Implication of system x_c^- in Complete Freund's Adjuvant-induced peripheral inflammation and associated nociceptive sensitization

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Keywords: Complete Freund's adjuvant, Hypersensitivity, Inflammation, Chronic pain, Slc7a11, Sulfasalazine, xCT.

List of abbreviations: CFA, Complete Freund's adjuvant; CNS, Central nervous system; GLAST, Glutamate aspartate transporter; GLT1, Glutamate transporter 1; IL-1β, Interleukin-1β; IL-6, Interleukin -6; i.p., Intraperitoneal; LPS, Lipopolysaccharides; PWL, Paw withdrawal latency; PWT, Paw withdrawal threshold; SAS, Sulfasalazine; TNF-α, Tumor necrosis factor α.

Abstract:

Background: Persistent inflammation leading to neuronal sensitization in pain pathways, are key features of chronic inflammatory pain. Alike macrophages in the periphery, glial cells exacerbate hypersensitivity by releasing proalgesic mediators in the central nervous system. Expressed by peripheral and central immune cells, the cystine-glutamate antiporter system x_c^- plays a significant role in inflammatory responses, but its involvement in chronic inflammatory pain remains underexplored. We herein investigated the contribution of this exchanger in nociceptive hypersensitivity triggered by a peripheral inflammatory insult.

Methods: Complete Freund's adjuvant (CFA) was injected into the left hind paw of wild-type C57Bl/6 female mice, of xCT-deficient mice (specific subunit of system x_c^-) and of mice receiving the system x_c^- inhibitor sulfasalazine. Paw edema was measured over three weeks and pain-associated behaviors were evaluated. Additionally, pro-inflammatory cytokine levels were assessed in blood samples.

Results: CFA injection led to a persistent increase in paw edema and hypersensitivity to mechanical and thermal stimuli, which were less pronounced in xCT-deficient mice. This reduced sensitivity was accompanied by lower systemic pro-inflammatory cytokine levels in xCT-deficient mice. Accordingly, pharmacological inhibition of system x_c^- with sulfasalazine, either before or after pain induction, efficiently reduced the algesic and inflammatory responses to CFA in wild-type mice.

Conclusion: Our findings reveal a critical role for system x_c^- in the pathophysiology of inflammatory pain. xCT deficiency reduces pain behaviors and peripheral inflammation, positioning system x_c^- as a promising therapeutic target for alleviating chronic inflammatory pain.

Keywords : System x_c^- ; SLC7A11 ; Inflammatory pain ; Hypersensitivity ; Sulfasalazine ;

Introduction:

With millions of affected individuals worldwide, chronic inflammatory pain represents a debilitating condition that causes significant global health challenges (Alotaibi et al. 2023, Rice, Smith and Blyth 2016). Current clinical treatments frequently offer inadequate relief, underscoring the urgent need to develop novel effective therapeutic approaches to tackle chronic inflammatory pain.

Upon peripheral tissular insult, macrophages play a critical role in orchestrating the inflammatory response that may lead to persistent pain. Indeed, once recruited at the lesion site, they release pro-inflammatory cytokines and chemokines, which in turn promote local inflammation and sensitize nociceptive sensory fibers (Linley et al. 2010). This macrophage-mediated inflammatory cascade contributes to the development and maintenance of pain which is part of the five cardinal symptoms of inflammation besides redness, heat, swelling, and loss of function (Lipnik-Stangelj 2013).

Peripheral inflammation is also documented to profoundly impact the modulation of nociception and pain perception, potentially leading to chronicity. Emerging evidence highlights a crucial role for glial cells of the central nervous system (CNS) in the pathogenesis of chronic pain with an inflammatory origin. Once activated by the peripheral trigger, microglia and astrocytes in the spinal cord or in the brainstem release a variety of proalgesic mediators that can facilitate signal transmission and pain. This is typically recapitulated in the widely used rodent model of inflammatory pain which consists in the single intraplantar injection of Complete Freund's Adjuvant (CFA). The production and release of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) by microglial cells is well-documented and promotes pain hypersensitivity following inflammatory insults (Lipnik-Stangelj 2013). Additionally, studies

have demonstrated that blocking microglial activation upstream of the inflammatory input can alleviate allodynia (pain due to a stimulus that does not usually provoke pain) and hyperalgesia (increased pain from a stimulus that usually provokes pain), common symptoms of chronic inflammatory pain (Baniasadi et al. 2020, Ledeboer et al. 2005).

Expressed on both peripheral (macrophages and lymphocytes) and central (astrocytes and activated microglia) immune cells, the cystine-glutamate antiporter system x_c^-, is known to participate in inflammatory responses. Its expression in the spleen and thymus is consistent with its prominent role in the modulation of both innate and adaptative immune responses (Taguchi et al. 2007). System x_c^- is a transmembrane Na^+-independent amino acid transporter that is structurally composed of two subunits: the heavy chain SLC3A2 known as 4F2hc, common to several amino acid transporters, and the light chain specific subunit SLC7A11 known as xCT (Sato et al. 1999). It transports L-cystine and L-glutamate in a 1:1 molecular ratio, preferentially importing cystine in exchange for glutamate release (Bannai and Kitamura 1980). By importing cystine needed for the biosynthesis of glutathione, system x_c^- contributes to protection against oxidative stress. xCT expression has been shown to be upregulated in macrophages and microglial cells during peripheral inflammation (Mesci et al. 2015, Sato et al. 1995), suggesting a role in immune cell survival (Nabeyama et al. 2010). Reinforcing the same notion, studies in aged mice lacking xCT showed attenuated systemic inflammatory responses and preserved brain function (Verbruggen et al. 2022). Moreover, the absence of system x_c^- led to reduced production of pro-inflammatory cytokines by activated microglia, while increasing anti-inflammatory markers (Mesci et al. 2015). Together these findings underscored that system x_c^- has a pivotal function in modulating macrophage/microglial activation and subsequent inflammatory responses, increasing the interest for its implication in inflammatory diseases such as chronic pain.

While importing cystine and regulating inflammatory responses, system x_c^- also elevates extracellular glutamate concentration (Lewerenz et al. 2013, Massie et al. 2015). Alteration in excitatory neurotransmission can significantly influence synaptic activity and contribute to pathological conditions. Hence, given the critical role of glutamate transmission in pain processing, a finely tuned regulation of its extracellular levels is essential. While most pain research has focused on synaptic glutamate uptake by astrocytic glutamate transporters (glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST)) which account for over 90% of glutamate clearance in the CNS (Danbolt 2001, Gegelashvili and Bjerrum 2017), the role of system x_c^-, responsible for a significant non-vesicular glutamate release, remains understudied in the pathophysiology of chronic inflammatory pain.

While a previous study from our laboratory has shown that xCT-deficient mice exhibited reduced pain sensitivity and decreased glial activation following peripheral sciatic nerve ligation (a surgical model of neuropathic pain) (Beckers et al. 2024), the exact role of system x_c^- in the inflammatory process leading to chronic pain remains overlooked. Given this, the current study aimed at specifically examining the potential implication of system x_c^- in modulating CFA-induced behavioral hypersensitivity and the associated peripheral inflammation.

Materials and methods:

1. Animals and Ethical statement

All experiments were conducted in strict accordance with the recommendations of the European commission and with the agreement of the Belgian Ministry of Agriculture (license number LA 1230618). The ethical committee of the Université catholique de Louvain for animal experiments specifically approved this study (aggregation number 2023/UCL/MD/50). Wild-type ($xCT^{+/+}$) and xCT knock-out ($xCT^{-/-}$) mice used in this study were high-generation descendants (with C57BL/6J background) of the strain previously described (Sato et al. 2005). These mice lacking xCT were generously provided by Dr. Hideyo Sato (Department of Medical Technology, Niigata University, Japan) and bred in the animal facilities of the Vrije Universiteit Brussel and the Université catholique de Louvain. All experiments combined homozygous animals obtained both from the breeding of heterozygous and homozygous animals. Homozygous breeders were always first-generation offspring of a heterozygous couple.

All animals were accommodated under standard laboratory conditions, receiving food and water ad libitum, and were housed in the animal facility at the Université catholique de Louvain, in a 12-12h light-dark cycle, controlled temperature and humidity conditions. Mice lacking xCT were genotyped by end-point PCR on genomic DNA extracted from an ear biopsy using specific primers for the Slc7a11 gene. PCR amplification products were analyzed by agarose-gel electrophoresis to identify bands revealing the intact or truncated gene (Sato et al. 2005). Since pain hypersensitivity is sex-dependent, only female mice were used to reduce variability.

2. Experimental design

Adult female mice (wild-type and $xCT^{-/-}$ mice) aged between 10 and 12 weeks were randomly subjected to subcutaneous plantar injection of CFA (1 mg/mL *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate, F5881, Sigma-Aldrich) or saline (NaCl 0.9%, Baxter). Briefly, mice were anesthetized with sevoflurane (Abbott) (4% for induction and 3% for maintenance with oxygen as a carrier gas). Under continuous anesthesia, mice were subcutaneously injected with 20 µL of CFA in the plantar surface of their left hind paw, referred to as ipsilateral in the current manuscript. Controls were injected with saline under the same conditions. Baseline measurement and daily follow-up after CFA injection was assessed for the body weight, spontaneous locomotion, and paw edema. Pain-related behavior (allodynia and hyperalgesia) was also evaluated on a daily basis throughout the duration of the experiment. For the pharmacological part of the study, mice were injected for 10 consecutive days with sulfasalazine (SAS, Sigma-Aldrich), a system x_c^- inhibitor, starting 2 days prior CFA injection. At different time-points (4, 7 or 21 days) following CFA injection, mice were sacrificed for blood collection.

3. Pain-related behavior testing

Pain is a complex experience that remains difficult to assess and measure in rodents. Hence, preclinical research commonly examines the response of animals to noxious stimuli which are thus interpreted as pain-related behaviors (Karimi, Zahra and Martin 2024). In the present study, mechanical hypersensitivity was assessed using the von Frey hair filament test as previously described (Bosier et al. 2015). Briefly, animals were placed in transparent chambers positioned on an elevated mesh floor. Acclimatization was allowed for 20 min. A set of 10 calibrated von Frey hair filaments (Stoelting Co.) was used (0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2,

4 and 6 g). To determine the mechanical sensitivity, the “up and down” method was used (Deuis, Dvorakova and Vetter 2017). The 50% paw withdrawal threshold (PWT) was then calculated using the formula previously described (Chaplan et al. 1994).

The thermal paw withdrawal latency (PWL) was used to assess hyperalgesia (Hargreaves et al. 1988) as previously described (Bosier et al. 2015). Briefly, mice were acclimated for 20 min in separated transparent, bottom-free plastic chambers placed over the glass enclosure of the Hargreaves paw thermal stimulator (University of California, San Diego). A heat source was positioned underneath the plantar surface of the hind paw and the time taken to withdraw from the heat source was automatically recorded by the apparatus. For behavioral analyses, the experimenter responsible for conducting the tests was blind to the treatment, the genotype and the injection performed on the mice.

4. Paw thickness measurement

The edema caused by inflammation was estimated by measuring the dorsal to plantar width of the hind paw as previously reported (Soyocak et al. 2019). Briefly, mice were held by the tail and the caliper was placed transversely to the ipsilateral hind paws to measure their width, expressed in millimeter (mm). Measurements were taken at baseline and at different time points following CFA injection.

5. General locomotion – open field

The spontaneous locomotor activity (velocity and distance moved) of the mice was assessed by placing them in an open field (60 cm x 60 cm x 60 cm) for 15 min. An automated integration system (Ethovision software, Noldus) was used to analyze the distance traveled and the average velocity, as previously described (Goursaud et al. 2015).

6. Sulfasalazine administration

Animals were randomly assigned to either the treatment or control group. An 80 mg/mL stock solution of SAS (Sigma-Aldrich) was prepared by dissolving the powder in DMSO. The final solution for administration was daily prepared by adding saline to reach the desired concentration of SAS. The pH was adjusted using a small volume of NaOH 0.1 M. Mice were daily injected intraperitoneally (i. p.) with 200 mg/kg of SAS (or vehicle that includes the same dilution of DMSO for the controls) (Tsuchihashi et al. 2016, Verbruggen et al. 2021) for 10 consecutive days starting 2 days prior or 7 days after the CFA injection.

7. Plasma cytokine analyses

Mice were euthanized with CO_2 4, 7 or 21 days after CFA or saline intraplantar injection. Blood was intracardially collected using a 26 g needle mounted on EDTA-coated syringe and transferred to heparin-coated collection tubes (365966A, D. Dutscher). The tubes were then centrifuged 3 min at 2700 g and the plasma fraction was collected and stored at -80°C. Plasma concentrations of pro-inflammatory cytokines (TNF-α and interleukin-6 (IL-6)) were determined using the Meso Scale Discovery U-PLEX customized mouse assay (MSD), according to the manufacturer's instructions. Briefly, biotinylated capture antibodies were coupled to their respective linker for 30 min. Subsequently, a coating solution was prepared by combining all U-PLEX linker-coupled primary antibodies. Next, the wells were coated with this solution and incubated at room temperature (RT) while shacking for 1 h before rinsing the plate with PBS-Tween (0.05%). During the incubation period, a calibration curve was prepared using the provided calibrator. Samples and calibrator standards were added to each well and incubated for 1 h at RT. Following a quick washing step, detection antibodies were introduced to the

wells and incubated for another hour. After a final washing step, MSD GOLD Read Buffer was added, and the plate was analyzed using the MSD Quickplex SQ120MM.

8. Statistical analyses

Data are presented as means with standard error of the mean (SEM). Outliers were identified using the Grubbs test and removed from the dataset when appropriate. For all analyses, a two-sided type I error of 0.05 was considered for statistical significance. Statistical analyses were performed using GraphPad Prism version 9.5.1 and R software.

For follow-up analyses, statistical comparisons were made relative to the corresponding baseline values to control for individual variability. Mixed-effects models were fitted to the data using genotype, time and treatment as the fixed effects. Repeated measurements were included in the model within individual animals. Two or three-way ANOVA were performed based on the experimental design and pairwise comparisons were conducted using the Tukey post-test. The normality of residuals was assessed using Shapiro-Wilk normality test, and equality of variances was tested using the Levene test to ensure the assumptions of the statistical tests.

Results:

1. xCT deletion attenuates edema without affecting body weight and general locomotion following intraplantar injection of CFA

The contribution of system x_c^- to chronic inflammatory pain was examined by subjecting female $xCT^{+/+}$ and $xCT^{-/-}$ mice to a single unilateral injection of CFA into the left hind paw. Mice were monitored for 21 days post-injection using a caliper to estimate the thickness of their ipsilateral paw. Prior to CFA injection, $xCT^{-/-}$ mice exhibited similar paw thicknesses compared to $xCT^{+/+}$ mice. Additionally, mice receiving an injection of saline maintained paw thicknesses to pre-injection levels. As depicted in Figure 1, CFA-injected mice displayed significant and prolonged paw edema, beginning as early as 4 h post-injection and persisting throughout the 21-day observation period. Paw thickness (width) peaked at 5.17 ± 0.11 mm in wild-type mice 24 h after CFA injection, compared to 2.06 ± 0.02 mm in mice injected with saline. $xCT^{-/-}$ mice also exhibited an increase in paw thickness over the course of the experiment, albeit considerably less pronounced (peaking at 4.46 ± 0.13 mm on day 1 post-injection) than observed in $xCT^{+/+}$ mice (significant difference at all time points tested). As expected, no change was observed for the contralateral paw (data not shown).

Daily monitored mouse body weight was unaffected by CFA injection or by genotype (Fig. 2A), indicating general well-being despite significant edema in the ipsilateral paw. Spontaneous locomotor activity was evaluated in an open field test. Measures of the distance traveled (Fig. 2B) and the average velocity (Fig. 2C) revealed no significant differences between $xCT^{+/+}$ and $xCT^{-/-}$ mice. Mice receiving CFA injection showed a transient (1 day) reduction in distance traveled and average velocity compared to baseline measurements, but the observed effects

were similar in both genotypes. Together, these results allow us to exclude a potential locomotor bias in the assessment of other parameters.

2. Mice lacking xCT present reduced hypersensitivity to mechanical and thermal stimuli following intraplantar injection of CFA

Peripheral inflammation is known to induce sensitization characterized by the development of enhanced sensitivity to mechanical and thermal stimulations. Therefore, female $xCT^{+/+}$ and $xCT^{-/-}$ mice were monitored for 21 days post-injection using the von Frey and Hargraeves tests to respectively evaluate CFA-induced allodynia and hyperalgesia. As illustrated in Figure 3A, prior to CFA injection, $xCT^{-/-}$ mice exhibited an equivalent mechanical sensitivity as compared to $xCT^{+/+}$ mice. Additionally, control mice (receiving saline injection) displayed transient hypersensitivity, as from the 3rd day post-injection, they regained a 50% PWT similar to that measured before the injection. This transient hypersensitivity appeared comparable between both genotypes. Besides, $xCT^{+/+}$ animals administered with CFA displayed significant and prolonged mechanical allodynia, evident as early as 4 h after CFA injection. The 50% PWT reached a minimum value of 0.002 ± 0.001 g on day 1 and persisted until day 18 (with a 50% PWT value of 0.052 ± 0.010 g), with some modest recovery on days 20 and 21 (0.16 ± 0.04 g and 0.20 ± 0.04 g, respectively) after injection. In $xCT^{-/-}$ mice, a decrease in 50% PWT was also observed following CFA administration which was less pronounced compared to that observed in $xCT^{+/+}$ animals. Moreover, the 50% PWT to mechanical stimulation tended to progressively recover as from day 15 post-injection (reaching 1.86 ± 0.23 g on day 21 post-injection), indicating that the hypersensitivity observed in $xCT^{-/-}$ animals tends to progressively resolve compared to that observed in $xCT^{+/+}$ mice. When testing the contralateral paw, a temporary reduction in the 50% PWT was noticed during the first 4 h for the saline-injected

mice and during the first 2 days for the CFA-injected mice (Fig. 3B). However, by day 4 after injection, all mice exhibited sensitivity to mechanical stimulation comparable to the baseline level.

When testing the response to a thermal stimulation using the Hargraeves test, $xCT^{+/+}$ mice receiving CFA injection developed a persistent hyperalgesia as indicated by the decreased latency for the ipsilateral paw withdrawal reaching 40% of the baseline value after only 4 h (Fig. 3C). This hypersensitivity to thermal stimuli persisted for the entire duration of the experiment. While $xCT^{-/-}$ also developed ipsilateral hyperalgesia after CFA administration, as soon as 3 days post-injection this decrease was significantly less pronounced compared to that observed in $xCT^{+/+}$ animals. No significant changes were observed when testing the contralateral paw for thermal sensitivity (Fig. 3D).

3. xCT deletion results in attenuated peripheral inflammation following intraplantar injection of CFA

After observing reduced CFA-induced hypersensitivity in mice lacking xCT along with a decrease in peripheral edema as compared to $xCT^{+/+}$ mice, we evaluated the impact of system x_c^- deletion on systemic inflammation induced by the peripheral insult. Considering that the absence of xCT had a predominant influence on the progressive recovery of pain sensitivity, we examined the difference in pro-inflammatory cytokine levels caused by the inflammatory insult in mice from both genotypes. As expected, in mice receiving a saline injection in the paw, plasma levels of the cytokines remained relatively unaffected over time, both in $xCT^{+/+}$ and $xCT^{-/-}$ mice (Fig. 4A&C). However, in $xCT^{+/+}$ animals receiving the CFA injection, the plasma IL-6 level increased over time, while conversely, a decreasing profile was observed in $xCT^{-/-}$ mice. A significant difference was indeed observed between the two genotypes at 21 days

after CFA injection (Fig. 4B). Plasma TNFα levels of $xCT^{-/-}$ mice receiving CFA appeared lower as compared to $xCT^{+/+}$ mice, reaching statistical significance at 7 and 21 days after CFA injection (Fig. 4D).

4. SAS exhibits anti-allodynic and anti-hyperalgesic properties through a system x_c^- dependent mechanism

A pharmacological approach was used to further examine the implication of system x_c^- on the development of inflammatory pain. Both $xCT^{+/+}$ and $xCT^{-/-}$ animals were subjected to CFA injection and treated for 10 consecutive days with either saline or SAS (200 mg/kg once per day, i.p.). The treatment was administered either preemptively to the induction of inflammatory pain (2 days before CFA injection) or once the pain was established (7 days after CFA injection). The mice were monitored over 3 weeks following the CFA administration.

a. Preemptive treatment with sulfasalazine

Baseline measurements revealed that SAS injection had no influence on paw thickness (Fig. 5A) or sensitivity to mechanical and thermal (Fig. 5B&C) stimuli, as thresholds remained unchanged following SAS treatment prior to CFA injection. When SAS treatment was initiated two days before CFA injection, a reduction in paw edema was observed in $xCT^{+/+}$ mice, as evidenced by decreased paw thickness compared to vehicle-treated mice. As mentioned above, the edema induced by CFA in $xCT^{-/-}$ mice was less pronounced as compared to $xCT^{+/+}$ mice, and no effect of SAS treatment was observed. Hence, it is noticeable that the paw edema observed in $xCT^{+/+}$ mice treated with SAS was similar to the edema observed in $xCT^{-/-}$ receiving or not SAS (Fig. 5A).

Mechanical hypersensitivity was relatively similar between vehicle-treated and SAS-treated $xCT^{+/+}$ mice, at least until day 13 post-injection. From that time point, a progressive recovery

was observed in SAS-treated $xCT^{+/+}$ animals as indicated by the increase in the 50% PWT, contrary to saline-treated $xCT^{+/+}$ mice. Therefore, SAS-treated $xCT^{+/+}$ mice displayed a profile similar to mice lacking xCT for the rest of the experiment (Fig. 5B).

As detailed before, $xCT^{+/+}$ mice injected with CFA developed significant hypersensitivity when tested for thermal stimulation, evidenced by a decreased latency before paw removal. As from the first time tested (2 days after CFA injection), $xCT^{+/+}$ mice receiving SAS exhibited significantly increased PWL compared to vehicle-treated counterparts, an effect maintained throughout the experimental period (Fig. 5C). When the same protocols were conducted in $xCT^{-/-}$ mice, no differences were observed in thermal and mechanical sensitivity between vehicle-treated and SAS-treated mice.

b. Curative treatment with sulfasalazine

To evaluate the effect of SAS on established inflammation and associated pain behaviors, the 10 days-treatment was initiated 7 days after CFA injection. Within a few days after initiating the treatment, $xCT^{+/+}$ mice receiving SAS exhibited a progressive reduction of the paw thickness as compared to vehicle-treated $xCT^{+/+}$ mice. This reduction was maintained throughout the treatment period and persisted beyond, with the paw thickness reaching levels similar to those seen in $xCT^{-/-}$ mice (Fig. 6A).

Regarding pain-related behaviors, the impact of initiating a treatment with SAS on hypersensitivity was mainly observed when assessing the response to thermal stimulation and less obvious with the mechanical stimulus. The mechanical sensitivity was comparable between vehicle-treated and SAS-treated $xCT^{+/+}$ mice up to day 7 after initiating the treatment with SAS. Thereafter, a modest recovery was noticed in SAS-treated $xCT^{+/+}$ mice, as indicated by an increase in the 50% PWT that did not reach statistical significance (Fig. 6B). When tested

333 for thermal hypersensitivity, $xCT^{+/+}$ mice receiving SAS after establishment of the
334 hypersensitivity show a time-dependent increase in PWL (Fig. 6C). After 10 days of treatment,
335 the PWL value measured in $xCT^{+/+}$ mice was similar to the value observed in $xCT^{-/-}$ mice
336 receiving or not SAS that indeed show reduced CFA-induced hypersensitivity.

337 To confirm that the observed effects of SAS were attributable to the inhibition of system x_c^-,
338 $xCT^{-/-}$ mice were subjected to the same treatment regimen. No significant differences were
339 observed in paw thickness, mechanical or thermal sensitivity between vehicle-treated and
340 SAS-treated mice (Fig. 6).

341

Discussion:

Inflammatory pain is a multifaceted biological response that engages the somatosensory, immune, neuronal, and vascular systems in reaction to tissue damage. Initially, acute inflammation, which results in pain perception, serves as a protective function by initiating the healing process and restoring tissue integrity. However, in some cases and for reasons that are still under investigation, inflammation and the accompanying pain may persist, evolving into maladaptive and chronic pain. The persistence of inflammation is associated with nervous sensitization in the pain pathways primarily assigned to the high levels of immune mediators such as pro-inflammatory cytokines including TNFα, IL-1β and IL-6 (Clark and Vissel 2016, Littlejohn 2015, Scholz and Woolf 2007, Zhang and An 2007). After binding to their receptors expressed on neurons within the central and peripheral nervous systems (Cook et al. 2018, Guo et al. 2007, Scholz and Woolf 2007, Szelényi 2001), these mediators modulate the expression and activity of ion channels and receptors responsible for pain transmission (Carlton and Coggeshall 1999, Kawasaki et al. 2008, Linley et al. 2010, Zhang, Bi and Gan 2018). Also, the inflammatory cytokine TNFα was shown to modulate the expression of astrocytic high-affinity glutamate transporters (GLT-1 and GLAST), in charge of maintaining low glutamate concentrations in the synaptic cleft and prevent hyperexcitability (Sitcheran et al. 2005). Chronic pain is indeed characterized by disrupted glutamatergic neurotransmission, partially attributed to altered GLT-1 and GLAST expression and functionality (Guo et al. 2019). In a mouse model of inflammatory pain induced by peripheral injection of CFA or formalin, a GLT-1 enhancer was found effective in reducing allodynia (Alotaibi et al. 2023, Alotaibi and Rahman 2019).

Alike glutamate transporters, emerging evidence suggests that system x_c^- could also be regulated by the presence of inflammatory mediators such as LPS, TNFα and IL-1β (Jackman et al. 2010, Sato et al. 1995). Since system x_c^- is responsible for glutamate release from activated glial cells and macrophages, it is considered as an indirect contributor to excitatory synaptic transmission (Kigerl et al. 2012, Massie et al. 2015). By increasing glutamate release and the consequent activation of glutamate receptors and their downstream pathways, enhanced activity of system x_c^- could participate in the dysregulated glutamate transmission, supporting the development of chronic pain. Expressed by immune and glial cells, system x_c^- is also a key player in modulating immune and inflammatory responses. Notably, the genetic invalidation of system x_c^- was shown to attenuate inflammation in various *in vivo* and *in vitro* models of neurological disorders (Albertini et al. 2018, Mesci et al. 2015, Verbruggen et al. 2022).

In both rats and mice, the intraplantar injection of CFA is a commonly used experimental model of persistent inflammatory pain (Fehrenbacher, Vasko and Duarte 2012). Shortly after CFA injection, rodents exhibit signs of peripheral inflammation with an important local edema associated with severe mechanical and thermal hypersensitivities that persist for several weeks (Fehrenbacher, Vasko and Duarte 2012, Stein, Millan and Herz 1988). This model has also been reported to trigger central plasticity with sustained neuroinflammation in the dorsal spinal cord (Li et al. 2005, Raghavendra, Tanga and DeLeo 2004). The present study aimed at elucidating the role of system x_c^- in the peripheral inflammatory response and the associated persisting pain hypersensitivity. Before evaluating the impact of xCT deletion on pain associated behaviors, the spontaneous locomotion was monitored using the open field test to exclude potential biases associated with CFA injection that could lead to a reduced mobility.

Such bias might otherwise interfere with paw withdrawal responses during the Von Frey or the Hargraeves tests. Our results demonstrated that, on average, throughout the duration of the experiment, neither $xCT^{+/+}$ nor $xCT^{-/-}$ mice exhibited significant changes in spontaneous locomotion following injection with CFA or saline. These results are in accordance with the meta-analysis of Burek and colleagues highlighting that unilateral intraplantar injection of CFA does not impact exploratory behavior tested in an open field up to 6 weeks post-injection (Burek et al. 2021).

We here report that the genetic invalidation of system x_c^- significantly influences the peripheral inflammation induced by CFA evidenced by the local edema at the injected paw. Compared to $xCT^{+/+}$, $xCT^{-/-}$ mice exhibited a noticeable reduction in paw edema both in the acute stage and during the entire animal follow-up (3 weeks). As described by Mesci and colleagues when studying the microglial response in a model of amyotrophic lateral sclerosis, this attenuated local inflammation in $xCT^{-/-}$ mice may be explained by the reduced release of pro-inflammatory mediators by activated macrophages recruited at the injection site (Mesci et al. 2015). Using a model of peripheral inflammation, the present observation strengthens the already established evidence underscoring the significant role of system x_c^- in regulating inflammation.

Beside a smaller edema, $xCT^{-/-}$ mice injected with CFA developed hypersensitivity to mechanical and thermal stimulation that differs from $xCT^{+/+}$ mice. While the behavioral tests revealed similar sensitization in the initial days following CFA injection in both genotypes, $xCT^{-/-}$ mice showed partial but substantial recovery within the first weeks following the injection whereas strong allodynia and hyperalgesia persisted for up to three weeks in $xCT^{+/+}$ animals. Since the behavior of mice lacking xCT only improved at later stages, this suggests that system

410 x_c^- activity participates in the maintenance of hypersensitivity rather than in its onset.
411 Nevertheless, it is noteworthy that the genetic suppression of xCT in mice also impacts on the
412 early immune responses, as shown by the reduced maximal paw thickness measured after the
413 CFA injection. This influence of xCT in the initiation of the tissue response leading to
414 sensitization is consistent with our previous observations in a model of neuropathic nerve
415 lesion (Beckers et al. 2024).

416 It is demonstrated that certain cytokines are not only involved in the initiation but also the
417 persistence of pathological pain through the continuous activation of nociceptive sensory
418 neurons in the periphery (Zhang and An 2007). Also, accumulating evidence indicates that
419 early biochemical modifications along nociceptive transduction pathways may have delayed
420 consequences in pain pathophysiology (Victoria and Murphy 2016). To fully understand the
421 role of system x_c^-, initiating its inhibition or invalidation a few days after triggering the
422 inflammatory insult would help to determine whether targeting this exchanger could reverse
423 established inflammatory pain.

424 The results obtained using the genetic model of constitutive xCT deletion were corroborated
425 using a pharmacological approach. We demonstrated that inhibition of system x_c^- by systemic
426 treatment with SAS was effective in reducing inflammatory pain-related behaviors. Indeed,
427 preventively and curatively treated $xCT^{+/+}$ mice exhibited a faster resolution of paw edema
428 and hypersensitivity to thermal and mechanical stimuli. Regarding mechanical allodynia, the
429 analgesic effect of SAS seems to appear at a later stage. However, this might be explained by
430 an experimental bias since CFA injection induces such a high degree of hypersensitivity that it
431 becomes difficult to validate differences between groups, as all animals respond positively to
432 the smallest Von Frey filament available. It is noteworthy that while SAS treatment appears

433 effective in both preventive and curative contexts, the kinetic of the response differs slightly
434 between these two approaches. In the case of preventive treatment, the reduction in edema
435 and thermal hypersensitivity is observed immediately, with significant effects already
436 apparent within the first days following CFA injection. Indeed, throughout the entire duration
437 of the experiment, SAS-treated $xCT^{+/+}$ mice exhibited a similar profile as compared to $xCT^{-/-}$
438 mice. This suggests that inhibition of system x_c^- may limit the intensity of the local
439 inflammation, leading to an early reduction in pain symptoms. In the curative setting, $xCT^{+/+}$
440 mice exhibited a similar profile after CFA injection until the initiation of SAS treatment. SAS
441 appears to also trigger early effects, with a rapid decrease in both edema and hypersensitivity
442 shortly after the treatment initiation. These findings highlight the implication of system x_c^- not
443 only in the initiation but also in the maintenance of inflammatory pain.

444 While the intact SAS behaves as an inhibitor of system x_c^-, the metabolites of SAS generated
445 in the gut exhibit anti-inflammatory properties via NF-κB inhibition (Gout et al. 2001, Wahl et
446 al. 1998). Nevertheless, it appears that the reduced edema of the inflamed paw and
447 attenuated pain-related behaviors observed in $xCT^{+/+}$ mice treated with SAS are not linked to
448 its action on NF-κB but rather to its inhibition of system x_c^-, as no difference in edema and pain
449 sensitivity were observed in $xCT^{-/-}$ mice. Although these data highlight the opportunity of
450 targeting system x_c^- in chronic inflammatory pain, it would be pertinent to develop and test
451 specific pharmacological inhibitors to confirm that the observed effects are indeed strictly
452 driven by system x_c^- inhibition. Nonetheless, these results consolidate our working hypothesis
453 that system x_c^- may play a role in both the onset and maintenance of chronic inflammatory
454 pain and therefore such inhibition would be beneficial as preemptive or curative treatment. It
455 is however likely that distinct and complementary biochemical mechanisms are involved in

456 the development and maintenance of inflammatory pain, and this could explain the different
457 time course of the influence of SAS on pain hypersensitivity when applying a preemptive or a
458 curative treatment.

459 Following CFA injection in the hind paw of mice, systemic inflammation is observed within a
460 few days and persists for several weeks. We here provide evidence that the kinetic profile of
461 this inflammatory response differs between $xCT^{+/+}$ and $xCT^{-/-}$ mice. Specifically, the expression
462 levels of pro-inflammatory cytokines tend to increase over time in $xCT^{+/+}$ mice, whereas they
463 stabilize or even decrease in mice lacking xCT. This could explain the impact of xCT deficiency
464 on pain perception, as increasing evidence revealed that pro-inflammatory cytokines recruit
465 immune cells and influence sensory transduction and synaptic plasticity along the entire
466 nociceptive axis (Ozaktay et al. 2002, Zhang and An 2007). Genetic knockout or neutralizing
467 these cytokines were proven efficient approaches to support analgesia (Clark and Vissel 2016,
468 Hess et al. 2011, Taves et al. 2013). Hence, plasma levels of selected cytokine have been
469 correlated with both neuroinflammation and pain and may serve as biomarkers for chronic
470 pain (Xu et al. 2018). Therefore, the altered profile of peripheral pro-inflammatory cytokines
471 in $xCT^{-/-}$ mice observed in our study appears as a putative mechanism explaining the early
472 recovery observed when measuring allodynia and hyperalgesia in mice lacking xCT or in mice
473 treated with SAS.

474 Alongside its impact on the inflammatory and immune response, system x_c^- plays a
475 predominant role in the modulation of glutamate levels in the brain parenchyma. In a mouse
476 model of cancer-induced pain where system x_c^- expression is increased, enhanced glutamate
477 release is observed which importantly contributes to the activation of sensory neurons (Zhu
478 et al. 2020). Furthermore, neuroinflammation was also shown to regulate both glutamate

receptor and glutamate transporters (Haroon, Miller and Sanacora 2017, Tilleux and Hermans 2007). Therefore, it would be essential to assess central and peripheral glutamate sensitization in $xCT^{-/-}$ mice following CFA injection to determine whether the observed effects are only related to reduced inflammation or also rely on the modulation of excitatory transmission.

Conclusion:

In conclusion, we here report for the first time that system x_c^- plays a role in the pathophysiology of chronic inflammatory pain. Our findings demonstrate that the absence of xCT moderates pain behaviors associated with CFA-induced peripheral inflammation. Specifically, $xCT^{-/-}$ mice exhibit diminished pain hypersensitivity, which correlates with reduced systemic inflammation and edema at the injection site. With consistent data obtained using SAS as a pharmacological inhibitor of system x_c^-, this study paves the way for the future development of specific drugs targeting this exchanger to alleviate chronic inflammatory pain in patients.

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Author's contribution:

Conceptualization of the study was primarily led by PBeckers, MC and EH. PBeckers, MC, LAR PBraconnier and ND performed the experiments. PBeckers and MC conducted the formal analysis and contributed to the interpretation of the data. PBeckers, MC, AM and EH wrote and edited the manuscript. EH supervised the entire project. Funding acquisition was managed by EH and AM. All authors provided critical feedback and approved the final version of the manuscript.

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Figure Legends:

Figure 1: Effect of unilateral intraplantar CFA injection on paw thickness in $xCT^{+/+}$ and $xCT^{-/-}$ mice

Change in ipsilateral paw thickness (mm) as an experimental readout of local inflammation was assessed at baseline and for 3 weeks following CFA or saline injection using a caliper. Data are presented as mean values with SEM (5 to 9 animals per group). Statistical analysis was performed through a three-way ANOVA with repeated measures followed by a Tukey's pairwise comparison. P-values for main effects are indicated in the graph (G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment). Results of the post hoc comparison are indicated on the graphs: * significant difference between CFA-injected $xCT^{+/+}$ mice and CFA-injected $xCT^{-/-}$ mice (***p<0.001, ****p<0.0001), # significant difference between CFA and genotype-matched saline animals (####p<0.0001). CFA, complete Freund's adjuvant; BL, baseline.

Figure 2: Effect of unilateral intraplantar CFA injection on mice weight and locomotion of $xCT^{+/+}$ and $xCT^{-/-}$ mice

Weight (A) was assessed at baseline and regularly for up to 3 weeks after CFA or saline injection. The distance traveled (B) and the average velocity (C) were recorded regularly using the open field test throughout the duration of the experiment. Data are presented as mean values with SEM (5 to 9 animals per group). For panel B and C, data are expressed as a percentage of baseline (before CFA injection). Statistical analyses were performed through a three-way ANOVA with repeated measures followed by a Tukey's pairwise comparison. P-values for main effects are indicated in the graph (G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment). CFA, complete Freund's adjuvant; BL, baseline.

Figure 3: Allodynia and hyperalgesia in $xCT^{+/+}$ and $xCT^{-/-}$ mice following intraplantar CFA injection

Pain hypersensitivity was assessed upon stimulation of the ipsilateral (A, C) and contralateral (B, D) hind paws at baseline and for up to 21 days after unilateral CFA or saline injection. The 50% PWT to mechanical stimulation (g) was used to determine allodynia (A, B), whereas the PWL to thermal stimulation (% of baseline) was used as a read-out of hyperalgesia (C, D). Data are presented as mean values with SEM (5 to 9 animals per group). Statistical analyses were performed through a three-way ANOVA with repeated measures followed by a Tukey's pairwise comparison. P-values for main effects are indicated in the graph (G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment). Results of the post hoc comparison are indicated on the graphs: * significant difference between CFA-injected $xCT^{+/+}$ mice and CFA-injected $xCT^{-/-}$ mice (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), # significant difference between CFA and genotype-matched saline animals (###p<0.001, ####p<0.0001). CFA, complete Freund's adjuvant; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; BL, baseline.

Figure 4: Plasma pro-inflammatory cytokine levels at 4, 7 and 21 days following unilateral intraplantar CFA injection in $xCT^{+/+}$ and $xCT^{-/-}$ mice

Plasma IL-6 (A,B) and TNFα (C,D) concentrations (pg/mL) were measured in saline- (A,C) and CFA-injected (B,D) $xCT^{+/+}$ and $xCT^{-/-}$ mice to assess systemic inflammation at different time points following the injection. Data are presented as mean values with SEM (5 to 10 animals per group). Statistical analyses were performed through a two-way ANOVA followed by a Tukey's pairwise comparison. P-values for main effects are indicated in the graph (G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment). Results of the post hoc comparison are indicated on the graphs (*p<0.05, **p<0.01, ***p<0.001).

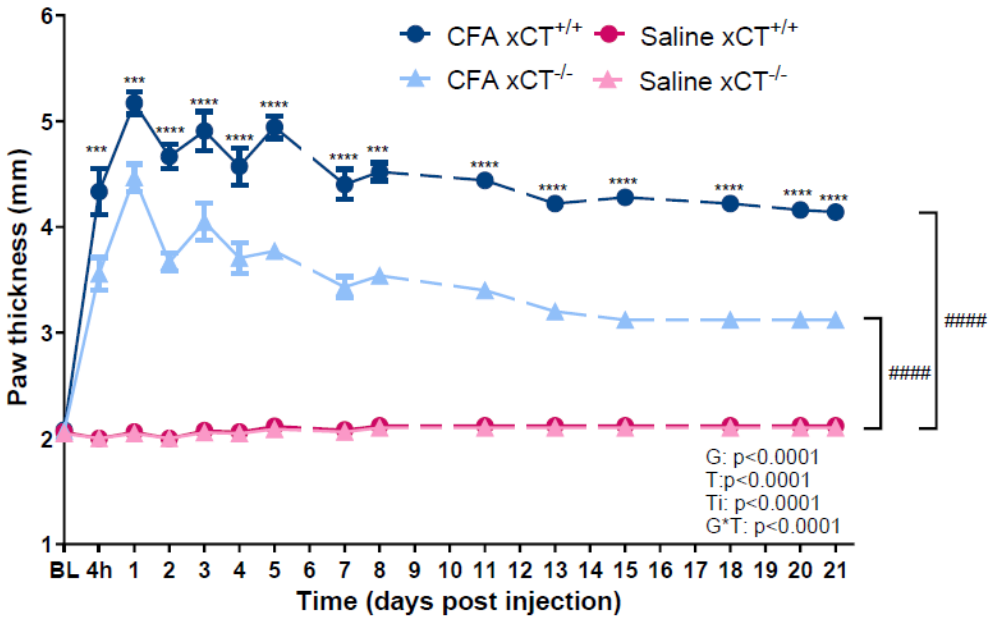
Figure 5: Effect of sulfasalazine preemptive treatment on the paw thickness and the pain hypersensitivity following unilateral intraplantar CFA injection

Mice were either treated with saline or with SAS (200mg/kg) daily for 10 consecutive days starting 2 days before CFA injection (grey zone on the graphs). Paw thickness (mm) was assessed using a caliper at baseline and for 3 weeks following CFA injection as a measure for local inflammation (A). Pain hypersensitivity was assessed for the ipsilateral hind paw at baseline and for up to 21 days after CFA injection. The 50% PWT to mechanical stimulation (g) was used to determine allodynia (B), whereas the PWL to thermal stimulation (% of baseline) was used as a read-out of hyperalgesia (C). Data are presented as mean values with SEM (6 animals per group). Statistical analyses were performed through a three-way ANOVA with repeated measures followed by a Tukey's pairwise comparison. P-values for main effects are indicated in the graph (G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment). Results of the post hoc comparison are indicated on the graphs: * significant difference between CFA-injected $xCT^{+/+}$ mice and CFA-injected $xCT^{-/-}$ mice (**p<0.01, ****p<0.0001). CFA, complete Freund's adjuvant; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; BL, baseline; SAS, sulfasalazine.

Figure 6: Effect of sulfasalazine post-treatment on the paw thickness and the pain hypersensitivity following CFA injection

Mice were either treated with saline or with SAS (200mg/kg) daily for 10 consecutive days between day 7 and day 16 post CFA injection (grey zone on the graphs). Paw thickness (mm) was assessed using a caliper at baseline and for 3 weeks following CFA injection as a measure for local inflammation (A). Pain hypersensitivity was assessed for the ipsilateral hind paw at baseline and for up to 21 days after CFA injection. The 50% PWT to mechanical stimulation was used to determine allodynia (B), whereas the PWL to thermal stimulation was used as a read-out of hyperalgesia (C). Data are presented as mean with SEM of 6 animals per group. Statistical analyses were performed through a three-way ANOVA with repeated measures followed by a Tukey's pairwise comparison. P-values for main effects are indicated in the graph (G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment). Results of the post hoc comparison are indicated on the graphs: * significant difference between CFA-injected $xCT^{+/+}$ mice and CFA-injected $xCT^{-/-}$ mice (***p<0.001, ****p<0.0001). CFA, complete Freund's adjuvant; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; BL, baseline; SAS, sulfasalazine.

767 Figure 1



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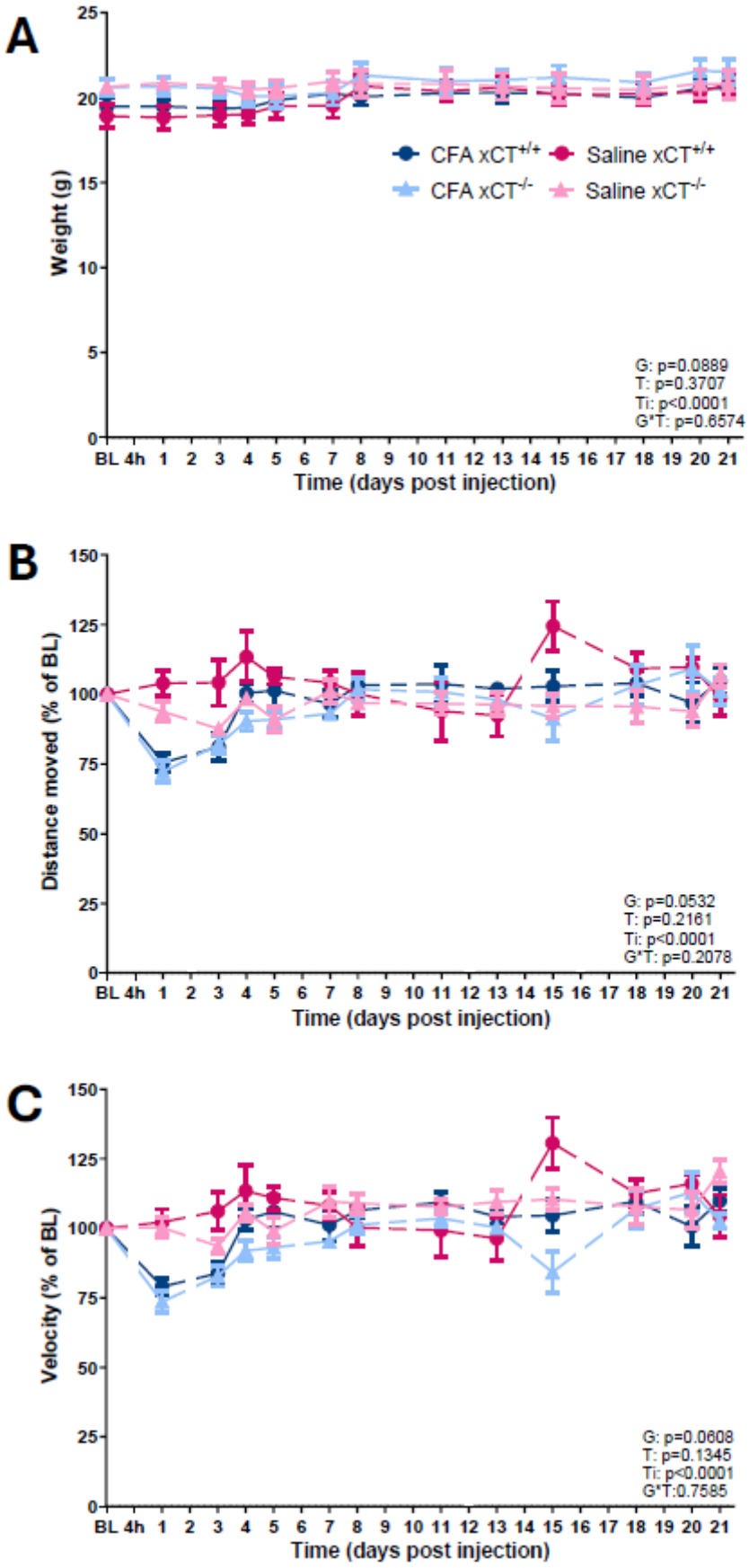
770 Figure 2



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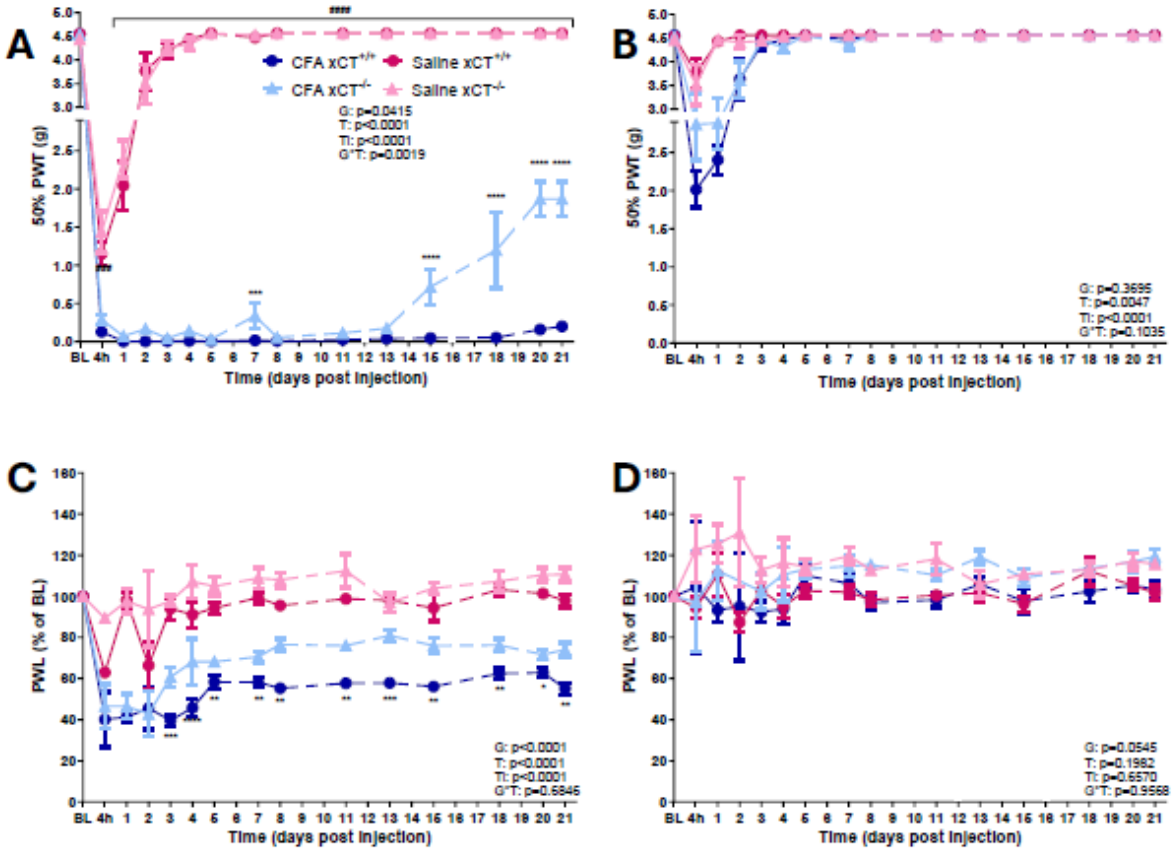

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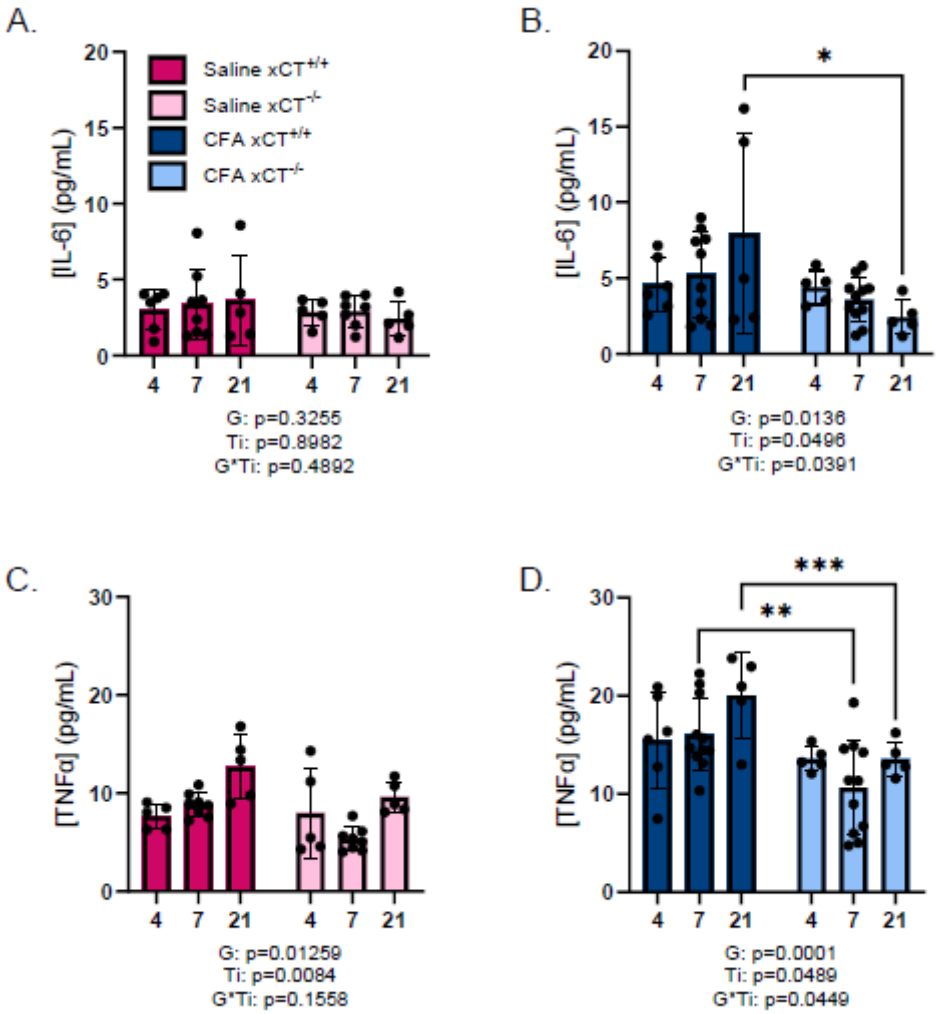
776 Figure 4



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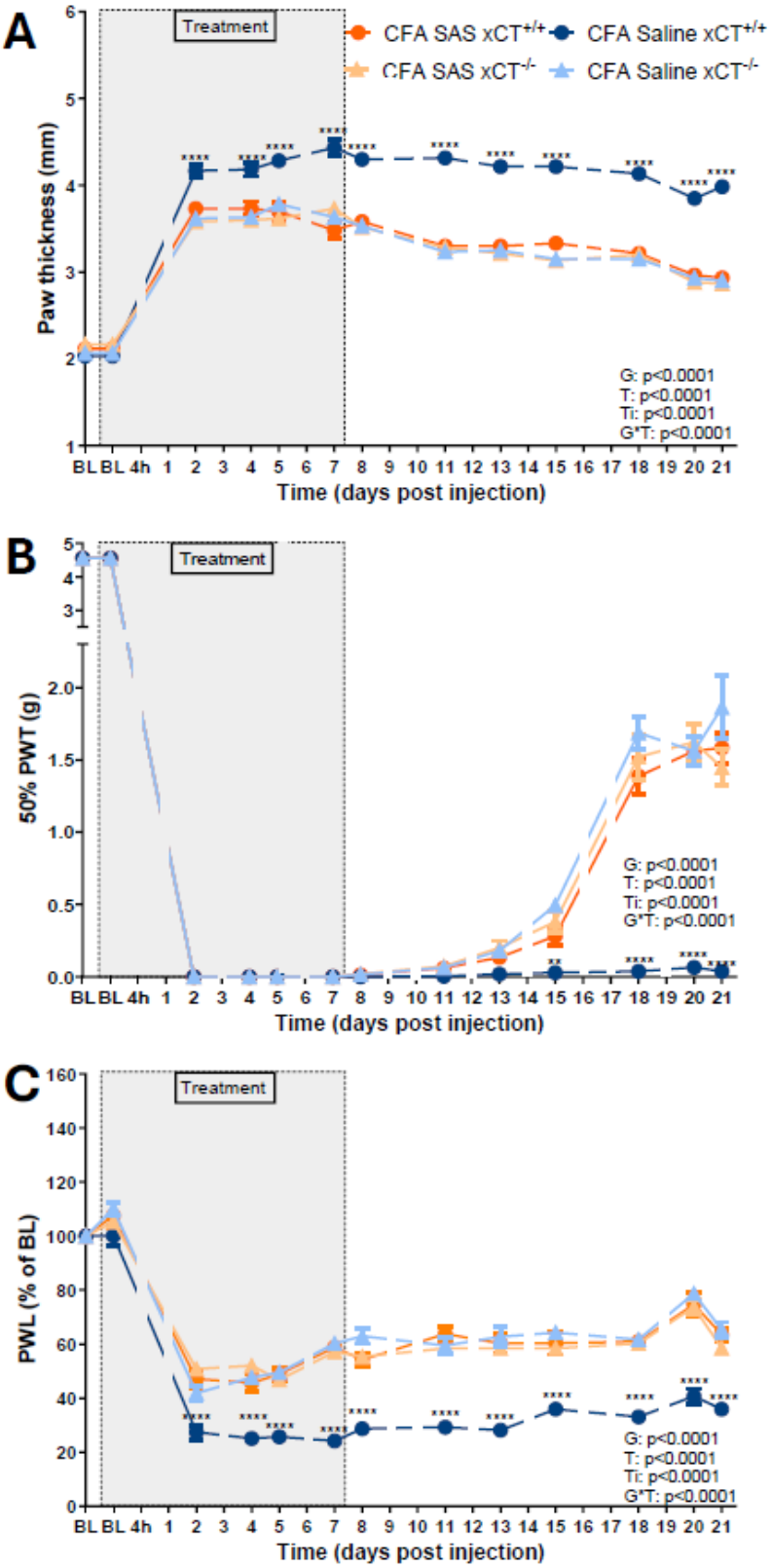
779 Figure 5



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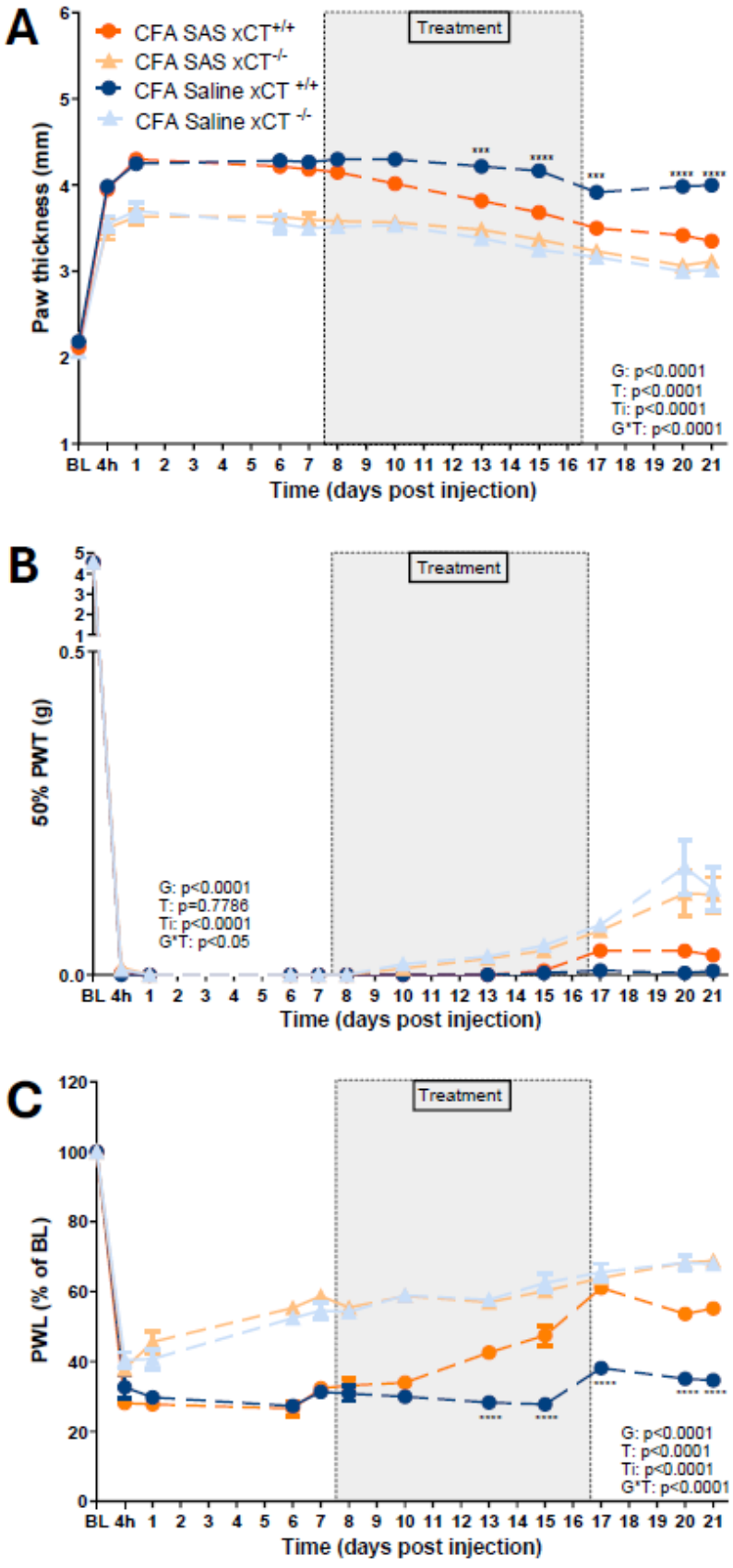
782 Figure 6



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