 inflammasome activation and inflammasome-related factors, such as IL-1 β , were significantly increased [46]. Furthermore, NLRP3-KO mice also exhibited better motor recovery after sciatic nerve injury compared to wild-type mice, confirming the crosstalk between neuropathy and inflammasome. NLRP3 inflammasome is enhanced by paclitaxel, triggering pro-inflammatory pathways and modulating pain *in vivo* [10,11]. Several reports demonstrated that C5a/C5aR1 axis was able to trigger inflammasome activation [47,48]. The formation of inflammasome complexes is activated by several conditions such as infections and metabolic dysfunctions, and its full activation induces expression of NLRP3 and pro-inflammatory mediators including NF- κ B and IL-1 β [49]. Our results

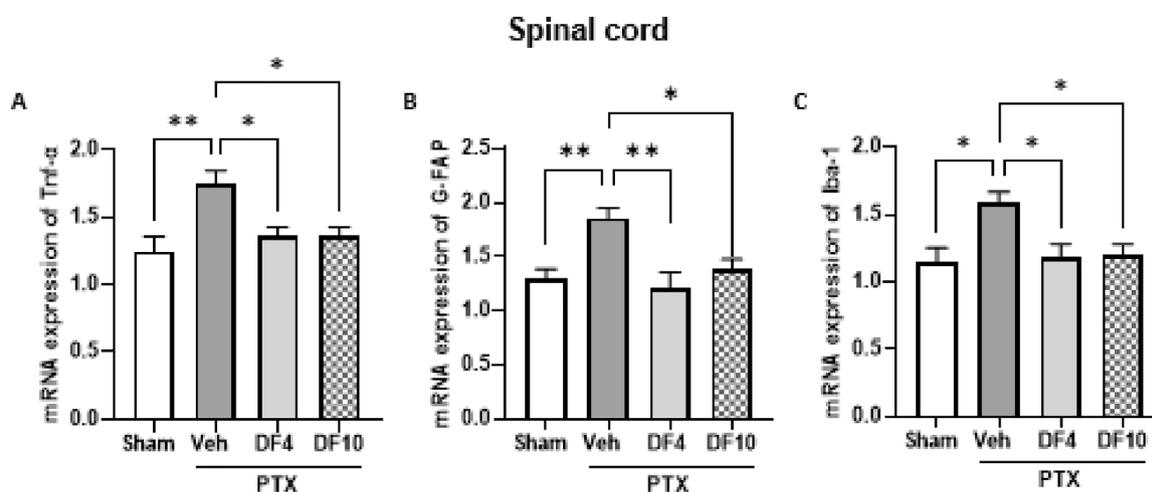


Fig. 4. Real-time PCR analysis of $TNF-\alpha$ (A) (one-way ANOVA, effect of treatment $F(3,36) = 6.609$; $p = 0.0011$ followed by Tukey's multiple comparisons test), GFAP (B) (one-way ANOVA, effect of treatment $F(3,36) = 6.777$; $p = 0.0010$ followed by Tukey's multiple comparisons test), and Iba-1 (one-way ANOVA, effect of treatment $F(3,36) = 4.534$; $p = 0.0085$; followed by Tukey's multiple comparisons test) expression levels in the spinal cord collected at day 27 after the first PTX administration. Sham (PTX vehicle), PTX + Veh (PTX + DF3966A vehicle), PTX + DF4 (PTX + DF3966A starting from day 4 after PTX) and PTX + DF10 (PTX + DF3966A starting from day 10 after PTX). ** $p < 0.01$ and * $p < 0.05$ vs respective the vehicle group. Data are expressed as mean \pm SEM; $n = 10$ per group.

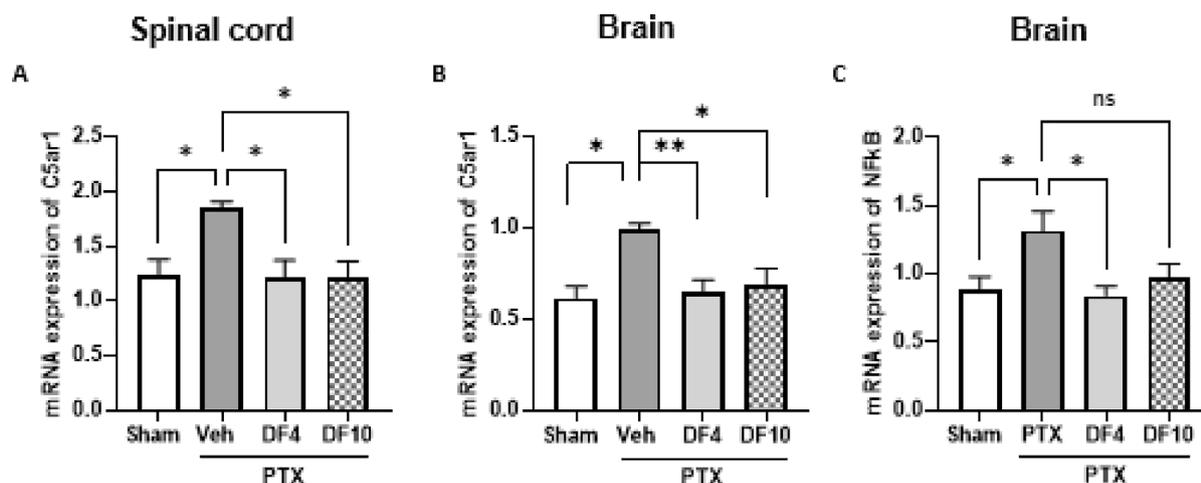


Fig. 5. Real-time PCR analysis of C5aR1 (A) (one-way ANOVA, effect of treatment $F(3,36) = 5.423$; $p = 0.0035$ followed by Tukey's multiple comparisons test) expression levels in the spinal cord, C5aR1 (B) (one-way ANOVA, effect of treatment $F(3,36) = 6.240$; $p = 0.0016$ followed by Tukey's multiple comparisons test), and (C) (one-way ANOVA, effect of treatment $F(3,36) = 4.131$; $p = 0.0129$; followed by Tukey's multiple comparisons test) expression levels in the brain collected at day 28 after the first PTX administration. Sham (PTX vehicle), PTX + Veh (PTX + DF3966A vehicle), PTX + DF4 (PTX + DF3966A starting from day 4 after PTX) and PTX + DF10 (PTX + DF3966A starting from day 10 after PTX). ** $p < 0.01$ and * $p < 0.05$ vs the respective vehicle group. Data are expressed as mean \pm SEM; $n = 10$ per group.

confirm that paclitaxel-induced C5aR1 activation stimulates inflammatory activation in the CIPN mouse model; in paclitaxel-vehicle mice, mRNA levels of NLRP3 were significantly increased compared to the vehicle group at peripheral level (DRG and sciatic nerve), and inflammatory mediators (IL-1 β , TNF- α) were also upregulated. DF3966A treatment restored this condition, reducing both inflammasome NLRP3 activation and cytokine production confirming a key role of C5aR1 activation. Paclitaxel is also reported to elicit pain by activating microglia and astrocytes, which in turn often release pro-inflammatory cytokines such as TNF- α , contributing to the maintenance of inflammatory and neuropathic pain [50]. Spinal administration of TNF- α is in fact sufficient to induce mechanical allodynia [51,52], while systemic TNF- α inhibitor treatment reduced paclitaxel-established mechanical and cold allodynia on both PNS and CNS [53,54]. In neuropathic injury, in addition to cytokines (IL-6, IL-1 β) [55] and neuropeptides (Iba-1, GFAP) [13] the expression of CCL2 in neurons and astroglia cells is also upregulated [56]. All these pathways are involved in paclitaxel-evoked

cold hyperalgesia [57]. Our results show that C5aR1 inhibition restores physiological conditions, reducing the expression of inflammatory mediators such as TNF- α , IL-1 β , and CCL2, and modulating the NLRP3 inflammasome signaling. Furthermore, DF3966A was able to modify GFAP and Iba-1 activation in the spinal cord, coherently with the observed efficacy on neuropathic pain development. Our work demonstrates the involvement of C5aR1 in pro-analgesic and neuro-inflammatory effects induced by paclitaxel at spinal and supraspinal levels. C5aR1 activation triggers multiple inflammatory pathways, such as stimulating cells to secrete inflammatory factors or recruiting and activating leukocytes [58]. Several studies show that C5aR1 activation triggers a cascade of events involved in the pathophysiology of PN and in the genesis of painful states of neuroinflammation [23,24] and we previously demonstrated the role of C5aR antagonist in neuroinflammation [26]. Our CNS results confirm the key role of the complement system in neuroinflammation. DF3966A reduced C5aR1 and NFkB expression in paclitaxel-treated mice, highlighting the important role of this system in

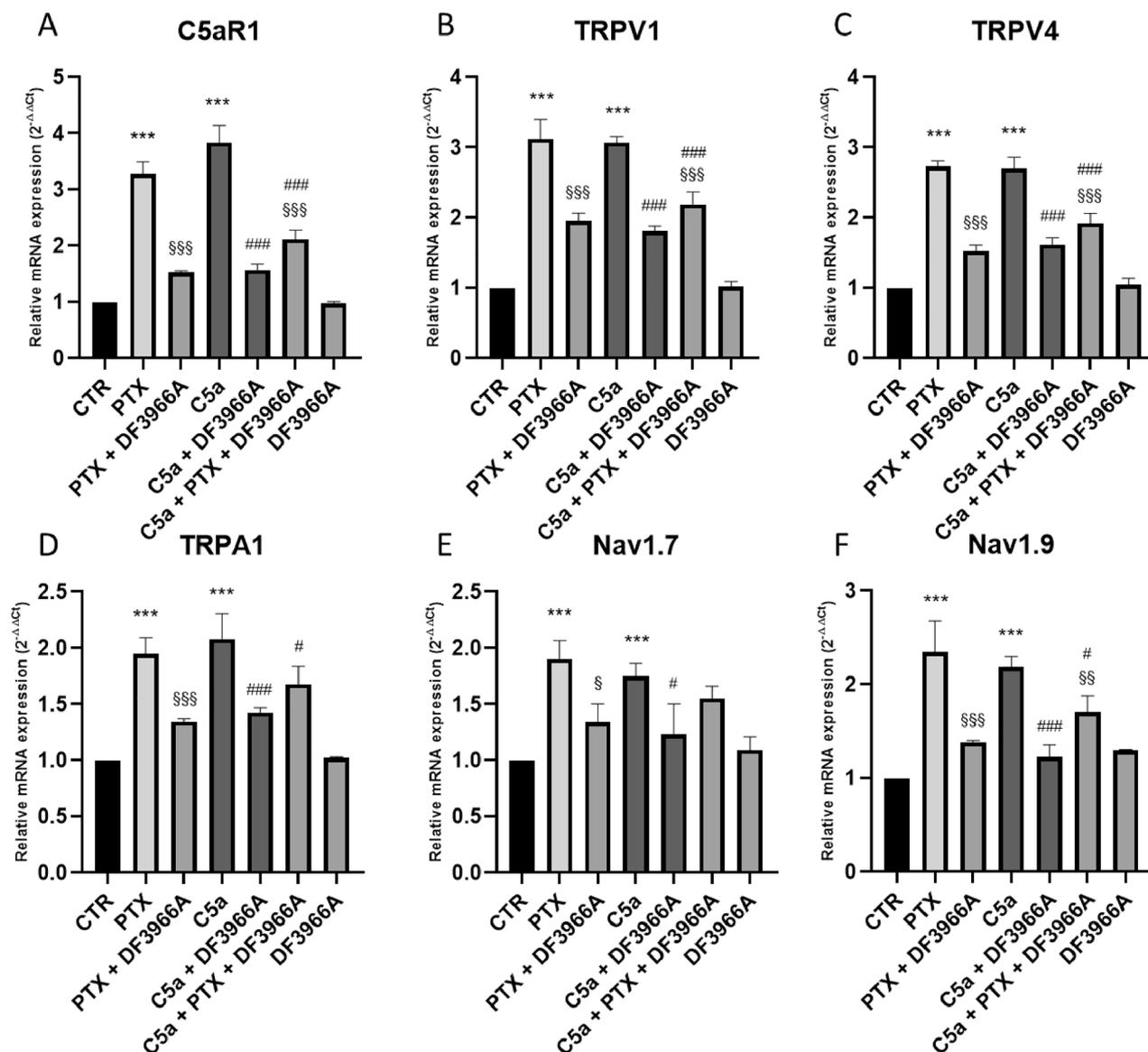


Fig. 6. Real-time PCR analysis of C5aR1 (A) TRPV1 (B) TRPV4 (C) TRPA1 (D) Nav1.7 (E) Nav1.9 (F) expression levels in sensory neurons treated for 12 h. *** $p < 0.0001$ vs CTR; §§§ $p < 0.0001$, §§ $p < 0.001$, § $p < 0.05$ vs Paclitaxel, ### $p < 0.0001$, # $p < 0.05$ vs C5a. Data are expressed as mean \pm SEM; $n = 3$ per group.

PNS and CNS. Of note, this pathway has been widely described as fundamental to sustain the production of pro-inflammatory cytokines underlying the development and progression of paclitaxel-induced neuropathic pain [59–63] and previous studies in which Avacopan and an anti-C5aR1 antibody were used, confirmed the specificity of DF3966A. [27] However, all the above discussed paves the way to new studies aimed at clarifying the link between the direct PTX-induced activation and pathways already described as associated with PIPN. Literature reports relevant correlations between mediators of homeostasis regulation that can help to clarify the cellular mechanisms underlying PIPN. TRPA1 ion channel has been shown to be involved in neuropathic pain induced by chemotherapy, in particular by paclitaxel, as demonstrated by studies on the effect of the drug on the increase of TRPA1 channel expression and activity in a cellular model [36] that suggests a role for the ion channel in the mechanism of PIPN, reinforced by the antinociceptive effect observed following administration of TRPA1 antagonists [64,65], even though specific molecular mechanisms are not yet known.

Expression and activation of TRPV4 ion channel, a transducer of inflammatory and nociceptive responses, are also involved in paclitaxel-

induced neuropathic pain mechanisms [66]. In particular, TRPV4, known to interact with NCS-1, a critical component of neuropathic pain induced by PTX, could be a key mediator of the signaling through which PTX induces cell damage and neuropathy [67], and could therefore be related to the regulation of NCS-1 described above. In this context, we evaluated in SH-SY5Y cells differentiated in sensory neurons, the expression of several mediators, including C5aR1, TRPV4, TRPV1 and TRPA1, Nav1.7 and Nav1.9 (Fig. 7) for both basal and paclitaxel-induced expression providing evidence that their expressions are increased by the chemotherapeutic agent, that this effect is induced by direct C5aR1 activation, as demonstrated by the comparison with C5a and by the effect of C5aR1 inhibitors, thus suggesting a link between C5aR1 activation and NCS-1.

All together these results reinforce the concept that paclitaxel/C5aR1 direct interaction and consequent receptor activation may represent the key event triggering the cascade of downstream signals already described as hallmarks of taxane-induced peripheral neuropathy.

In summary, the findings obtained using a pharmacological approach in this study validate the key role of C5aR1 activation, directly induced by paclitaxel binding to the receptor, in the induction and

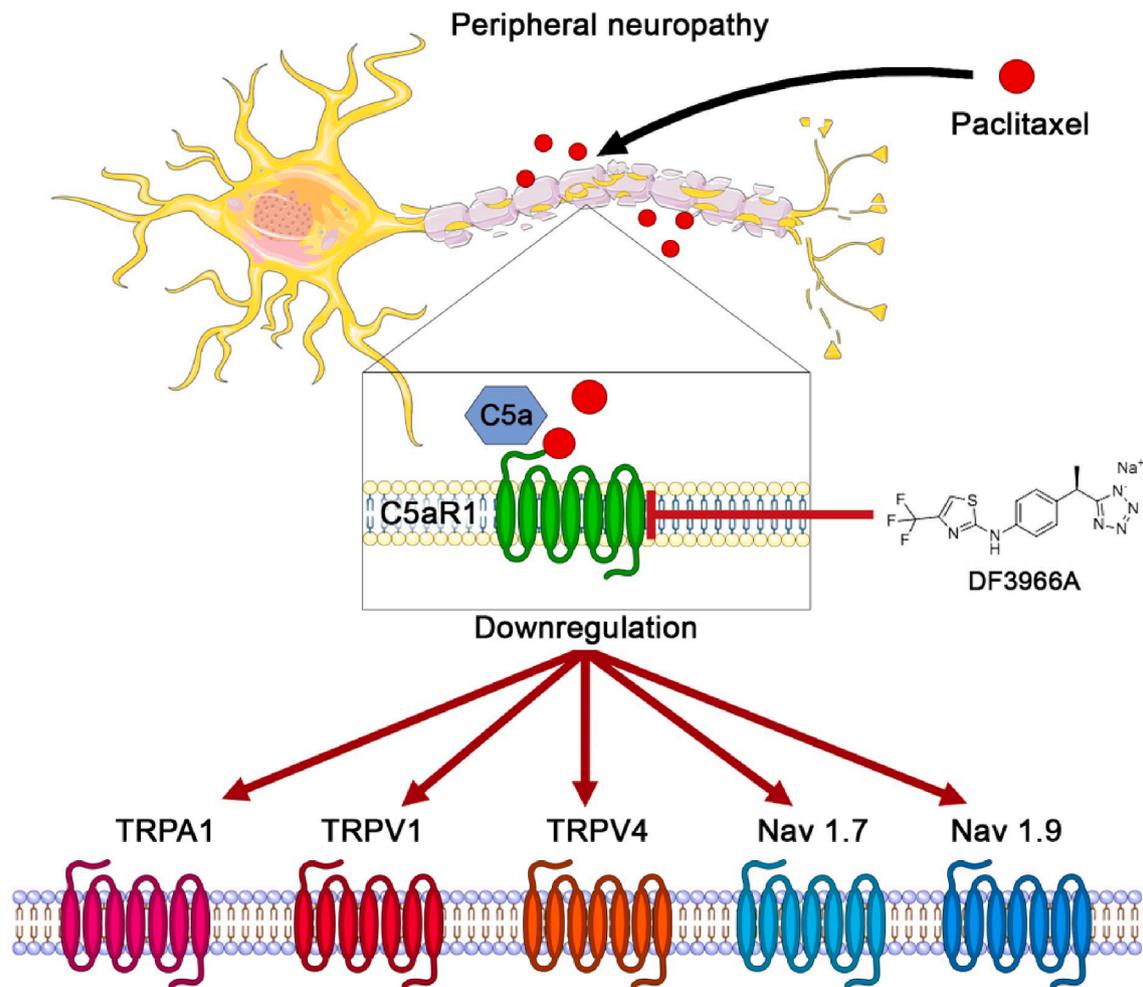


Fig. 7. Schematic representation of the potential effects of DF3966A in PIPN. Specifically, C5aR1 inhibition through DF3966A is crucial in the downregulation of pain receptors including ion channels TRPA1, TRPV1, TRPV4, Nav1.7 and Nav1.9.

maintenance of taxane-induced neuropathy, thus underscoring the therapeutic potential of targeting C5aR1 not only for the prevention but also for the treatment of patients with established symptoms of peripheral neuropathy induced by taxanes.

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CRediT authorship contribution statement

C. Cristiano: Methodology, Software, Formal analysis, Writing – original draft. **C. Giorgio:** Conceptualization, Methodology, Writing – original draft. **P. Cocchiario:** Methodology, Writing – original draft. **S. Boccella:** Methodology, Writing – original draft. **M.C. Cesta:** Writing – review & editing. **V. Castelli:** . **F.M. Liguori:** Methodology, Validation. **M.R. Cuozzo:** Methodology, Validation. **L. Brandolini:** Conceptualization, Writing – review & editing, Supervision. **R. Russo:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision. **M. Allegretti:** Conceptualization, Validation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Marcello Allegretti reports a relationship with Dompé farmaceutici S.p.A. that includes: employment. Maria Candida Cesta reports a relationship with Dompé farmaceutici S.p.A. that includes: employment. Laura Brandolini reports a relationship with Dompé farmaceutici S.p.A. that includes: employment. Pasquale Cocchiario reports a relationship with Dompé farmaceutici S.p.A. that includes: employment. Cristina Giorgio reports a relationship with Dompé farmaceutici S.p.A. that includes: employment. Serena Boccella reports a relationship with Dompé farmaceutici S.p.A. that includes: employment. Marcello Allegretti has patent #C5aR inhibitors for use in the treatment of chemotherapy-induced iatrogenic pain issued to Dompé farmaceutici S.p.A. Laura Brandolini has patent #C5aR inhibitors for use in the treatment of chemotherapy-induced iatrogenic pain issued to Dompé farmaceutici S.p.A.

Data availability

Data will be made available on request.

References

- [1] C. Gioroiu, L.H. Weimer, Update on Chemotherapy-Induced Peripheral Neuropathy, *Curr. Neurol. Neurosci. Rep.* 17 (6) (2017) 47.
- [2] A.M. Barbuti, Z.S. Chen, Paclitaxel Through the Ages of Anticancer Therapy: Exploring Its Role in Chemoresistance and Radiation Therapy, *Cancers (Basel)* 7 (4) (2015) 2360–2371.
- [3] A.S. Jaggi, N. Singh, Mechanisms in cancer-chemotherapeutic drugs-induced peripheral neuropathy, *Toxicology* 291 (1–3) (2012) 1–9.
- [4] I. Klein, H. Lehmann, Pathomechanisms of Paclitaxel-Induced Peripheral Neuropathy, *Toxics* 9 (10) (2021) 229.

- [5] Y. Tanabe, K. Hashimoto, C. Shimizu, A. Hirakawa, K. Harano, M. Yunokawa, K. Yonemori, N. Katsumata, K. Tamura, M. Ando, T. Kinoshita, Y. Fujiwara, Paclitaxel-induced peripheral neuropathy in patients receiving adjuvant chemotherapy for breast cancer, *Int. J. Clin. Oncol.* 18 (1) (2013) 132–138.
- [6] C.D. Scripture, W.D. Figg, A. Sparreboom, Peripheral neuropathy induced by paclitaxel: recent insights and future perspectives, *Curr. Neuropharmacol.* 4 (2) (2006) 165–172.
- [7] M.C. Spera, M.C. Cesta, M. Zippoli, G. Varrassi, M. Allegretti, Emerging Approaches for the Management of Chemotherapy-Induced Peripheral Neuropathy (CIPN): Therapeutic Potential of the C5a/C5aR Axis, *Pain Ther.* 11 (4) (2022) 1113–1136.
- [8] R.R. Ji, T. Berta, M. Nedergaard, Glia and pain: is chronic pain a gliopathy? *Pain* 154 (Suppl 1(0 1)) (2013). S10–S28.
- [9] E. Burgos, D. Gomez-Nicola, D. Pascual, M.I. Martin, M. Nieto-Sampedro, C. Goicoechea, Cannabinoid agonist WIN 55,212-2 prevents the development of paclitaxel-induced peripheral neuropathy in rats. Possible involvement of spinal glial cells, *Eur. J. Pharmacol.* 682 (1–3) (2012) 62–72.
- [10] Q.-Z. Zeng, F. Yang, C.-G. Li, L.-H. Xu, X.-H. He, F.-y. Mai, C.-Y. Zeng, C.-C. Zhang, Q.-B. Zha, D.-Y. Ouyang, Paclitaxel Enhances the Innate Immunity by Promoting NLRP3 Inflammation Activation in Macrophages, *Front. Immunol.* 10 (2019) 72.
- [11] M. Jia, C. Wu, F. Gao, H. Xiang, N. Sun, P. Peng, J. Li, X. Yuan, H. Li, X. Meng, B. o. Tian, J. Shi, M. Li, Activation of NLRP3 inflammasome in peripheral nerve contributes to paclitaxel-induced neuropathic pain, *Mol. Pain* 13 (2017), 174480691771980.
- [12] G. Chen, M.H. Shaw, Y.-G. Kim, G. Nuñez, NOD-like receptors: role in innate immunity and inflammatory disease, *Annu. Rev. Pathol.* 4 (1) (2009) 365–398.
- [13] H. Zhang, J.A. Boyette-Davis, A.K. Kosturakis, Y. Li, S.-Y. Yoon, E.T. Walters, P. M. Dougherty, Induction of monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2 in primary sensory neurons contributes to paclitaxel-induced peripheral neuropathy, *J. Pain* 14 (10) (2013) 1031–1044.
- [14] J.P. Cata, H.R. Weng, P.M. Dougherty, The effects of thalidomide and minocycline on taxol-induced hyperalgesia in rats, *Brain Res.* 1229 (2008) 100–110.
- [15] N.P. Staff, J.C. Fehrenbacher, M. Caillaud, M.I. Damaj, R.A. Segal, S. Rieger, Pathogenesis of paclitaxel-induced peripheral neuropathy: A current review of in vitro and in vivo findings using rodent and human model systems, *Exp. Neurol.* 324 (2020) 113121.
- [16] E.Y. Ibrahim, B.E. Ehrlich, Prevention of chemotherapy-induced peripheral neuropathy: A review of recent findings, *Crit. Rev. Oncol./Hematol.* 145 (2020) 102831.
- [17] R.L. Rodwin, N.Z. Siddiq, B.E. Ehrlich, M.B. Lustberg, Biomarkers of Chemotherapy-Induced Peripheral Neuropathy: Current Status and Future Directions, *Front. Pain Res. (Lausanne)*, 3 (2022), 864910.
- [18] G.R. Boeckel, B.E. Ehrlich, NCS-1 is a regulator of calcium signaling in health and disease, *Biochimica et Biophysica Acta (BBA) – Mol. Cell Res.* 1865 (11) (2018) 1660–1667.
- [19] M. Mo, I. Erdelyi, K. Szigeti-Buck, J.H. Benbow, B.E. Ehrlich, Prevention of paclitaxel-induced peripheral neuropathy by lithium pretreatment, *FASEB J.* 26 (11) (2012) 4696–4709.
- [20] R.J. Wadia, M. Stolar, C. Grens, B.E. Ehrlich, H.H. Chao, The prevention of chemotherapy induced peripheral neuropathy by concurrent treatment with drugs used for bipolar disease: a retrospective chart analysis in human cancer patients, *Oncotarget* 9 (7) (2018) 7322–7331.
- [21] C.A. Warwick, A.L. Keyes, T.M. Woodruff, Y.M. Usachev, The complement cascade in the regulation of neuroinflammation, nociceptive sensitization, and pain, *J. Biol. Chem.* 297 (3) (2021) 101085.
- [22] J. Xu, L. Zhang, M. Xie, Y. Li, P. Huang, T.L. Saunders, D.A. Fox, F. Lin, Role of Complement in a Rat Model of Paclitaxel-Induced Peripheral Neuropathy, *J. Immunol.* 200 (12) (2018) 4094–4101.
- [23] C. Giorgio, M. Zippoli, P. Cocchiario, V. Castelli, G. Varrassi, A. Aramini, M. Allegretti, L. Brandolini, M.C. Cesta, Emerging Role of C5 Complement Pathway in Peripheral Neuropathies: Current Treatments and Future Perspectives, *Biomedicines*. 9 (4) (2021) 399.
- [24] A.U. Quadros, T.M. Cunha, C5a and pain development: An old molecule, a new target, *Pharmacol. Res.* 112 (2016) 58–67.
- [25] R.S. Griffin, M. Costigan, G.J. Brenner, C. Him Eddie Ma, J. Scholz, A. Moss, A. J. Allchorne, G.L. Stahl, C.J. Woolf, Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity, *J. Neurosci.* 27 (32) (2007) 8699–8708.
- [26] L. Brandolini, M. Grannonico, G. Bianchini, A. Colanardi, P. Sebastiani, A. Paladini, A. Piroli, M. Allegretti, G. Varrassi, S. Di Loreto, The Novel C5aR Antagonist DF3016A Protects Neurons Against Ischemic Neuroinflammatory Injury, *Neurotox. Res.* 36 (1) (2019) 163–174.
- [27] L. Brandolini, M. d'Angelo, R. Novelli, V. Castelli, C. Giorgio, A. Sirico, P. Cocchiario, F. D'Egidio, E. Benedetti, C. Cristiano, A. Bugatti, A. Ruocco, P. G. Amendola, C. Talarico, C. Manelli, D. Iaconis, A. Beccari, A.U. Quadros, T. M. Cunha, A. Caruso, R. Russo, A. Cimini, A. Aramini, M. Allegretti, Paclitaxel binds and activates C5aR1: A new potential therapeutic target for the prevention of chemotherapy-induced peripheral neuropathy and hypersensitivity reactions, *Cell Death Dis.* 13 (5) (2022) 500.
- [28] N. Percie du Sert, V. Hurst, A. Ahluwalia, S. Alam, M.T. Avey, M. Baker, W. J. Browne, A. Clark, I.C. Cuthill, U. Dirnagl, M. Emerson, P. Garner, S.T. Holgate, D.W. Howells, N.A. Karp, S.E. Lázic, K. Lidster, C.J. MacCallum, M. Macleod, E. J. Pearl, O.H. Petersen, F. Rawle, P. Reynolds, K. Rooney, E.S. Sena, S. D. Silberberg, T. Steckler, H. Würbel, I. Boutroun, The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, *PLoS Biol.* 18 (7) (2020) e3000410.
- [29] R.C. Polomano, A.J. Mannes, U.S. Clark, G.J. Bennett, A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel, *Pain* 94 (3) (2001) 293–304.
- [30] M. Cuzzo, V. Castelli, C. Avagliano, A. Cimini, M. d'Angelo, C. Cristiano, R. Russo, Effects of Chronic Oral Probiotic Treatment in Paclitaxel-Induced Neuropathic Pain, *Biomedicines*. 9 (4) (2021) 346.
- [31] B. Laura, B. Elisabetta, R.P. Adelchi, R. Roberto, C. Loredana, A. Andrea, D. Michele, C. Vanessa, G. Antonio, A. Marcello, C. Annamaria, CXCR1/2 pathways in paclitaxel-induced neuropathic pain, *Oncotarget* 8 (14) (2017) 23188–23201.
- [32] C. Cristiano, C. Avagliano, M. Cuzzo, F.M. Liguori, A. Calignano, R. Russo, The Beneficial Effects of Ultramicronized Palmitoylethanolamide in the Management of Neuropathic Pain and Associated Mood Disorders Induced by Paclitaxel in Mice, *Biomolecules* 12 (8) (2022) 1155.
- [33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* 25 (4) (2001) 402–408.
- [34] A. Chukyo, T. Chiba, T. Kambe, K. Yamamoto, K. Kawakami, K. Taguchi, K. Abe, Oxaliplatin-induced changes in expression of transient receptor potential channels in the dorsal root ganglion as a neuropathic mechanism for cold hypersensitivity, *Neuropeptides* 67 (2018) 95–101.
- [35] M. Naziroglu, N. Braidly, Thermo-Sensitive TRP Channels: Novel Targets for Treating Chemotherapy-Induced Peripheral Pain, *Front. Physiol.* 8 (2017) 1040.
- [36] J.C. Sanchez, L.V. Munoz, M.L. Galindo-Marquez, A. Valencia-Vasquez, A. M. Garcia, Paclitaxel Regulates TRPA1 Function and Expression Through PKA and PKC, *Neurochem. Res.* 48 (1) (2023) 295–304.
- [37] J. Burgess, M. Ferdousi, D. Gosal, C. Boon, K. Matsumoto, A. Marshall, T. Mak, A. Marshall, B. Frank, R.A. Malik, U. Alam, Chemotherapy-Induced Peripheral Neuropathy: Epidemiology, Pathomechanisms and Treatment, *Oncol. Ther.* 9 (2) (2021) 385–450.
- [38] E. Ting, A.T.G. Guerrero, T.M. Cunha, W.A. Verri, S.M. Taylor, T.M. Woodruff, F. Q. Cunha, S.H. Ferreira, Role of complement C5a in mechanical inflammatory hypernociception: potential use of C5a receptor antagonists to control inflammatory pain, *Br. J. Pharmacol.* 153 (5) (2008) 1043–1053.
- [39] A. Moriconi, T.M. Cunha, G.R. Souza, A.H. Lopes, F.Q. Cunha, V.L. Carneiro, L. G. Pinto, L. Brandolini, A. Aramini, C. Bizzarri, G. Bianchini, A.R. Beccari, M. Fanton, A. Bruno, G. Costantino, R. Bertini, E. Galliera, M. Locati, S.H. Ferreira, M.M. Teixeira, M. Allegretti, Targeting the minor pocket of C5aR for the rational design of an oral allosteric inhibitor for inflammatory and neuropathic pain relief, *PNAS* 111 (47) (2014) 16937–16942.
- [40] M.R. de Zoete, N.W. Palm, S. Zhu, R.A. Flavell, Inflammasomes, *Cold Spring Harb. Perspect. Biol.* 6 (12) (2014) a016287.
- [41] Q.X. Yue, X. Liu, D.A. Guo, Microtubule-binding natural products for cancer therapy, *Planta Med.* 76 (11) (2010) 1037–1043.
- [42] W. Li, J. Liang, S. Li, L. Wang, S. Xu, S. Jiang, M. Song, H. Meng, D. Zhai, L. Tang, Y. Yang, L.i. Zhang, B. Zhang, Research progress of targeting NLRP3 inflammasome in peripheral nerve injury and pain, *Int. Immunopharmacol.* 110 (2022) 109026.
- [43] Y. Nakamura, N. Kambe, M. Saito, R. Nishikomori, Y.-G. Kim, M. Murakami, G. Nuñez, H. Matsue, Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria, *J. Exp. Med.* 206 (5) (2009) 1037–1046.
- [44] T. Zhang, Z. Fang, K.G. Linghu, J. Liu, L. Gan, L. Lin, Small molecule-driven SIRT3-autophagy-mediated NLRP3 inflammasome inhibition ameliorates inflammatory crosstalk between macrophages and adipocytes, *Br. J. Pharmacol.* 177 (20) (2020) 4645–4665.
- [45] E.L. Goldberg, J.L. Asher, R.D. Molony, A.C. Shaw, C.J. Zeiss, C. Wang, et al., beta-Hydroxybutyrate Deactivates Neutrophil NLRP3 Inflammasome to Relieve Gout Flares, *Cell Rep.* 18 (9) (2017) 2077–2087.
- [46] M. Cui, J. Liang, D. Xu, L. Zhao, X. Zhang, L.i. Zhang, S. Ren, D. Liu, X. Niu, Y.-J. Zang, B. Zhang, NLRP3 inflammasome is involved in nerve recovery after sciatic nerve injury, *Int. Immunopharmacol.* 84 (2020) 106492.
- [47] M.K. Mamik, C. Power, Inflammasomes in neurological diseases: emerging pathogenic and therapeutic concepts, *Brain* 140 (9) (2017) 2273–2285.
- [48] L.L. An, P. Mehta, L. Xu, S. Turman, T. Reimer, B. Naiman, et al., Complement C5a potentiates uric acid crystal-induced IL-1beta production, *Eur. J. Immunol.* 44 (12) (2014) 3669–3679.
- [49] E.I. Elliott, F.S. Sutterwala, Initiation and perpetuation of NLRP3 inflammasome activation and assembly, *Immunol. Rev.* 265 (1) (2015) 35–52.
- [50] Y. Jung, J.H. Lee, W. Kim, S.H. Yoon, S.K. Kim, Anti-allodynic effect of Buja in a rat model of oxaliplatin-induced peripheral neuropathy via spinal astrocytes and pro-inflammatory cytokines suppression. *BMC Complement, Altern. Med.* 17 (1) (2017) 48.
- [51] Y.-J. Gao, L. Zhang, O.A. Samad, M.R. Suter, K. Yasuhiko, Z.-Z. Xu, J.-Y. Park, A.-L. Lind, Q. Ma, R.-R. Ji, JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain, *J. Neurosci.* 29 (13) (2009) 4096–4108.
- [52] Y. Kawasaki, Z.-Z. Xu, X. Wang, J.Y. Park, Z.-Y. Zhuang, P.-H. Tan, Y.-J. Gao, K. Roy, G. Corfas, E.H. Lo, R.-R. Ji, Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain, *Nat. Med.* 14 (3) (2008) 331–336.
- [53] Z. Wu, S. Wang, I. Wu, M. Mata, D.J. Fink, Activation of TLR-4 to produce tumour necrosis factor-alpha in neuropathic pain caused by paclitaxel, *Eur. J. Pain* 19 (7) (2015) 889–898.
- [54] S. Al-Mazidi, M. Alotaibi, T. Nedjadi, A. Chaudhary, M. Alzoghbi, L. Djouhri, Blocking of cytokines signalling attenuates evoked and spontaneous neuropathic pain behaviours in the paclitaxel rat model of chemotherapy-induced neuropathy, *Eur. J. Pain* 22 (4) (2018) 810–821.

- [55] J. Khan, N. Noboru, Y. Imamura, E. Eliav, Effect of Pregabalin and Diclofenac on tactile allodynia, mechanical hyperalgesia and pro inflammatory cytokine levels (IL-6, IL-1beta) induced by chronic constriction injury of the infraorbital nerve in rats, *Cytokine* 104 (2018) 124–129.
- [56] F.A. White, J. Sun, S.M. Waters, C. Ma, D. Ren, M. Ripsch, J. Steflik, D.N. Cortright, R.H. LaMotte, R.J. Miller, Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion, *PNAS* 102 (39) (2005) 14092–14097.
- [57] M. Pevida, A. Lastra, A. Hidalgo, A. Baamonde, L. Menendez, Spinal CCL2 and microglial activation are involved in paclitaxel-evoked cold hyperalgesia, *Brain Res. Bull.* 95 (2013) 21–27.
- [58] A.F. Ducruet, B.E. Zacharia, S.A. Sosunov, P.R. Gigante, M.L. Yeh, J.W. Gorski, M. L. Otten, R.Y. Hwang, P.A. DeRosa, Z.L. Hickman, P. Sergot, E.S. Connolly, S. Di Giovanni, Complement inhibition promotes endogenous neurogenesis and sustained anti-inflammatory neuroprotection following reperfused stroke, *PLoS One* 7 (6) (2012) e38664.
- [59] K. Janes, C. Wahlman, J.W. Little, T. Doyle, D.K. Tosh, K.A. Jacobson, D. Salvemini, Spinal neuroimmune activation is independent of T-cell infiltration and attenuated by A3 adenosine receptor agonists in a model of oxaliplatin-induced peripheral neuropathy, *Brain Behav. Immun.* 44 (2015) 91–99.
- [60] X.-T. Ji, N.-S. Qian, T. Zhang, J.-M. Li, X.-K. Li, P. Wang, D.-S. Zhao, G. Huang, L. Zhang, Z. Fei, D. Jia, L.e. Niu, F. Gallyas, Spinal astrocytic activation contributes to mechanical allodynia in a rat chemotherapy-induced neuropathic pain model, *PLoS One* 8 (4) (2013) e60733.
- [61] T. Doyle, Z. Chen, C. Muscoli, L. Bryant, E. Esposito, S. Cuzzocrea, C. Dagostino, J. Ryerse, S. Rausaria, A. Kamadulski, W.L. Neumann, D. Salvemini, Targeting the overproduction of peroxynitrite for the prevention and reversal of paclitaxel-induced neuropathic pain, *J. Neurosci.* 32 (18) (2012) 6149–6160.
- [62] H. Kabata, D. Artis, Neuro-immune crosstalk and allergic inflammation, *J. Clin. Invest.* 129 (4) (2019) 1475–1482.
- [63] Y. Han, T. He, D.R. Huang, C.A. Pardo, R.M. Ransohoff, TNF-alpha mediates SDF-1 alpha-induced NF-kappa B activation and cytotoxic effects in primary astrocytes, *J. Clin. Invest.* 108 (3) (2001) 425–435.
- [64] A.S. de Almeida, F.K. Rigo, S.-T. De Prá, A.M. Milioli, G.C. Pereira, D. D. Lückemeyer, C.T. Antoniazzi, S.Q. Kudsí, D.M.P.A. Araújo, S.M. Oliveira, J. Ferreira, G. Trevisan, Role of transient receptor potential ankyrin 1 (TRPA1) on nociception caused by a murine model of breast carcinoma, *Pharmacol. Res.* 152 (2020) 104576.
- [65] C.T.D.D. Antoniazzi, R. Nassini, F.K. Rigo, A.M. Milioli, F. Bellinaso, C. Camponogara, C.R. Silva, A.S. de Almeida, M.F. Rossato, F. De Logu, S. M. Oliveira, T.M. Cunha, P. Geppetti, J. Ferreira, G. Trevisan, Transient receptor potential ankyrin 1 (TRPA1) plays a critical role in a mouse model of cancer pain, *Int. J. Cancer* 144 (2) (2019) 355–365.
- [66] J.C. Sanchez, B.E. Ehrlich, Functional Interaction between Transient Receptor Potential V4 Channel and Neuronal Calcium Sensor 1 and the Effects of Paclitaxel, *Mol. Pharmacol.* 100 (3) (2021) 258–270.
- [67] J.A. Boyette-Davis, E.T. Walters, P.M. Dougherty, Mechanisms involved in the development of chemotherapy-induced neuropathy, *Pain Manag.* 5 (4) (2015) 285–296.