Macrophages transfer mitochondria to sensory neurons to resolve inflammatory pain

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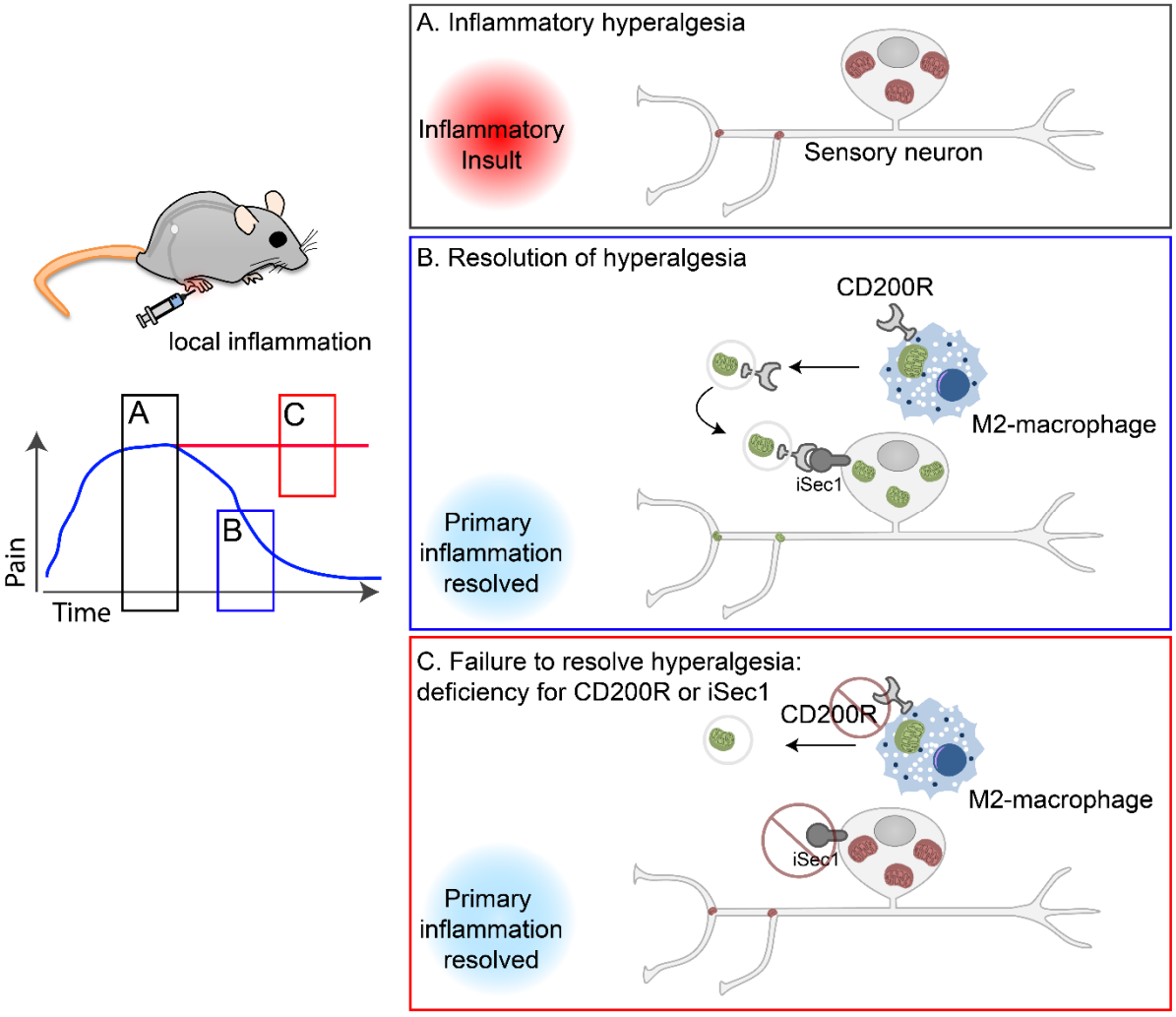
Graphical Abstract



Abstract

The current paradigm states that inflammatory pain passively resolves following the cessation of inflammation. Yet, in a substantial proportion of patients with inflammatory diseases, resolution of inflammation is not sufficient to resolve pain, resulting in chronic pain. Mechanistic insight as to how inflammatory pain is resolved is lacking. Here we show that macrophages actively control resolution of inflammatory pain remotely from the site of inflammation by transferring mitochondria to sensory neurons. During resolution of inflammatory pain in mice, M2-like macrophages infiltrate the dorsal root ganglia that contain the somata of sensory neurons, concurrent with the recovery of oxidative phosphorylation in sensory neurons. The resolution of pain and the transfer of mitochondria requires expression of CD200 Receptor (CD200R) on macrophages and the non-canonical CD200R-ligand iSec1 on sensory neurons. Our data reveal a novel mechanism for active resolution of inflammatory pain and suggests a new direction for treatment of chronic pain.

Introduction

Pain and pain hypersensitivity (hyperalgesia) are functional features of inflammation that serve to protect the tissue from further damage. At the site of inflammation, immune cells and inflammatory mediators, such as IL-1β, TNF, and bradykinin, sensitize and activate sensory neurons, which cause pain and hyperalgesia[1,2]. While the initiation of inflammatory pain is relatively well understood[3,4], the mechanisms of inflammatory pain resolution are less well characterized. Resolution of inflammatory pain is often considered to be the direct result of waning of inflammation. However, in a substantial proportion of patients with inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, spontaneous or treatment-induced resolution of inflammation does not reduce pain[5-9]. Basic discovery research to understand mechanisms of endogenous pain-resolution may help us understand how chronic pain develops when resolution pathways fail[10].

Macrophages are one of the most plastic cells of the immune system and are well known for their ability to induce tissue healing and resolution of inflammation[11]. Macrophages are strongly imprinted by their tissue of residence[12,13]. Peripheral nervous tissue shapes resident macrophages to have unique features compared to microglia and/or macrophages outside the nervous system[14-16]. After nerve damage, monocyte-derived macrophages engraft nervous tissue[14], are skewed by sensory neurons into an M1-like phenotype[17], and accumulate in the DRG to initiate and maintain neuropathic pain[18]. Thus, nervous tissue macrophages contribute to neuropathic pain. However, because macrophages can contribute to tissue healing and resolution of inflammation, we here set out to better understand the endogenous mechanisms for resolution of inflammatory pain and the role of macrophages in this process, using transient inflammatory pain models.

Results

We injected carrageenan into the hind paw of mice (intraplantar; i.pl.) as a model for transient inflammatory pain (Supplemental data fig. 1A)[19]. Treated mice displayed signs of pain, such as allodynia/hyperalgesia as assessed by the von Frey and Hargreaves tests, and postural changes measured with dynamic weight bearing. Carrageenan-induced hyperalgesia resolved within ~3-4 days (Figs. 1A and supplemental data fig. 1B). We analysed the cellular composition of lumbar (L3-L5) dorsal root ganglia (DRG) which contain the somata of sensory neurons innervating the hind paw and observed an accumulation of macrophages. Macrophage numbers peaked at day 3 and returned to baseline levels after resolution of inflammatory hyperalgesia (Fig. 1B/C, supplemental data figs. 1C/D, and supplemental data movies 1 and 2). Infiltration of macrophages was specific to the DRG that innervate the inflamed paw, and was not observed at the contralateral side (supplemental data fig. 1D). In contrast, during the entire course of inflammatory hyperalgesia, T cells, B cells or other $CD45^+$ immune cell numbers in the DRG did not change significantly (Figs. 1B and supplemental data fig. 1C). To address the function of these macrophages in pain resolution, we selectively depleted monocytes and macrophages by intraperitoneal (i.p.) injection of diphtheria toxin (DT) in *Lysm*^{cre} x *Csf1r* $^{LsL\text{-}DTR}$ mice[20] (from here on referred to as 'MM^{dtr}', supplemental data fig. 2). DT administration depleted monocytes and macrophages in the DRG but did not affect the number and morphology of microglia in spinal cord (Supplemental data Figs. 2G-J). The induction and magnitude of carrageenan-induced hyperalgesia in these mice was normal. However, MM^{dtr} mice failed to resolve inflammatory mechanical hyperalgesia (Fig. 1D), thermal hyperalgesia (Supplemental data fig. 3A) and postural changes related to inflammatory pain for at least six days (Fig. 1E) in both male and female mice (Fig. 1F). Similarly, MM^{dtr} mice failed to resolve Complete Freund's Adjuvant

(CFA)-induced transient inflammatory hyperalgesia for at least 12 days (Fig. 1G, and supplemental data fig. 3B).

To directly target monocytes to the DRG[21], we intrathecally (i.t.) injected wildtype (WT) bone-marrow-derived $CD115^+$ monocytes into MM^{dtr} mice (Supplemental data fig. 4A), which reconstituted macrophages in the ipsilateral DRG (Supplemental data fig. 4B). Within hours, i.t. injection of WT monocytes sustainably rescued the defective resolution of hyperalgesia in MM^{dtr} mice (Fig. 2A and supplemental data fig. 4C). The pain-resolving capacity of monocytes was independent of their origin (bone marrow or spleen; supplemental data fig. 4C-E) or Ly6C expression ('classical' $Ly6C^+$ or 'non-classical' $Ly6C^-$; supplemental data figs. 4F-H). These data show that monocytes are essential to resolve inflammatory pain and sufficient when specifically grafted into the DRG.

Macrophages that reside in peripheral nerve tissue are different from microglia and non-nervous residing macrophages[16,22]. It was shown that after nerve injury, monocyte-derived macrophages engraft in the pool of resident peripheral nervous system macrophages[14], and are programmed by vesicles secreted by sensory neuron[17]. We determined whether monocytes/macrophages that infiltrate the DRG during inflammatory pain, had an inflammatory ('M1') - or resolution ('M2')-like phenotype. At day 3, the number of $CD206^+$ M2-like or tissue-repair macrophages[23,24] was increased in the DRG, whereas the number of $iNOS^+$ M1-like or inflammatory macrophages did not significantly change (Fig. 2B). Consistent with the dominant presence of $CD206^+$ macrophages, i.t. injection of bone-marrow derived macrophages (from here on referred to as 'macrophages') differentiated *in vitro* with IL-4 ('M2') rescued resolution of hyperalgesia in MM^{dtr} mice. In contrast, inflammatory macrophages differentiated with LPS and

IFNγ ('M1') induced a transient hyperalgesia in the saline treated paws and were incapable of resolving inflammatory hyperalgesia in the carrageenan treated paws (Figs. 2C and supplemental data fig. 4I). Macrophages resolved pain through a pathway independent of neuronal IL10 receptor (IL10R) signalling because *Nav1.8*^{Cre}*Il10r*^{flox} mice, which are deficient for the IL10 receptor in pain-sensing sensory neurons that mediate inflammatory hyperalgesia[25], recovered normally (Supplemental data fig. 4J).

Metabolically, M2 macrophages depend on oxidative phosphorylation (OxPhos), while M1 macrophages are glycolytic (Supplemental data fig. 5)[26]. Neurons have a very high metabolic demand[27]. We previously demonstrated that a deficiency in mitochondrial function in sensory neurons prevents the resolution of inflammatory pain[21]. Moreover, in chronic pain neuronal mitochondrial functions, such as OxPhos and Ca^{2+} buffering, are impaired[28,29]. Indeed, oxygen consumption in DRG neurons was reduced during the peak of inflammatory pain and resolved at day 3 (Fig. 3A). Therefore, we posited that to resolve inflammatory pain, sensory neurons have to re-establish OxPhos by restoring a functional mitochondrial pool. Given that after ischemic stroke neurons can take up mitochondria released by adjacent astrocytes[30], we hypothesized that during pain resolution monocytes/macrophages aid neurons by supplying new mitochondria.

We stained mitochondria from macrophages with MitoTracker Deep Red (MTDR) that binds covalently to mitochondrial proteins[31] and co-cultured live macrophages, or an equivalent volume of sonicated macrophages, with the neuronal cell line Neuro2a (N2A). After 2 hours, macrophage-derived $MTDR^{+}$-mitochondria were detectable in N2A cells by flow cytometry and image stream (Supplemental data figs. 6A/B). Macrophages transduced with mitochondria-targeted DsRed (mitoDsRed) also transferred mitochondria to primary sensory neurons upon co-

culture in vitro, excluding that the signal was due to MTDR leak from macrophages to neurons (Supplemental data fig. 6C). Transfer of mitochondria from macrophages to neurons also occurred *in vivo*. During the resolution of pain, we detected a significantly higher percentage of MitoDentra2 positive sensory neurons in the lumbar ipsilateral DRG of *LysM*^{Cre}-MitoDendra2^{flox} mice compared to the contralateral DRG (Figs. 3B/C). In contrast, MitoDendra2^{flox} mice or *LysM*^{Cre}-GFP^{flox} mice did not have any MitoDendra2 or GFP positive neurons (Supplemental data fig. 6D), suggesting that monocytes/macrophages transfer mitochondrial content to neurons during resolution of inflammatory pain. In addition, i.t. injection of MTDR-labelled macrophages in MM^{dtr} mice at day 6 after carrageenan injection increased the MTDR intensity in sensory neurons of mice with persisting inflammatory hyperalgesia (Figs. 3D/E), but not in control treated mice or in WT mice that had resolved inflammatory hyperalgesia. Injection of sonicated MTDR-labelled macrophages did not result in accumulation of MTDR in sensory neurons (Fig. 3D), confirming mitochondrial transfer to sensory neurons. Using flow cytometry, we found that macrophages released CD45^{+} extracellular vesicles that stained positive for macrophage plasma membrane proteins, such as MHC class II, CD11b and CD200 Receptor 1 (CD200R), and the mitochondrial dye MTDR (Supplemental data fig. 7A/B). In line with the MTDR staining in vesicles, in the supernatant of MitoDendra2^{+} macrophages we detected CD45^{+}MitoDentra2^{+} vesicles (Supplemental data fig. 7C). The vesicles had a broad range in size (Supplemental data fig. 7D).

We hypothesized that the mitochondria-containing vesicles released by macrophages were sufficient to resolve pain. Indeed, i.t. administration of mitochondria-containing extracellular vesicles isolated from macrophage supernatant rapidly but transiently resolved inflammatory hyperalgesia in MM^{dtr} mice. However, injection of sonicated extracellular vesicles did not affect

hyperalgesia (Fig. 3F, supplemental data fig. 7E). Taken together, this suggests that functional mitochondria, but not their individual components, are sufficient to resolve pain. Furthermore, monocytes that have distressed mitochondria and significantly reduced mitochondrial DNA (mtDNA) content due to a heterozygous deletion of the Transcription Factor A/*Tfam*[32] failed to resolve inflammatory hyperalgesia in MM^{dtr} mice (Supplemental data figs. 8A/B). Finally, we obtained artificial vesicles containing mitochondria from macrophage cell bodies (MitoAV). MitoAV stained positive for macrophage plasma membrane markers and MTDR and had active OxPhos (Supplemental data figs. 8C and 11B). I.t. injection of MitoAV rapidly but transiently resolved inflammatory hyperalgesia in MM^{dtr} mice (Fig. 3G and supplemental data fig. 8D). In contrast, MitoAV in which oxidative phosphorylation was blocked by complex III inhibitor myxothiazol[33] failed to resolve hyperalgesia (Fig. 3G and supplemental data fig. 8D). Thus, to resolve inflammatory pain, macrophages transfer vesicles containing mitochondria that are functional in their oxidative phosphorylation capacity.

For efficient transfer of mitochondria, we hypothesized that docking of extracellular vesicles to sensory neurons requires receptor-ligand interactions. Macrophages, predominantly those with an M2 phenotype[34], and macrophages-derived extracellular vesicles expressed CD200R (Fig Supplemental data fig. 7A), while neurons are known to express its ligand CD200 (ref. [35]). In line with this reasoning, *Cd200r*$^{-/-}$ mice completely failed to resolve inflammatory hyperalgesia, which persisted for at least 16 days (Fig. 4A and Supplemental data fig. 9A). Place-preference conditioning with the fast-working analgesic gabapentin[36], a drug that relieves neuropathic and inflammatory pain[37,38], confirmed ongoing spontaneous pain in *Cd200r*$^{-/-}$ mice for at least 2 weeks after carrageenan injection (Fig. 4B, supplemental data fig. 9B). Of note, acute inflammation and the resolution of inflammation at the site of carrageenan injection in *Cd200r*$^{-/-}$

mice did not differ from that of WT mice (Figs. 4C and 4D). This further supports the role of CD200R in the resolution of acute inflammatory pain and prevention of chronic pain.

I.t. injection of *Cd200r*$^{-/-}$ monocytes did not resolve inflammatory hyperalgesia in MM^{dtr} mice (Figs 5A, supplemental data fig. 10A). Consistent with these data, WT monocytes or macrophages did resolve persisting inflammatory hyperalgesia in *Cd200r*$^{-/-}$ mice, whereas injection of additional *Cd200r*$^{-/-}$ monocytes or macrophages did not (Fig. 5B; supplemental data fig. 10B). These data indicate an intrinsic defect in the pain-resolution capacity of *Cd200r*$^{-/-}$ monocytes and macrophages independent from effects of macrophages at the site of local inflammation.

We found no evidence for a defect in mitochondrial respiration or vesicle release in *Cd200r*$^{-/-}$ macrophages (Supplemental data figs. 11A-C) and *Cd200r*$^{-/-}$ macrophages were normal in their capacity to migrate into the DRG and had a similar phenotype to WT macrophages (Supplemental data figs. 11D-H). This suggested instead that there was a defect in mitochondrial transfer between *Cd200r*$^{-/-}$ macrophages and neurons. MTDR transferred normally from intrathecally injected MTDR-labelled macrophages to neurons from *Cd200r*$^{-/-}$ mice (Fig. 5C). In contrast, *Cd200r*$^{-/-}$ macrophages failed to transfer MTDR-labelled mitochondria to sensory neurons of *Cd200r*$^{-/-}$ mice (Fig. 5C) and extracellular vesicles isolated from *Cd200r*$^{-/-}$ macrophage culture supernatant did not resolve inflammatory hyperalgesia in MM^{dtr} mice (Fig. 5D; supplemental data fig. 12). Thus, CD200R expression on monocytes/macrophages and their mitochondria containing extracellular vesicles is required for transfer of mitochondria to sensory neurons and for the resolution of inflammatory pain.

CD200 is the best-known ligand for CD200R and in inflammatory models, such as arthritis, *Cd200*$^{-/-}$ and *Cd200r*$^{-/-}$ mice have a similar phenotype[39,40]. However, in sharp contrast to *Cd200r*$^{-/-}$ mice, *Cd200*$^{-/-}$ mice completely resolved inflammatory pain with similar kinetics to WT mice (Fig. 6A; supplemental data fig. 13A). This suggests the involvement of an alternative CD200R ligand. In 2016, iSec1/*Gm609* was described as a CD200R ligand expressed specifically in the gut[41]. We found that iSec1/*Gm609* mRNA is also expressed in DRG, along with CD200 (Supplemental data figs. 13B/C). Repetitive i.t. injections of iSec1/*Gm609* targeting antisense oligodeoxynucleotides (ASO)[42] silenced iSec1 mRNA expression in the DRG of WT mice (Fig. 6B and supplemental data fig. 13D) and partially prevented resolution of inflammatory hyperalgesia (Fig. 6C; Supplemental data fig. 13E). In *Cd200*$^{-/-}$ mice, i.t. injections of *iSec1/Gm609*-ASO completely prevented the resolution of hyperalgesia (Fig. 6D; supplemental data fig. 13F). Next, we injected Herpes Simplex Virus (HSV) encoding iSec1 i.pl. to specifically target sensory neurons innervating the inflamed area[21]. Expression of iSec1/*gm609* that was mutated to resist ASO treatment in sensory neurons (HSV-iSec1^{res}, Fig. 6E) completely rescued the ability of iSec1/*Gm609*-ASO treated *Cd200*$^{-/-}$ mice to resolve pain, while an empty vector (HSV-e) did not (Fig. 6F; Supplemental data fig. 13G). We conclude that monocyte/macrophage expression of CD200R and sensory neuron expression of its ligand iSec1 are required for the transfer of macrophage-derived mitochondria to sensory neurons *in vivo* to resolve inflammatory pain.

Discussion

We identified a previously unappreciated role for macrophages which transfer mitochondria to somata of sensory neurons to resolve inflammatory pain. Previous studies showed that

respiratory competent mitochondria are present in human whole blood[43], and that tissue-resident cells can transfer mitochondria[30,44]. We now show that non-tissue resident monocytes are recruited to the DRG, acquire a M2/tissue-repair like phenotype and transfer mitochondria to sensory neurons via a CD200R:iSec1 interaction in order to resolve pain. In contrast to M2 macrophages, inflammatory M1 macrophages induced pain. Thus, a DRG-milieu that skews local macrophages towards a M1 phenotype could contribute to the development of chronic pain[45-48].

Previous studies have implicated macrophages in resolution of inflammatory pain at the site of the primary inflammatory insult by secretion of IL-10[49,50], or by clearance of the inflammatory agent zymozan[51]. We now found that macrophages are necessary to resolve pain distant from the primary inflammatory insult and restore neuronal homeostasis independent of the anti-inflammatory capacities of macrophages at the site of the primary insult. Importantly, resolution of inflammatory pain was independent of IL-10 Receptor signalling in sensory neurons, excluding a direct effect of IL-10 on neurons in resolution of inflammatory pain.

Our data show that transfer of mitochondria by macrophages in the DRG is required for resolution of inflammatory pain. However, it is possible that macrophages have additional roles in other areas of the nervous system, including nerves or nerve endings. Also, we cannot exclude other cells to contribute to resolution of inflammatory pain. For example, Csf1r/LysM negative macrophages at the site of the primary inflammatory insult or satellite glial cells in the DRG that surround the soma of sensory neurons may play additional roles[49-51].

CD200 has long been thought of as the only ligand for CD200R. Although previous studies implicated CD200 as a checkpoint for microglia cell activation in neuropathic pain by ligating

microglial CD200R[52,53], we show that *Cd200*$^{-/-}$ mice fully resolve inflammatory pain. Furthermore, we found that iSec1/*gm609* is expressed in DRG neurons and we demonstrated that sensory neuron-iSec1 is required to resolve inflammatory pain. Of note, iSec1/*gm609* knockdown did have a greater effect on pain resolution in *Cd200*$^{-/-}$ mice than in WT mice, suggesting that the function of these ligands is partially redundant.

Various chronic pain states, such as chemotherapy-induced pain and neuropathic pain caused by trauma or diabetes, are associated with mitochondrial defects[54-57]. We show here that oxidative phosphorylation is reduced during the peak of transient inflammatory pain but is restored when inflammatory hyperalgesia resolves. Hence, we postulate that resolution of inflammatory pain requires the restoration of mitochondrial homeostasis in sensory neurons and that DRG macrophages facilitate this process.

Given that the injection of isolated extracellular vesicles only transiently resolves pain, a more durable resolution of pain requires a prolonged flux of mitochondria and/or additional signals from intact macrophages. These mitochondria could replace mitochondria in neurons that have incurred mitochondrial damage. Future work should assess how exactly neuronal mitochondrial homeostasis is restored by macrophage-derived mitochondria.

Why would sensory neurons require external help to restore the integrity of their mitochondrial network? Sensory neurons face unique challenges in maintaining a functional mitochondrial network because of their exceptional architecture and their intense demand for energy to support energetically expensive processes such as resting potentials, firing action potentials and calcium signalling[27,58]. Stressed neurons, e.g. during inflammatory pain, turn to anabolic metabolism[59]. In the face of this high energy demand during stress, an energy consuming process such as rebuilding the mitochondrial network is difficult to support.

Moreover, maintaining an excess mitochondrial pool that is capable of handing the stress of inflammatory pain would come at a high fitness cost because it would require more energy intake for the organism. Thus, we propose that dispensable monocytes/macrophages supply mitochondria to stressed indispensable neurons.

Together, our data show that pain is actively resolved by an interaction between the immune and neuronal systems that is separate from the cessation of inflammation within the peripheral tissue. Novel therapeutic strategies to resolve chronic pain may focus on the restoration of mitochondrial homeostasis in neurons or on enhancing the transfer of mitochondria from macrophages.

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Data and materials availability: All data is available in the manuscript or supplementary materials. Raw data and materials will be made available upon reasonable request. Some materials used in this manuscript are subject to Material Transfer Agreement (MTA).

Supplementary Materials:

Materials and Methods

Figures S1-S13

Movies S1-S2

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Figure Legends

Figure 1. Monocytes/macrophages are required to resolve inflammatory pain

(A) Course of mechanical hyperalgesia after i.pl. injection of 1% carrageenan in the left hind paw and saline in the right hind paw.

(B) Absolute number of $CD45^+$ cells classified to subset per lumbar dorsal root ganglia (DRG, L3-L5) that contain sensory neurons innervating the hind paw of mice that received 1% carrageenan. See Supplemental data fig. 1C for gating strategy.

(C) Light-sheet render showing macrophage (F4/80, red) dispersed throughout an ipsilateral lumbar DRG isolated 1 day after saline or 1% carrageenan injection. See supplemental movies S1 and S2. Scale bar: 150µm.

(D) Course of mechanical hyperalgesia in WT and MM^{dtr} littermates injected with 1% carrageenan in the left hind paw and saline in the right hind paw.

(E) Course of weight bearing of the left hind paw (as % of total body weight) in WT and MM^{dtr} littermates injected with 1% carrageenan in the left hind paw and saline in the right hind paw.

(F) Course of carrageenan-induced mechanical hyperalgesia in male versus female in WT and MM^{dtr} littermates.

(G) Course of CFA-induced mechanical hyperalgesia in WT and MM^{dtr} littermates.

Significance was determined by (A, B, D, E, G, H) two-way repeated measurement ANOVA followed by indicated post hoc test, (E) two-way ANOVA with Dunnets post hoc test. Asterisks indicate significance in these comparisons: (A) saline versus carrageenan, Sidak, (B) number of cells per subset compared to day 0, Dunnett; (D) WT and MM^{dtr} mice, both with carrageenan, Sidak; (E) difference from t=0, within genotype. (F) Female vs Male MMdtr mice both with carrageenan (n.s.), Tukey; (G) WT and MM^{dtr} mice, both with carrageenan, Sidak;

Figure 2. Monocytes/macrophages are required to resolve inflammatory pain

(A) Course of mechanical hyperalgesia in WT and MM^{dtr} mice after i.pl. injection of 1% carrageenan in the hind paws and i.t. injection of PBS or WT $CD115^{+}$ monocytes.

(B) Phenotype of F4/80 positive macrophages in DRG of mice i.pl injected with carrageenan in the hind paw at indicated time points. Gating strategy is indicated in Fig. S1C.

(C) Course of mechanical hyperalgesia in MM^{dtr} mice after i.pl. injection of 1% carrageenan in the left hind paw and i.t. injection of M0, M1 or M2 macrophages.

Significance was determined by two-way repeated measurement ANOVA followed by indicated post hoc test. Asterisks indicate significance in these comparisons: (A) MMdtr mice injected with PBS or monocytes, Sidak; (B) number of cells per subset compared to day 0, Dunnett; (C) M1 versus M2 macrophages, both with carrageenan, Sidak.

Figure 3. Macrophages migrate into the DRG and transfer mitochondria to neurons

(A) Basal oxygen consumption rates in sensory neuron cultures obtained from lumbar DRG isolated from mice at indicated days post carrageenan injection in the hind paw. Analysis show pooled DRG cultures from 3 mice at each day.

(B, C) (B) Quantification and (C) example images of percentage of MitoDendra2^{+} neurons in the contra- or ipsilateral DRG of *LysM*^{cre}-MitoDendra2^{flox} mice three days after carrageenan injection. scale bar: 150 µm. n=6.

(D, E) (D) Analysis and (E) example images of MTDR signal in sensory neurons in the DRG of MM^{dtr} and WT mice. At day 6 after 1% carrageenan (ongoing pain in MM^{dtr}, resolved pain in WT mice) or saline injection, PBS, MTDR-labelled macrophages (Mφ), or sonicated MTDR-labelled macrophages (son) were injected intrathecally. After 18h, lumbar DRG were isolated for immunofluorescence analysis and counter-stained with β3-tubulin (green, neurons) and DAPI (blue, nuclei). White arrowheads indicate MTDR+ neurons. Scale bar: 50µm.

(F) Course of mechanical hyperalgesia in MM^{dtr} mice injected with carrageenan. At day 6 mice were injected i.t. with intact or sonicated macrophage-derived vesicles.

(G) Course of mechanical hyperalgesia in MM^{dtr} mice injected with carrageenan in the left hind paw, and saline in the right hind paw. At day 6 mice were injected i.t. with artificially generated vesicle containing mitochondria (MitoAV) with functional or myxothiazol (complex III)-inhibited mitochondria.

Significance was determined by (A, B) student t test; (D) one-way ANOVA Tukey post hoc test; or (D, E) repeated measurement 2-way ANOVA with Dunnett post hoc test. Asterisks indicate significance in these comparisons (F) intact and sonicated vesicles, both in mice injected with carrageenan, and (G) functioning versus inhibited mitochondria, both in mice injected with carrageenan.

Figure 4. Monocytes require CD200R to resolve inflammatory pain

(A) Course of mechanical hyperalgesia in *Cd200r*$^{-/-}$ or WT mice that were unilateral injected with 1% carrageenan in the hind paw.

(B) Gabapentin-induced place preference conditioning at day 16 after unilateral 1% carrageenan injection in the hind paws. Conditioning efficiency is depicted as the difference in time (seconds, s) spent in a white room pre- and post-conditioning.

(C) Paw swelling of the carrageenan-injected paw in WT and *Cd200r*$^{-/-}$ mice. Significance tested: RM 2-way ANOVA, Sidak.

(D) mRNA expression of *Il1* and *Il6* in the carrageenan-injected paw of WT and *Cd200r*$^{-/-}$ mice. Significance tested: RM 2-way ANOVA, Sidak, not significant.

Significance was determined by (A, C, D) repeated measurement 2-way ANOVA with indicated post hoc; (B) t Test. Asterisks indicate significance between (A) WT and *Cd200r*$^{-/-}$ mice, Sidak; (C) WT vs *Cd200r*$^{-/-}$ mice, Sidak, not significant, and (D) WT versus *Cd200r*$^{-/-}$ mice, Sidak, not significant.

Figure 5. Cd200r deficient monocytes/macrophages fail to resolve inflammatory pain

(A) Course of mechanical hyperalgesia in MM^{dtr} mice that were injected unilateral with 1% carrageenan and saline. At day 6, WT or *Cd200r*$^{-/-}$ $CD115^{+}$ monocytes were i.t. injected.

(B) Course of mechanical hyperalgesia in *Cd200r*$^{-/-}$ mice that were injected unilateral with 1% carrageenan and saline. At day 6, WT or Cd200r$^{-/-}$ macrophages were i.t. injected.

(C) *In vivo* MTDR transfer from WT or *Cd200r*$^{-/-}$ MTDR-labelled macrophages to DRG neurons of *Cd200r*$^{-/-}$ mice. At day 6 after carrageenan injection, macrophages or PBS were injected i.t. and after 18h DRG were isolated and stained as described for Fig. 2C.

(D) Course of mechanical hyperalgesia in MM^{dtr} mice injected unilateral with 1% carrageenan and saline. At day 6, vesicles isolated from WT or *Cd200r*$^{-/-}$ macrophage culture supernatant, or the supernatant of the vesicle pellet (cleared supernatant) was i.t. injected.

Significance was determined by (A, B, D) repeated measurement 2-way ANOVA with indicated post hoc; or (C) one-way ANOVA with Holm-Sidak post hoc. Asterisks indicate significance between (A) WT and *Cd200r*$^{-/-}$ injected monocytes, both with carrageenan, Sidak; (B) WT and *Cd200r*$^{-/-}$ macrophages, both with carrageenan, Dunnett; and (D) WT versus *Cd200r*$^{-/-}$ vesicles with carrageenan, Dunnett.

Figure 6 – iSec1 is required for resolution of pain

(A) Course of carrageen-induced mechanical hyperalgesia in WT or Cd200-/- littermates.

(B) iSec1/gm609 mRNA expression after silencing of iSec1/gm609 in DRG of WT mice treated with mismatch (MM-ASO) or iSec1-targeting Antisense Oligo nucleotides (iSec1-ASO).

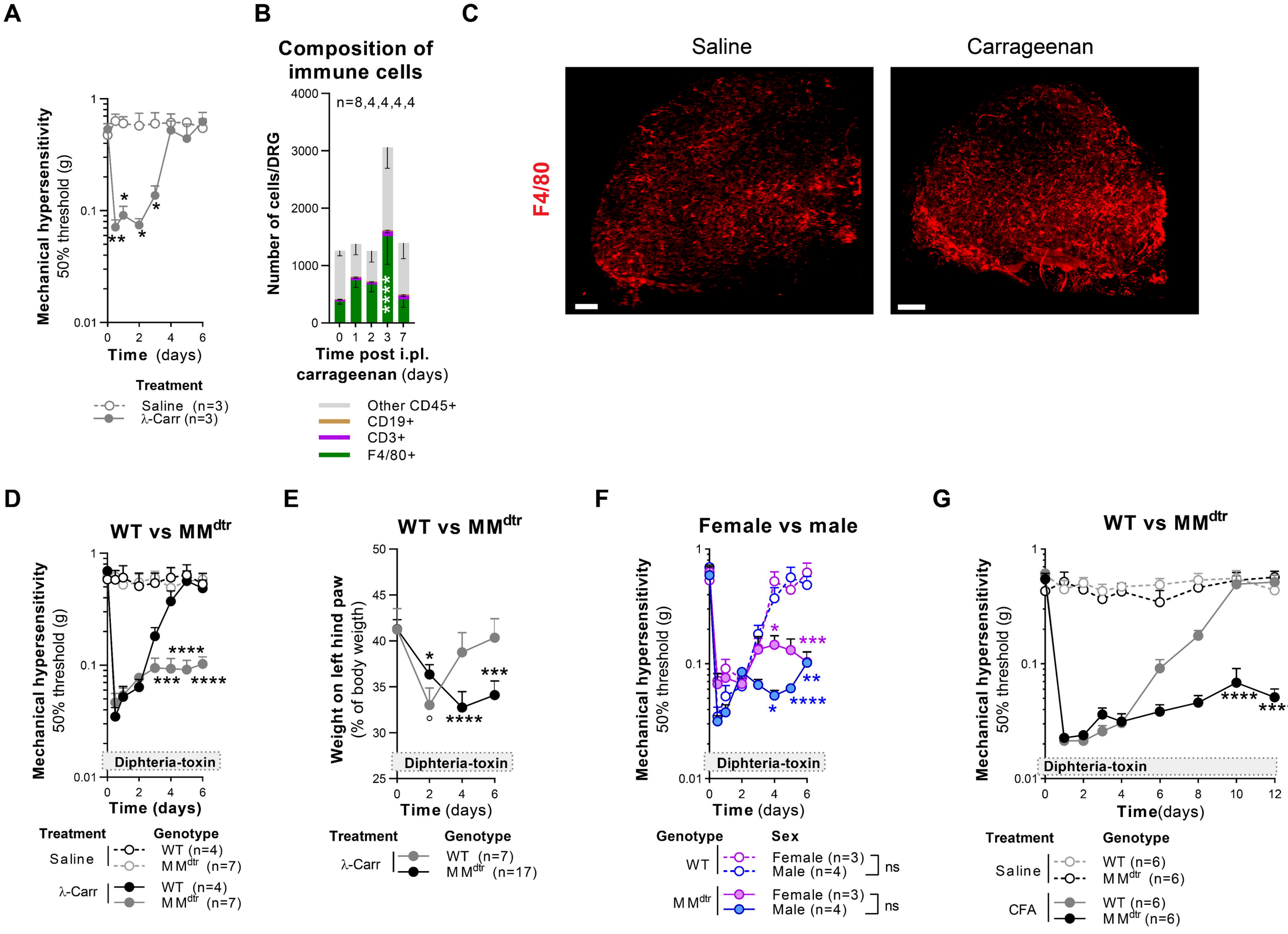
(C-D) Course of carrageen-induced mechanical hyperalgesia in (C) WT mice or (D) Cd200-/- littermates injected with mismatch (MM-ASO) or iSec1-specific antisense oligonucleotides (iSec1-ASO).

(E) Expression of iSec1^{wt}-flag (n=3 from 1 experiment) and iSec1^{res}-flag (n=6 from 2 experiments) as assessed by flow cytometry.

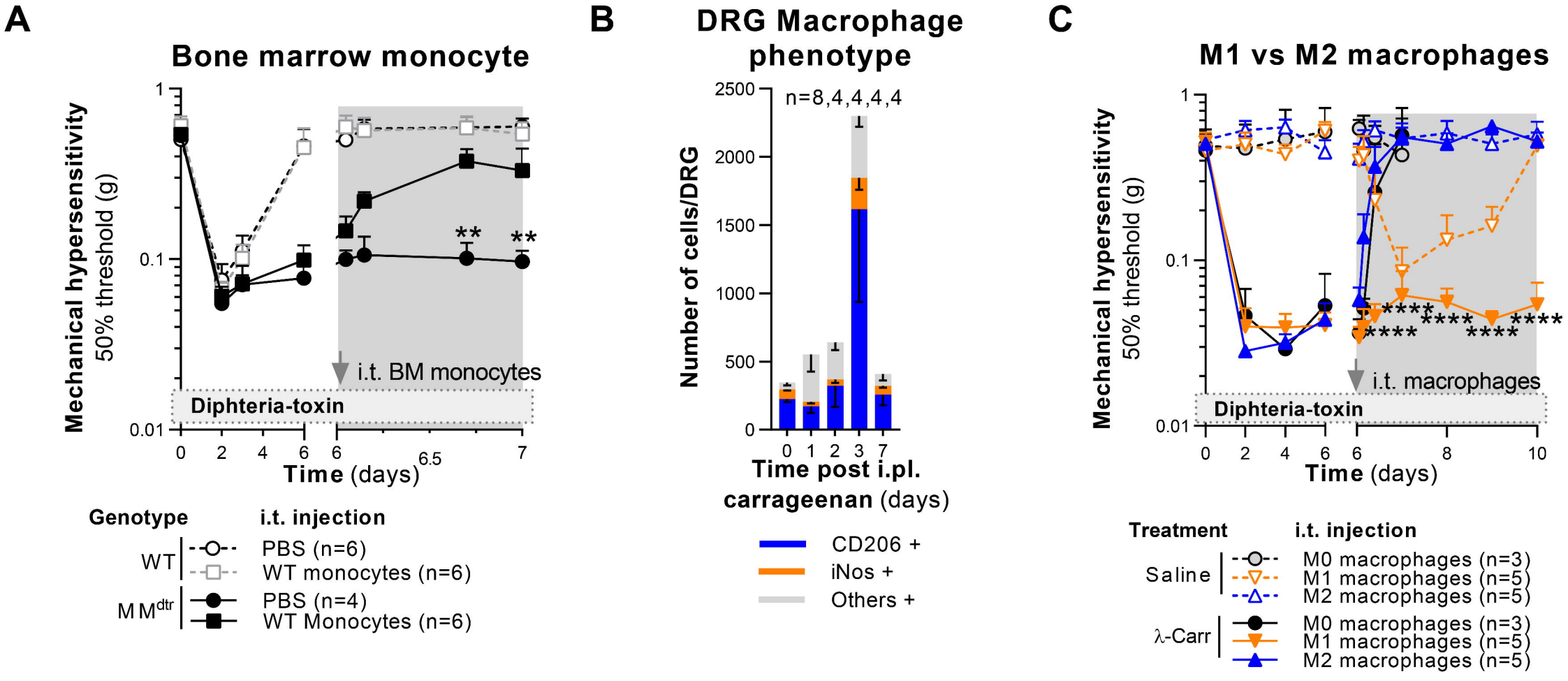
(F) Cd200-/- mice were intraplantar (i.pl) injected with HSV-e or HSV-iSec1^{res} before i.pl. carrageenan injection, treated with iSec1-specific ASO injected i.t.

Significance was determined by repeated measurement (A, C, D, E, F) 2-way ANOVA with Sidak post hoc, or (B) a student's T test. Asterisks indicate significance between (A) WT and Cd200-/- mice, both with carrageenan – not significant; (C, D) MM versus iSec1 ASO, both with carrageenan; (E) HSV-e versus HSV-Isec1

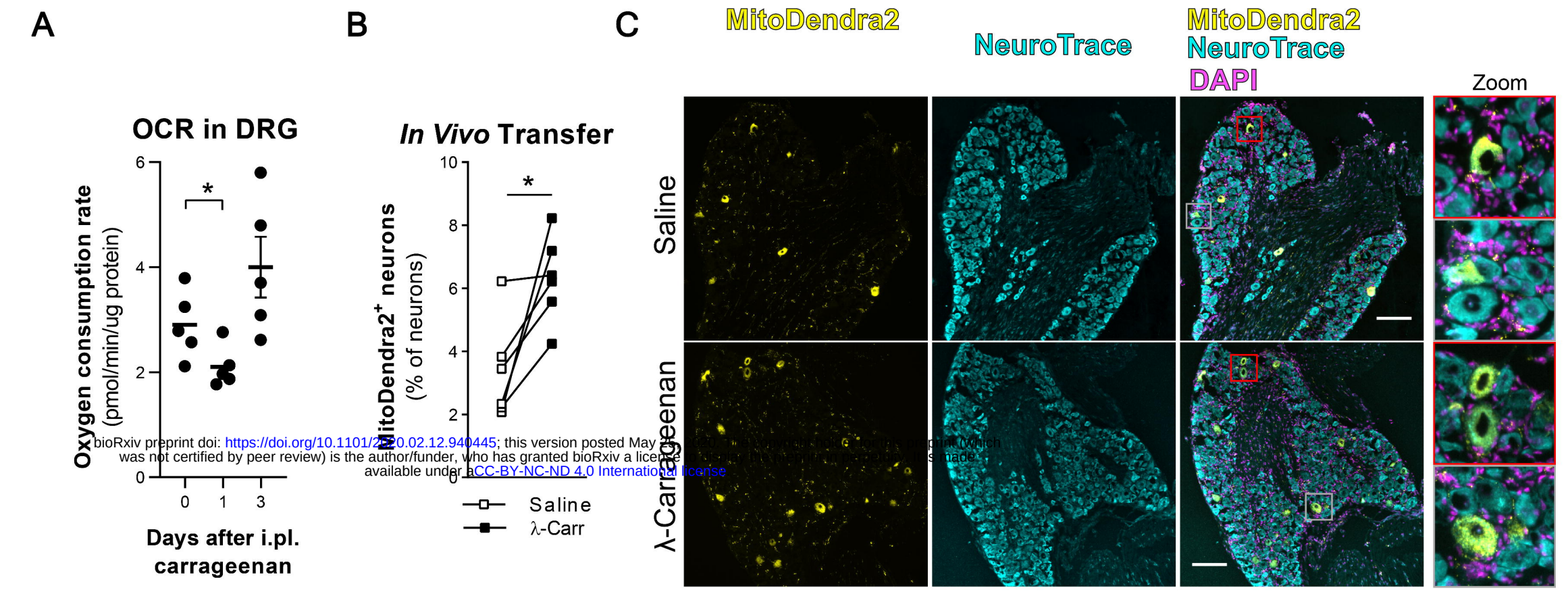
Raoof fig 1. macrophage infiltration and depletion

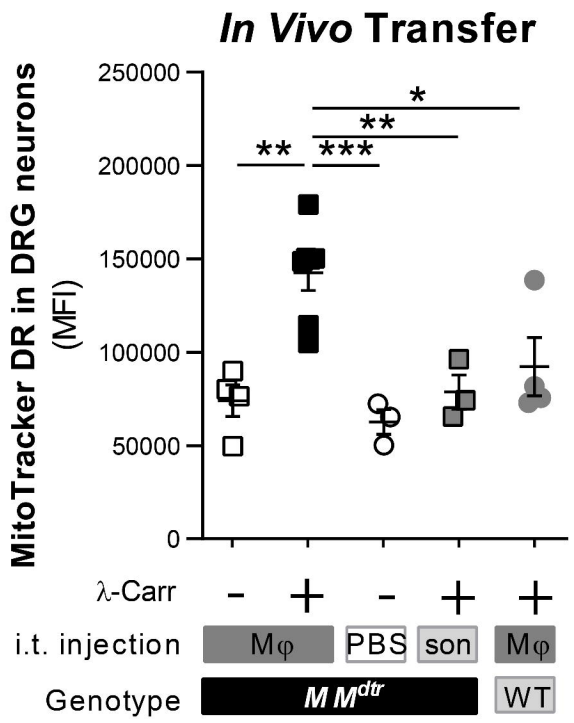


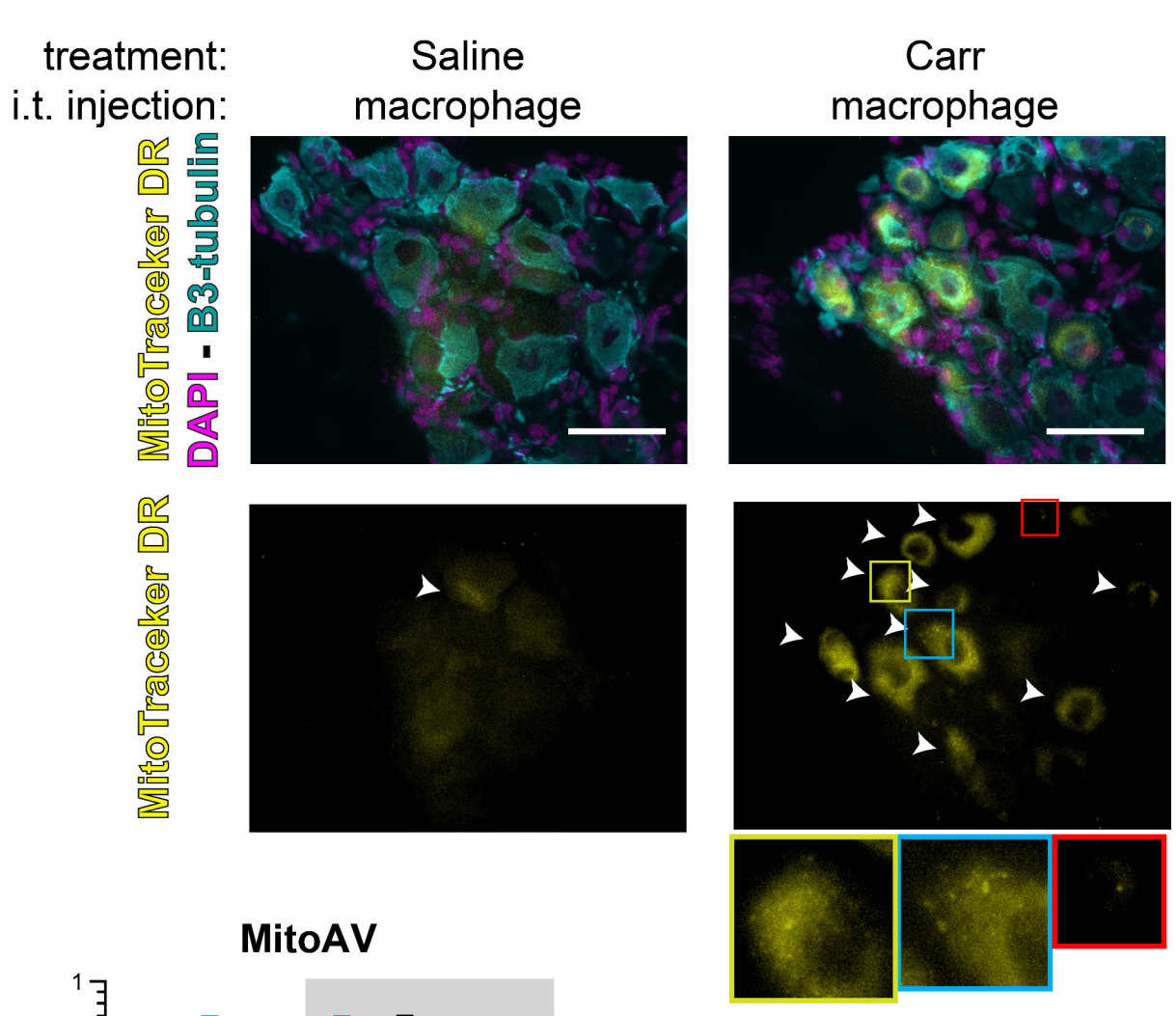
Raoof. fig 2 - reconstitution of macrophages

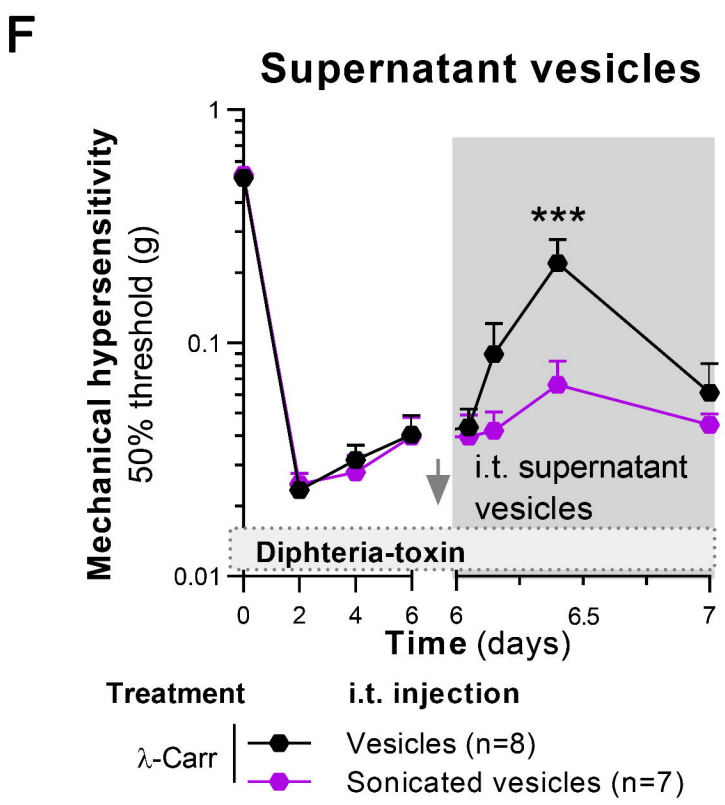


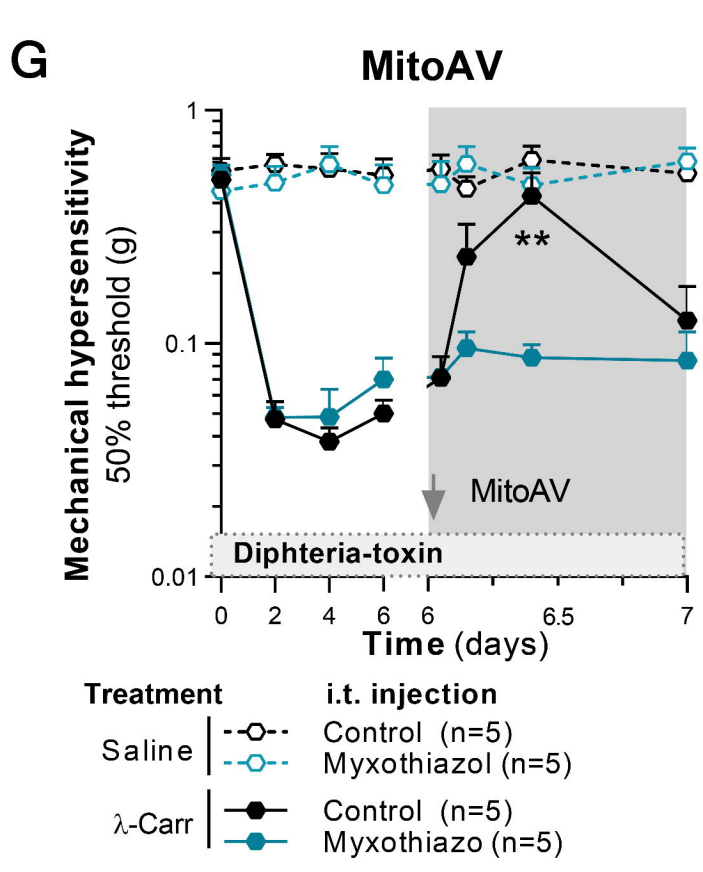
Raoof fig 3. transfer of mitochondria and vesicles



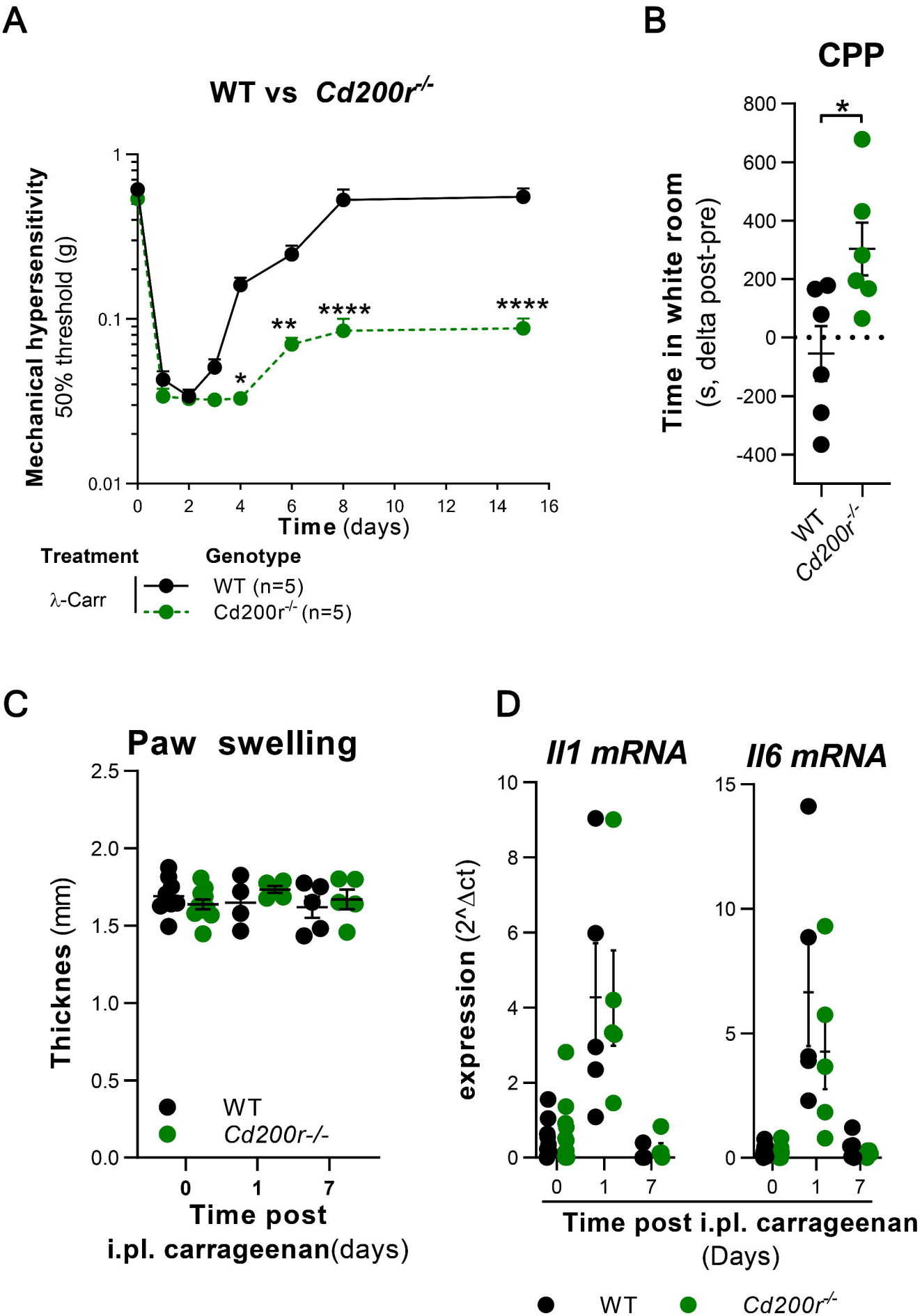




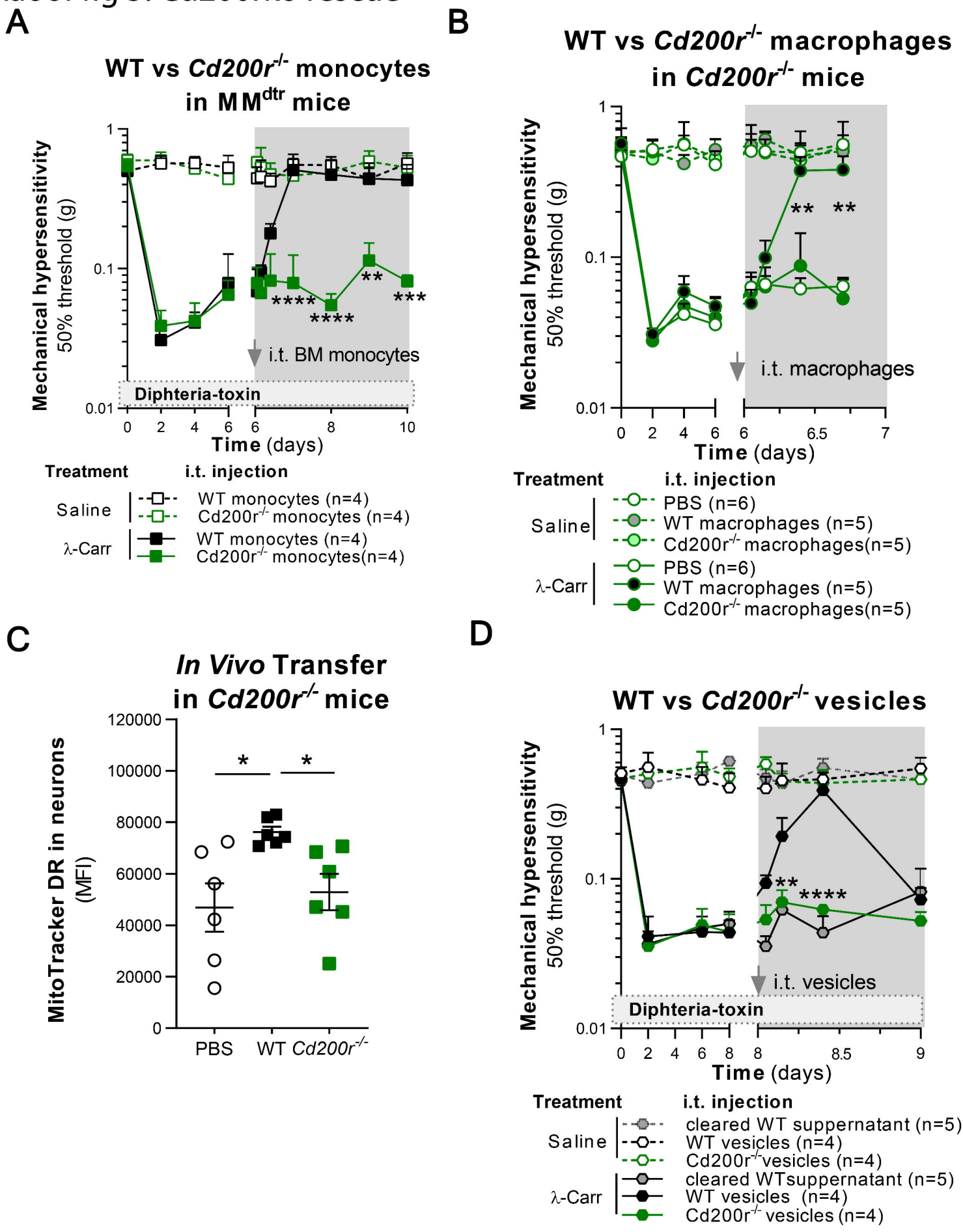


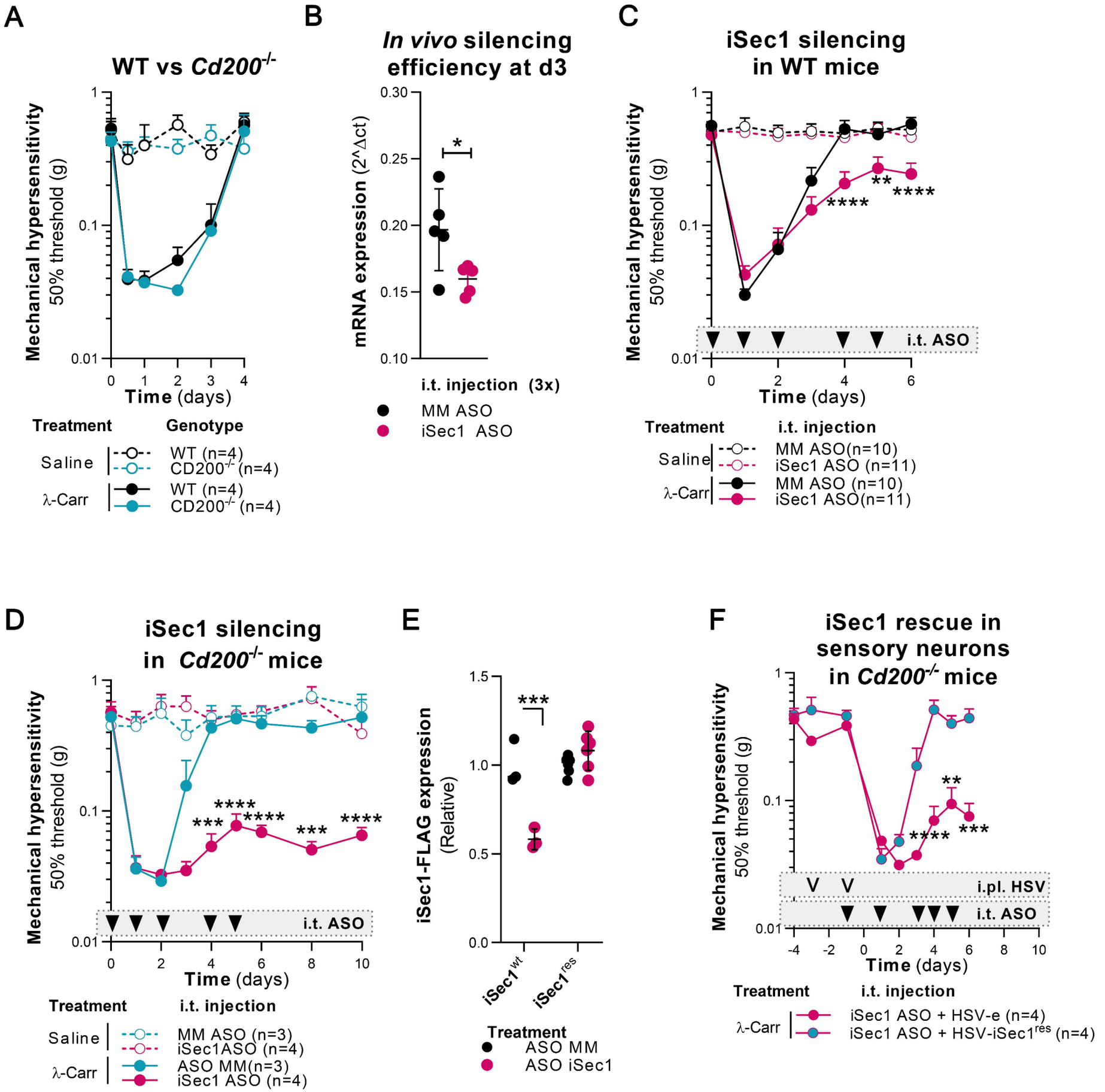


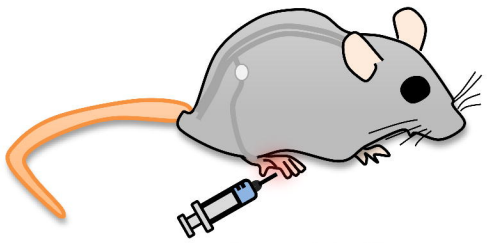
Raoof fig 4. Cd200rko



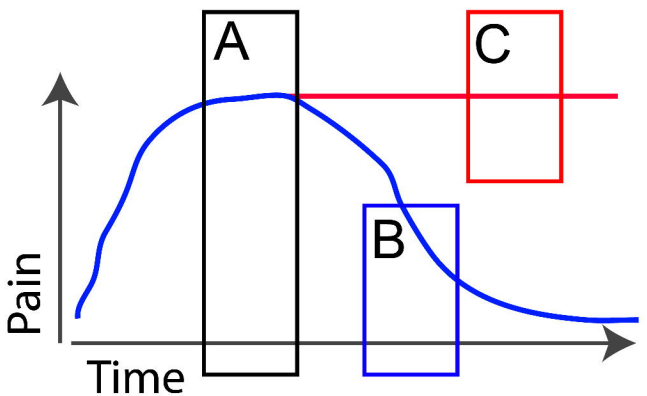
Raoof fig 5. Cd200rko rescue







local inflammation



A. Inflammatory hyperalgesia

Inflammatory Insult

Sensory neuron

B. Resolution of hyperalgesia

CD200R

M2-macrophage

iSec1

Primary inflammation resolved

C. Failure to resolve hyperalgesia: deficiency for CD200R or iSec1

CD200R

M2-macrophage

iSec1

Primary inflammation resolved