Supplemental data for:

Macrophages transfer mitochondria to sensory neurons to resolve inflammatory pain

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Materials and Methods

Animals

All experiments were performed in accordance with international guidelines and approved by the experimental animal committee of University Medical Center Utrecht (2014.I.08.059) or by the local experimental animal welfare body and the national Central Authority for Scientific Procedures on Animals (CCD, AVD115002015323).
Adult (age 8–15 weeks) male and female C57Bl/6J, *Lysm*^{Cre} x *Csf1r* $^{LsL\text{-}DTR}$(Jackson laboratories #024046)(MM^{dtr}), *Nav1.8*^{Cre}*Il10r*^{flox}, *Cd200*$^{-/-}$(ref. 1), *Cd200r*$^{-/-}$ (ref. 2), *Lysm*^{Cre} x PhAM^{flox} (LysM^{MitoDendra2}, Jackson laboratories #18397) mice in a CD57Bl/6 background were used and maintained in the animal facility of the University of Utrecht. *Nav1.8*^{cre} mice were kindly donated by Dr. Wood (University College London, UK). *Il10r*^{flox} mice were backcrossed from *Lysm*^{cre}*Il10r*^{flox} mice that were kindly donated to us by Dr. Muller (University of Manchester, UK). *CD200R*$^{-/-}$ were kindly donated by Dr. R.J. Soberman (Harvard Medical School). Mice were housed in groups under a 12h:12h light:dark cycle, with food and water available *ad libitum*. The cages contained environmental enrichments including tissue papers and shelter. Mice were acclimatized to the experimental setup for at least 1 week prior to the start of each experiment. To minimize bias, animals were randomly assigned to the different groups prior to the start of experiment, and experimenters were blinded for the treatments and genotypes.

Transient inflammatory pain models

For the carrageenan model, mice received an intraplantar injection of 5 µl λ-carrageenan (1% w/v, Sigma-Aldrich) in one or both hind paws[3]. For the transient complete Freund's adjuvant (CFA) model, mice received intraplantar injection of 2.5 µl mix of 1:1 saline and CFA (Sigma-Aldrich). In experiments where mice received a unilateral intraplantar injection, latency times and 50% thresholds of each paw was considered as an independent measure, while in experiments with bilateral intraplantar injection the average of the left and right paw were considered as an independent measure.

Pain behavioral tests

Heat withdrawal latency times were determined using the Hargreaves test (IITC Life Science)[4,5]. Mechanical thresholds were assessed using the von Frey test (Stoelting, Wood Dale, IL, US) with the up-and-down method to determine the 50% threshold[5,6]. Changes in weight bearing were evaluated using the dynamic weight bearing (DWB) apparatus (Bioseb, Vitrolles, France)[7] and the following parameters were used for the analysis. (i) Low weight threshold of 0.5g, (ii) High weight threshold of 1g, (iii) Surface threshold of 2 cells, (iv) Minimum 5 images (0.5 seconds) for stable segment detections. The weight bearing of the affected paw is expressed as percentage of body weight. Spontaneous pain was assessed by the conditioned place preference test (CPP, Stoelting, Wood Dale) as described previously[8,9]. CPP was calculated by subtracting the mean time spent in the white room during preconditioning (day 1) from the time spent in the white room after 3 days of conditioning (day 5) with daily intraperitoneal injections of gabapentin (100 mg/kg, Sigma-Aldrich).

Depletion of monocytes and macrophages

To deplete monocytes and macrophages in vivo, MM^{dtr} mice received a first intraperitoneal injection of 20 ng/g body weight diphtheria toxin (DT) (Sigma-Aldrich) followed by daily intraperitoneal injections of 4 ng/g body weight on all subsequent days as described previously[10].

Monocyte isolation and in vitro differentiation

To obtain bone marrow-derived monocytes, the tibia and femur bones were flushed with phosphate buffered saline (PBS). For spleen-derived monocytes, spleens were mechanically excised and minced in PBS and passed through a 70 μm cell strainer (Corning). After erythrocyte lysis with RBC lysis buffer (eBioscience), cells were centrifuged on a Ficoll density gradient (GE Healthcare) for 22 min at 1100RCF at 22°C to obtain mononuclear cells. Finally, $CD115^{+}$ monocytes were isolated with biotin labeled anti-CD115 antibody and streptavidin-coupled magnetic beads according to the manufacturer's instructions (Miltenyi Biotec).

To obtain classical ($Ly6c^{hi}$) or non-classical ($Ly6c^{low}$) monocytes for adoptive transfer, monocytes were FACS sorted (FACS Aria III, BD) using CD115 and Ly6c antibodies (see

subheading ‘antibodies’). *Tfam*$^{+/-}$ splenocytes and bone-marrow cells were isolated at Yale School of Medicine and shipped frozen in 10% DMSO to the Netherlands.

For monocyte-derived macrophage generation, 10 million bone-marrow cells were seeded in a 75 cm^2 non-treated tissue culture flasks (VWR, Radnor, PA) for 7 days in macrophage medium (High-glucose Dulbecco’s Modified Eagle medium (DMEM; Cat# 31966-021, Gibco) and DMEM/F12 (Cat#31331-028, Gibco) (1:1), supplemented with 30% L929 cell-conditioned medium (see cell lines and primary cell cultures), 10% fetal bovine serum (FBS; Cat# 10270-106, Gibco), 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (200 mM, ThermoFisher).

To polarize macrophages toward M1- or M2-like macrophages, cells were stimulated with 20 ng/ml IFNγ and 100 ng/ml LPS, or 20 ng/ml of IL-4 for 24 hours, respectively.

Adoptive Transfer of monocytes and macrophages

Cells were injected intrathecal (30.000 cells/5 µl per mouse) under light isoflurane anesthesia as described previously[5,11]. For some experiments, 2 million macrophages were labeled with 20nM of MitoTracker DeepRed FM (MTDR; Thermo Fisher Scientific) in 400µl macrophage-medium for 30 minutes at 37°C, followed by 3 washes before intrathecal injection of 30.000 cells. For some experiments, cells were sonicated with a sonicator (soniprep150, MSE, UK) at a frequency of 23 kHz, 3 times for 15 seconds on ice before injection.

Isolation and injection of extracellular vesicles

To isolate macrophages-derived extracellular vesicles, 10 million macrophages were in a 75 cm^2 flask the day prior to the isolation of vesicles. The supernatant from 10 flasks were collected and centrifuged at 2000RCF for 10 minutes to pellet large debris[12]. The supernatant was divided in 1.5 ml Eppendorf tubes followed by a centrifugation at 17,000RCF for 30 minutes at 4°C. Supernatant was discarded, except the last 50 µl to resuspend the pellets and pool them together. The pooled supernatant was centrifuged at 17.000RCF for 30 minutes. The final supernatant was collected as ‘cleared supernatant’, and the pellet was resuspended in 100 µl PBS and injected intrathecally (5 µl per mouse).

To destroy vesicles, supernatant vesicles were sonicated 3 times at a frequency of 23 kHz for 15 seconds on ice before injection.

NTA analysis of extra cellular vesicles

For analysis of extracellular vesicles, 4 million macrophages were seeded in a T75 flask and cultured for 4 days, then they were washed and 7 ml of plain Opti-Mem (Gibco, 31985062) was added for an additional 24h. As control, mock-treated medium that was treated identically except for the presence of macrophages. Supernatant was harvested, spun down at 2000G to remove cell debris, followed by centrifugation at 17.000G to pellet the vesicles. The pellets were resuspended in PBS and measured in a Nanosight NS500 analyser (Melvern Intruments) equipped with a 405 nm laser. NTA analysis was acquired in NTA 3.3 Dev Build 3.3.104, the camera level was set at 10, and detection threshold was set at 5, all other settings were automated.

Isolation and injection of mitochondria

10 million macrophages were seeded in a 75 cm^2 flask the day prior to the isolation of mitochondria. To inhibit mitochondrial oxidative phosphorylation, macrophages were cultured for 10 minutes with 1μM myxothiazol (Sigma-Aldrich) prior to intrathecal injection. Subsequently, macrophages were detached, and mitochondria were isolated from 1.2 million macrophages as previously described[13] and resuspended in 200 μl of PBS and injected intrathecally (5 μl per mouse).

Cell lines and primary cell cultures

Mouse neuroblastoma N2A cells were kept in cell culture-medium: DMEM (Cat# 31966-021, Gibco) plus 10% FBS (Cat# 10270-106, Gibco) and 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (200 mM, ThermoFisher).

To obtain L929 cell-conditioned medium, 10 million L929 cells were seeded in a 75 cm^2 flask with cell culture-medium supplemented with 1% non-essential amino acids (Sigma-Aldrich) for a week. L929 cells were passaged to a 162 cm^2 flask with 50 ml medium and after a week the supernatants were collected and filtered through a 0.2-μm filter and stored at −20°C (L929-drived M-CSF).

Over-expression and cloning

We amplified CD200 cDNA derived from DRG (mCD200-BamHI-fwd: TAAGCAGGATCCGCCGCCACCATGGGCAGTCTGGTATTCAG; mCD200-SalI-rev: TGCTTAGTCGACTCATTATTTCATTCTTTGCATCC; mCD200-NotI-rev:TGCTTAGCGGCCGCTCATTATTTCATTCTTTGCATCC) and ligated the PCR product into pMXc after digestion with BamHI and ApoI, or into pLenti-MP2 (ref. 14) after digestion with BamHI and SalI. All cDNA inserts were verified using sanger sequencing.

Standard transduction protocols were performed to generate stable CD200 and iSec1 expression in N2A using pMX-iSec1-IRES-GFP[15] and pLenti-MP2-CD200. MitoDsRed expression in macrophages were made by standard transduction protocols using pLV-MitoDsRed[16].

We generated a bicistronic herpes simplex virus (HSV) construct by cloning iSec-Flag, under control of the α4 promotor and GFP under control of the α22 promoter (HSV-iSec). We used sewing-PCR to introduce silent mutations in iSec1-Flag to resist antisense oligonucleotide-mediated knockdown (see table below for sequence of primers). The first PCR products were made by combining primers '*iSec1-FLAG forward*' with 'iSec1^{mutASO1}_mid_reverse', and '*iSec1^{mutASO1}_mid_forward*' with '*iSec1^{mutASO2}-reverse*'. The right length products were excised from agarose gel, purified and combined in a next PCR using primers '*iSec1-FLAG forward*' with '*iSec1^{mutASO2}-reverse*'. The resulting iSec1^{mut} was digested with HINDIII and purified from agarose gel and ligated into HSV as described before[17], and validated using sanger sequencing. Control empty HSV (HSV-e) only expresses GFP. HSV was produced as previously described[17]. Mice received 35000 pfu/paw (8 µl) intraplantar HSV-e or –iSec1^{mut} at days -3 and -1 prior to carrageenan.

Isec1^{mut} was tested for ASO resistance *in vitro* by transfecting Isec1^{wt} or iSec1^{mut} into Neuro 2A cells with Lipofectamin 2000, followed by transfection with mis-match (MM) ASO or iSec-targetting ASO (See below: *Antisense oligonucleotide-mediated knockdown*).

Mutagenesis primer	**sequence**
iSec1-FLAG forward	cgcgcgcg*AAGCTT*ccaccgcatggctcctgccatggaatc
iSec1^{mutASO2}-reverse	gcgcgcgc*AAGCTT*ttaAGTCTCGCTCTGACAGTGATGTCGCAttaagttctcagtctctgttgatgg
iSec1^{mutASO1}_mid_reverse	TGTCAAACATGTCTGCCCTCCatggctgccatgtggaaaagtg
iSec1^{mutASO1}_mid_forward	GGAGGGCAGACATGTTTGACAattataactgtatctgaactagtaactga

Italic capital sequence: HINDIII site, underlined capital: mutated sequence to resist iSec-ASO.

Antisense oligonucleotide-mediated knockdown

For in vitro knockdown, we used lipofectamine 2000 (Thermofisher) to transfect N2A-Cd200-iSec1 cells with Mismatch (MM) or iSec1-targetting phosphorothioated antisense oligonucleotides (ASO) according to manufacturer protocol. After 24h, mRNA was isolated with a RNeasy mini kit (Qiagen) and cDNA was generated with iScript (Biorad) according to manufacturer's protocol. To knockdown iSec1 in sensory neurons in vivo, mice received intrathecal injections of 5 µl iSec-ASO mix (total concentration of 3 µg/µl constituting 1:1 mix of iSec-ASO 1 and 2; Sigma-Aldrich) at day 0, 1, 2, 4, and 5. A MM-ASO mix was used as control[11,18]. The following phosphorothioated ASO sequences, that specifically target iSec1/gm609, were used:

Name	**Sequence (5'-3')**
iSec-ASO 1	[mG]*[mG]*[mU]*[mG]*[mA]*G*G*C*A*G*G*T*T*T*G*[mU]*[mC]*[mC]*[mU]*[mC]
iSec-ASO 2	[mA]*[mC]*[mU]*[mG]*[mG]*C*A*G*T*G*A*T*G*T*C*[mU]*[mC]*[mA]*[mU]*[mU]
MM-ASO 1	[mG]*[mU]*[mG]*[mC]*[mG]*A*T*G*T*A*T*G*C*C*G*[mG]*[mU]*[mG]*[mC]*[mU]
MM-ASO 2	[mG]*[mU]*[mA]*[mU]*[mU]*G*T*T*A*A*C*G*C*G*T*[mA]*[mU]*[mG]*[mC]*[mC]

[mA] [mG] [mC] [mU] = 2'OMethyl nucleotides to prevent breakdown/toxicity

Flow cytometry analysis

DRGs (L3–L5) were collected to analyze infiltrating immune cells. In brief, tissues were gently minced and digested at 37 °C for 30 minutes with an enzyme cocktail (5 mg collagenase type I with 2.5 mg trypsin, Sigma Aldrich) in 5 ml DMEM. Cells were stained with various combinations of fluorochrome-labeled antibodies (see subheading 'antibodies').

Blood was collected in EDTA tubes (Greiner Bio-One) following heart puncture and erythrocytes were lysed (RBC lysis buffer, eBioscience) before FACS staining.

For vesicles (see isolation of extracellular vesicles), pellets were pooled and stained for CD45, CD11b and CD200 Receptor 1 (CD200R), and MTDR (see subheading 'antibodies'). Before samples were acquired by LSRFortessa flow cytometer (BD Biosciences) and analyzed with FACSDIVA software, counting beads were added. On average the recovery rate of counting beads (eBioscience) was 44%±3. For all cellular analysis, we used FSC as trigger to identify events. For vesicle analysis, we used FSC or CD45-PB as trigger to identify events.

Transfer of mitochondria

Bone marrow-derived macrophages were harvested and 2 million cells were labelled with 20 nM MTDR in 500 µl culture medium. Cells were washed 3 times, counted (NucleoCounter NC-200; Chemometec) and resuspended at a concentration of 120.000 cells/ml medium.

N2As (30.000 cells) were seeded in a 24-well plate and 24h later co-cultured with 12.000 MTDR pre-stained macrophages for 2h and harvested using 1X Trypsin-EDTA (Gibco). Cells were stained for F4/80 and CD11b (see subheading 'antibodies'). MTDR signal in N2A's was assessed using the ImageStream MkII (Millipore, Burlington, MA) or flow cytometer (4 laser BD Fortessa, 3 laser BD Canto II).

Primary DRG neurons were cultured as described before[19] and co-cultured with MitoDsRed-expressing macrophages. After 16h, co-cultures were fixed and imaged with a Zeiss Axio Observer microscope (Zeiss, Oberkochen).

Immunofluorescent staining and detection of mitochondrial transfer in vivo

To monitor mitochondrial transfer in vivo, 24 hours after intrathecal injection of MTDR-labelled macrophages, mice were killed by cervical dislocation and lumbar spinal cords and DRGs were collected. Tissues were post-fixed in 4% paraformaldehyde (PFA), cryoprotected in sucrose overnight and embedded in optimal cutting temperature (OCT) compound (Sakura, Zoeterwoude, the Netherlands), and frozen at −80°C.

For immunofluorescence, cryosections (10 µm) of lumbar DRGs or spinal cords, were stained with primary antibodies overnight at 4°C followed by 2 hours incubation with fluorescent-tagged secondary antibodies (see subheading 'antibodies'). Nuclei were counterstained with or without 4,6-diamidino-2-phenylindole (DAPI). Immunostaining images were captured with a Zeiss Axio Observer microscope (Zeiss, Oberkochen, Germany) using

identical exposure times for all slides within one experiment. Fluorescence intensity was analyzed with ImageJ software.

iDisco, clearing procedure and light sheet imaging

DRGs from adult mice were cleared using iDISCO protocol as described before[20]. Briefly, animals were perfused with 4% PFA, lumbar dorsal root ganglia were dissected and samples were dehydrated in increasing concentrations (20%, 40%, 60%, 80%, 100%) of methanol solutions. Samples were bleached and rehydrated in decreasing concentrations of methanol solutions. After blocking for 48 hours, samples were incubated with the primary antibodies for 48 hours followed by incubation with secondary antibody for another 48 hours (see subheading 'antibodies'). After samples were embedded in agarose, they were dehydrated in increasing concentrations of methanol solutions. Samples were incubated overnight in 1 volume of 100% methanol/2 volumes 100% dichloromethane (DCM) anhydrous, washed with 100% DCM and incubated in 100% dibenzyl ether (DBE) for at least one day before imaging. Samples were imaged with an Ultramicroscope II (LaVision BioTec) lightsheet microscope equipped with Inspector (version 5.0285.0) software (LaVision BioTec). The microscope consists of an Olympus MVX-10 Zoom Body (0.63-6.3x) equipped with an Olympus MVPLAPO 2x Objective lens, which includes, dipping cap correction optics (LV OM DCC20) with a working distance of 5.7mm. Images where taken with a Neo sCMOS camera (Andor) (2560x2160 pixels. Pixel size: 6.5 x 6.5 um2). Samples were scanned with a sheet NA of 0.148348 (results in a 5 µm thick sheet) and a step-size of 2.5 µm using the horizontal focusing light sheet scanning method with the optimal number of steps and using the contrast blending algorithm. The following laser filter combinations were used: Coherent OBIS 561-100 LS Laser with 615/40 filter and Coherent OBIS 647-120 LX with 676/29 filter.

Real-time RT-PCR

Total RNA was isolated from freshly isolated DRGs (L3-L5) or hind paws using TRizol and RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using iScript reverse transcription supermix, according to manufacture protocol (Bio-Rad, Hercules, CA). Quantitative real-time PCR reactions were performed using an I-cycler iQ5 (Bio-Rad, Hercules, CA) as described[21] or on a QuantStudio 12K Flex or a StepOnePlus Realtime PCR system (AB

Instruments) with SYBR Select Master Mix (Life Technologies). We used 1-5 ng cDNA input per qPCR reaction.

mRNA expression is represented as relative expression = 2^(Ct(average of reference genes)-Ct(target)). For N2As we used the average Ct values of Gapdh and B2M as reference, for mRNA expression in DRG we used the average of 18S, TBP and Rictor as reference. #1 primers were used for silencing validation in vitro, #2 primers were used for ex vivo mRNA.

Target	**Fwd primer**	**Rev primer**
Cd200 #1	AAGGATGGGCAGTCTGGTATTC	CATGCCCCAAATCAGGCTGT
Cd200 #2	GGGGTGAATCATCACAGGGG	CAAATCCCTCACAGGCTCGT
iSec1/Gm609 #1	TCAAGGAGGTACCACGAATCC	TGATGGCTCGGGCATGTTAT
iSec1/Gm609 #2	CTCTTTGAAAACTGCGAGGTC	CAGTTTAAACAAGGATTCGTGGTA
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
Tnf	GCGGTGCCTATGTCTCAG	GCCATTTGGGAACTTCTCATC
Tgfb1	CAGAGCTGCGCTTGCAGAG	GTCAGCAGCCGGTTACCAAG
Il10	GCACCCACTTCCCAGTCG	GCATTAAGGAGTCGGTTAGCAG
Il1	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il6	TCTAATTCATATCTTCAACCAAGAGG	TGGTCCTTAGCCACTCCTTC
TBP	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA
B2M	TGGTCTTTCTGGTGCTTGTCT	ATTTTTTTCCCGTTCTTCAGC
Rictor	TGCGATATTGGCCATAGTGA	ACCCGGCTGCTCTTACTTCT

Measurement of mitochondrial respiration by Seahorse

Macrophages of WT and *Cd200r*$^{-/-}$ mice were seeded on non-coated XF24 (125k cells) or XF96 (50k cells) plates (Seahorse Bioscience), and grown overnight at 37°C. Next day, cells were washed and placed in Seahorse XF-assay base media (pH 7.4) supplemented with 10 mM glucose and 1 mM sodium pyruvate at 37°C to degas. The Seahorse Bioscience XFe24 Analyzer (Seahorse Bioscience) was used to measure oxygen consumption rates (OCR) under basal conditions, and after sequential addition of oligomycin (1 μM), FCCP (0.2 μM), and rotenone (0.5 μM), which were injected after cycle 4, 8, and 12, respectively. Each assay cycle consisted of 1.5 minute of mixing, 2 minutes waiting, and 3 minutes of OCR measurements. For each condition, three cycles were used to determine the average OCR under given condition. The

measured OCR was normalized for protein content. Five independent experiments were run, each consisting of 3 or more replicates.

DRGs were isolated day 0, 1 and 3 after intraplantar carrageenan. Primary DRG neurons were cultured as described before[19] and seeded on poly-d-ornithine/laminin coated XF24 wells plate (15K) and grown overnight at 37°C. Next day, cells were washed and placed in Seahorse XF-assay base media (pH 7.4) supplemented with 4 mM Glutamine, 25 mM glucose and 1 mM sodium pyruvate. OCR was measured under basal conditions. Three independent experiments were run, each consisting duplo's.

Mitochondria from WT and *Cd200r*$^{-/-}$ macrophages were isolated according to Iuso et al.[13]. To measure complex I and complex II driven respiration, 15 μg and 5 μg mitochondria were added in a non-coated XF24 plates, respectively. To measure complex II driven respiration, MAS buffer (220 mM d-Mannitol, 70 mM sucrose, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, and 0.2% (w/v) of fatty acid-free BSA, pH 7.2) was supplemented with 10 mM succinate and 2 μM rotenone. For complex I specific respiration, MAS buffer was supplemented with 5 mM malate and 10 mM glutamate. OCR levels were measured under basal conditions, and after sequential addition of ADP (2 mM), oligomycin (3,2 μM), FCCP (4 μM), and antimycin A (4 μM). Each assay cycle consisted of 1 minute of mixing and 3 minutes of OCR measurements. For each condition, three cycles were used to determine the average OCR under given condition.

Statistical analysis

All data are presented as mean ± SEM and were analyzed with GraphPad Prism version 8.3 using unpaired two-tailed t tests, one-way or two-way ANOVA, or as appropriate two-way repeated measures ANOVA, followed by post-hoc analysis. The used post-hoc analyses are indicated in each figure. A p value less than 0.05 was considered statistically significant, and each significance is indicated with * or °: $p < .05$; ** or °° :$p < .01$; *** or °°°: $p < .001$; **** or °°°°: $p < .0001$.

All results from statistical analysis are available in the supplemental data.

Antibodies

Target	Clone	Fluorophore	Vendor	Catalogue #
Flow cytometry and ImageStream				
CD115	AFS98	APC	eBioscience	1277550
CD115	AFS98	PE-eF610	eBioscience	61-1152-80
CD11b	M1/70	PerCP-Cy5.5	BioLegend	101227(8)
CD11b	M1/70	PE	BD Bioscience	553311
CD11c	N418	BV785	BioLegend	117336
CD19	6D5	PE	BioLegend	115508
CD200	OX90	PE	eBioscience	12-5200-82
CD200R	OX110	AF488	Bio-Rad	MCA2281A488
CD206	C068C2	BV650	eBioscience	1308615
CD3	17A2	APC	BioLegend	100236
CD45	30-F11	BV711	BioLegend	103147
CD45	30-F11	PB	BioLegend	103126
CD45	30-F11	APC-eF780	eBioscience	47-0451-82
F4/80	BM8	FITC	BioLegend	123108
F4/80	BM8	BV510	BioLegend	123135
iNOS	CXNFT	APC	eBioscience	17-5920-80
Ly6C	AL-21	BV421	BD Bioscience	562727
Ly6G	1A8	BV785	BioLegend	127645
MHCII	M5/114.15.2	PerCP	BioLegend	107624
FLAG	L5	APC	BioLegend	637308
Immune fluorescent microscopy and iDISCO				
F4/80	C1:A3-1 (rat)	None	Cedarlane	CL8940AP
CD206	Polyclonal goat	None	R&D	AF2535
IbaI	Polyclonal rabbit	None	Wako	019-19741
β3-Tubulin	Polyclonal rabbit	None	Abcam	ab18207
Neurofilament M	Polyclonal rabbit	None	Biolegend	841001
Anti-Goat	Donkey	AF488	LifeTech	A11055
Anti-Rabbit	Donkey	AF594	LifeTech	A21207
Anti-Rabbit	Donkey	AF568	LifeTech	A10042
Anti-Rat	Goat	AF647	LifeTech	A21247

Supplemental figure legends

Fig. S1. Carrageenan-induced transient inflammatory pain model

(A) Schematic representation of the carrageenan-induced transient inflammatory pain model. Baseline thresholds were determined three times the first week prior to carrageenan injection and averaged. After intraplantar (i.pl.) carrageenan injection, we measured pain-associated behaviors and signs.

(B) Course of inflammatory thermal hyperalgesia in WT mice that were injected i.pl. with carrageenan in the left hind paw, and saline in the right hind paw. Significance tested: RM-2-way ANOVA, Bonferroni.

(C) Gating strategy for monocyte/macrophage phenotyping and T- and B-cells in the DRG as displayed in Fig. 1B. A time gate unified the acquisition analysis, followed by a rough separation of events based on CD45 expression but excluding the counting beads. Cells were further gated by FCS/SSC, single cells and CD45 expression before analysis of CD11b, and F4/80. F4/80+ cells were further assessed for expression of iNOS and CD206; or excluding CD11b+ cells and assessing CD3 and CD19 expression on the CD11b negative cells.

(D) 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.

(E) F4/80 positive macrophage influx in DRG of control animals or the contralateral lumbar DRG at day 3 after i.pl. injection of carrageenan.

Fig. S2. Depletion controls in MM^{dtr} mice

(A) Gating strategy for depletion analysis in blood.

(B, C) Flow cytometry data to validate monocyte depletion in MMdtr mice treated with Diptheria toxin (DT). (B) Percentage of CD115+ monocytes in blood to confirm depletion of monocytes, and (C) percentage of non-myeloid cells in blood (CD45+CD11b- cells) to confirm depletion specificity.
Significance tested: (B) one-way ANOVA, Dunnett; (C) t Test.

(D) Percentage of F4/80+ macrophages in the DRG to confirm partial depletion of tissue resident macrophages, for gating see Fig. S1C.
Significance tested: t Test.

(E, F) Immunofluorescent microscopy analysis of depletion specificity of macrophages versus microglia cells in MM^{dtr} mice after DT treatment. (E) Quantification and (F) example images of F4/80 expression in DRG of MM^{dtr} mice treated with saline or DT. Blue: nuclei, Green: F4/80. Scale bar: 50µM.

(G) Red square in schematic drawing of lumbar spinal cord indicates the area of dorsal horn analyzed for subfigures H, I and J.

(H-J) Spinal microglia are not depleted after DT treatment. (H, I) Quantification and (J) example images of Iba1+ microglia in the spinal cord of MM^{dtr} mice treated with saline or DT. Blue: nuclei, Green: Iba1. Scale bar: 50µM.
Significance tested: t Test.

Fig. S3. Pain response in MM^{dtr} mice

(A, B) Course of thermal (A) hyperalgesia, and weight bearing of the left hind paw (B) in WT and MM^{dtr} mice injected with 1% carrageenan in the left hind paw and saline in the right hind paw.

(C) Course of carrageenan-induced mechanical hyperalgesia in male and female MM^{dtr},mice.
Significance tested: saline versus carrageenan, within males or females, RM 2-way ANOVA, Tukey.

(D, E) Course of CFA-induced mechanical (D) and thermal (E) hyperalgesia in MM^{dtr} mice. Significance tested: WT versus MM^{dtr} mice, both either with CFA, RM 2-way ANOVA, Sidak.

Fig. S4. Depletion of monocytes/macrophages prevents resolution of pain

(A) Schematic representation of the depletion, intraplantar (i.pl.) and intrathecal (i.t.) injections in the pain models.

(B) Flow cytometry analysis of lumbar DRG (L3-L5) after i.t injection of WT monocytes in MM^{dtr} mice. Gating strategy is indicated in Fig. S2.
Significance tested: T test.

(C) Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice injected i.t. with $CD115^+$ bone marrow monocytes at day 6.
Significance tested: PBS and WT monocytes, both with carrageenan, RM-2way ANOVA with Sidak.

(D, E) Course of carrageenan-induced mechanical (D) and thermal(E) hyperalgesia in MM^{dtr} mice injected i.t. with splenic $CD115^+$ monocytes at day 6.
Significance tested: PBS and WT monocytes, both with carrageenan, RM-2way ANOVA with Sidak.

(F) Course of carrageenan-induced mechanical hyperalgesia in MM^{dtr} mice injected i.t. with 'classical' $Ly6C^+$ or 'non-classical' $Ly6C^-$ bone marrow monocytes at day 6.
Significance tested: $Ly6C^+$ and $Ly6C^-$ bone marrow monocytes, both with carrageenan RM-2way ANOVA with Sidak, not significant;

(G, H) Course of carrageenan-induced mechanical (G) and thermal (H) hyperalgesia in MM^{dtr} mice injected i.t. with 'classical' $Ly6C^+$ or 'non-classical' $Ly6C^-$ splenic monocytes or PBS at day 6.
Significance tested: $Ly6C^+$ (*) or $Ly6C^-$ (°) versus PBS all with carrageenan, RM-2way ANOVA with Tukey

(I) 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.
Significance tested: one-way ANOVA, indicated days compared to day 0, Dunnett.

(J) Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice injected i.t. with LPS/IFNγ-treated 'M1', IL4-treated 'M2' macrophages or PBS.
Significance tested: M1 versus M2 macrophages, both with carrageenan, Sidak;

(K) Course of carrageenan-induced mechanical hypersensitivity in $Nav1.8^{cre}$-$Il10r^{-/-}$ mice.
Significance tested: WT versus $Nav1.8^{cre}$-$Il10r^{-/-}$ mice, Sidak, not significant.

Fig. S5. Macrophages transfer mitochondria to neurons

(A) Oxygen consumption rate of macrophages (M0), macrophages differentiated with LPS and IFNγ (M1), or with IL4 (M2). One representative experiment of 3.

(B) 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.

Fig. S6. Macrophages transfer mitochondria to neurons

(A) Analysis of MitoTracker Deep Red (MTDR) signal in N2A-neurons after co-culture of MTDR-labelled macrophages (macs), or sonicated MTDR-labelled macrophages (Son.) with MTDR-negative N2A-neurons. Co-cultures were stained with CD11b and CD45 to identify macrophages and N2A cells and analysed by flow cytometry.

(B) Macrophages labeled with MTDR were co-cultured with N2A for 2 hours and analyzed by image stream (n=2). Co-cultures were stained for CD11b and F4/80 to identify macrophages, and neurons were identified by negative selection. Scale bar: 7µm.

(C) Macrophages transduced with Mito-dsRed were co-cultured with primary mouse sensory neuron cultures for 16h. Co-cultures were analyzed with immune fluorescence for macrophage-derived mitochondria (red), neurons (β3 tubulin, green), and nuclei (DAPI, blue). Macrophages are indicated with pink arrow heads. Neurons positive for Mito-dsRed are indicated by white arrow heads. n=1. Scale bar: 50µM.

(D) Example image of MitoDenra2^{+} presence in naïve MitoDendra2^{flox} mouse, or GFP^{+} neurons in *Lysm*^{cre}-*GFP*^{flox} mouse.

(E) Analysis of MTDR signal in sensory neurons in the DRG of MM^{dtr} mice. At day 6 after 1% carrageenan or saline injection. 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.

Fig. S7. Characterization of macrophage-derived extra cellular vesicles.

(A) Expression of CD11b, F4/80, CD200R and MHCII on CD45^{+}MTDR^{+} vesicles.

(B) Gating strategy for subpanel (C). Events were captured based on FSC or CD45-PB-signal and gated for CD45 expression, followed by a FFC/SSC gate.
Dotted line: unstained, solid black: WT, solid green: *Cd200r*$^{-/-}$.

(C) Expression of Mitochondrion-targeted PhAM in CD45^{+} vesicles of macrophages, quantified and example histogram.

(D) Nanoparticle Tracking Analysis (NTA) of supernatant vesicles from WT macrophages. Data are SEM from 6 technical replicates.

Fig. S8. Functional mitochondria are required to resolve inflammatory pain

(A, B) Course of carrageenan-induced (A) mechanical and (B) thermal hyperalgesia in MM^{dtr} mice injected intra thecal (i.t.) with WT or $Tfam^{+/-}$ $CD115^{+}$ monocytes.
Significance tested: WT vs. $\underline{Tfam}^{+/-}$ monocytes, all with carrageenan, RM-2way ANOVA, Sidak.

(C) Flow cytometry analysis of isolated mitochondria. Events were gated on FSC/SSC, followed by FCS/FCS-H for singles. Dotted line: unstained, solid black: specific stain.

(D) Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice i.t. injected with functional isolated mitochondria, or mitochondria inhibited with complex III inhibitor myxothizaol.
Significance tested: WT vs. $\underline{Tfam}^{+/-}$ monocytes, all with carrageenan, RM-2way ANOVA, Sidak.

Fig. S9. CD200R is required for the resolution of inflammatory pain

(A) Course of carrageenan-induced thermal hyperalgesia in WT and $Cd200r^{-/-}$. Significance tested by RM 2-way ANOVA, Sidak.

(B, C) Gabapentin-induced place preference conditioning at day 16 after unilateral 1% carrageenan injection (B) or saline injection (C) in the hind paws. Conditioning efficiency is depicted as the difference in time spent in a white room pre- and post-conditioning. Significance tested: t Test, n=6.

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

(E) 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.

(H) Course of carrageenan-induced mechanical hyperalgesia in $Cd200r^{-/-}$ mice i.t injected with WT of $Cd200r^{-/-}$ $CD115^{+}$ monocytes. Significance tested: WT vs $Cd200r^{-/-}$ monocytes, both with carrageenan, RM 2-way ANOVA, Sidak.

(F, G) Course of carrageenan-induced mechanical (F) and thermal (G) hyperalgesia in MMdtr mice intra thecal (i.t.) injected with WT of Cd200r-/- CD115+ monocytes. Significance tested: WT vs Cd200r-/- monocytes, both with carrageenan, RM 2-way ANOVA, Sidak.

Fig. S10. $CD200R^{-/-}$ have normal vesicles, mitochondrial respiration and macrophage infiltration into DRG

(A) Mitochondrial respiration in WT or $Cd200r^{-/-}$ macrophages.

(B) Mitochondrial respiration in MitoAV from WT or $Cd200r^{-/-}$ macrophages assessed by extracellular flux assay.

(C) Analysis of vesicle content of WT or $Cd200r^{-/-}$ macrophage-conditioned medium.

(D, E) Flow cytometry analysis of monocytes/macrophages infiltrating the DRG. Significance tested: RM 2-way ANOVA, Sidak, not significant.

(F, G, H) Quantification and example image of F4/80 and CD206 expression in DRG by immune fluorescence in naïve WT and *Cd200r*$^{-/-}$ mice. Scale bars: 50µm.

Fig. S11. *Cd200r*$^{-/-}$ macrophages derived vesicles cannot resolve inflammatory pain.

(A) Course of carrageenan-induced thermal hyperalgesia in *Cd200r*$^{-/-}$ mice i.t injected with vesicles derived from WT of *Cd200r*$^{-/-}$-macrophage-conditioned medium. Significance tested: WT vs *Cd200r*$^{-/-}$ vesicles, both with carrageenan, RM 2-way ANOVA, Dunnett.

Fig. S12. iSec1 is required for resolution of inflammatory pain

(A) Course of carrageenan-induced thermal hyperalgesia in WT and *Cd200*$^{-/-}$. Significance tested by RM 2-way ANOVA, Sidak.

(B) *Cd200* mRNA expression in DRG of WT mice at indicated days after intra plantar (i.pl) injection of carrageenan. Significance tested by 1-way ANOVA, Dunnett

(C) *iSec1/gm690* mRNA expression in DRG of WT mice at indicated days after intra plantar (i.pl) injection of carrageenan. Significance tested by 1-way ANOVA, Dunnett

(D) *Cd200* and *iSec1/gm609* mRNA expression after silencing of iSec1/gm609 in neuronal N2A cells expressing Cd200 and iSec1/gm609. n=5 from 2 experiments.

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

(F) Course of carrageenan-induced thermal hyperalgesia in WT mice that are control- or iSec1/gm609 silenced by ASO. Significance tested by RM 2-way ANOVA, Sidak.

(G) Course of carrageenan-induced thermal hyperalgesia in *Cd200*$^{-/-}$mice that are control- or iSec1/gm609 silenced by ASO. Significance tested by RM 2-way ANOVA, Sidak.

(H) Expression of iSec1-flag (WT, n=3 from 1 experiment) and iSec1^{mut}-flag (Mutant, n=6 from 2 experiments) as assessed by flow cytometry.

(I) Course of carrageenan-induced thermal hyperalgesia in *Cd200*$^{-/-}$ mice that are iSec1/gm609 silenced by ASO, while i.pl. infected with HSV-e or HSV-iSec1. Significance tested by RM 2-way ANOVA, Sidak.

Movie S1. 3D render of control DRG

Video render of a lumbar DRG from a saline treated animal. iDISCO immunofluorescence staining for macrophage marker F4/80 (red) or neuronal marker Neurofilament M (green). The effective magnification for all images was 13.6x (zoombody * objective+dipping lens = 6.3x*2.152x).

Movie S1. 3D render of carrageenan DRG

Video render of a lumbar DRG 1 day after 1% carrageenan injection. iDISCO immunofluorescence staining for macrophage marker F4/80 (red) or neuronal marker Neurofilament M (green). The effective magnification for all images was 13.6x (zoombody * objective+dipping lens = 6.3x*2.152x).

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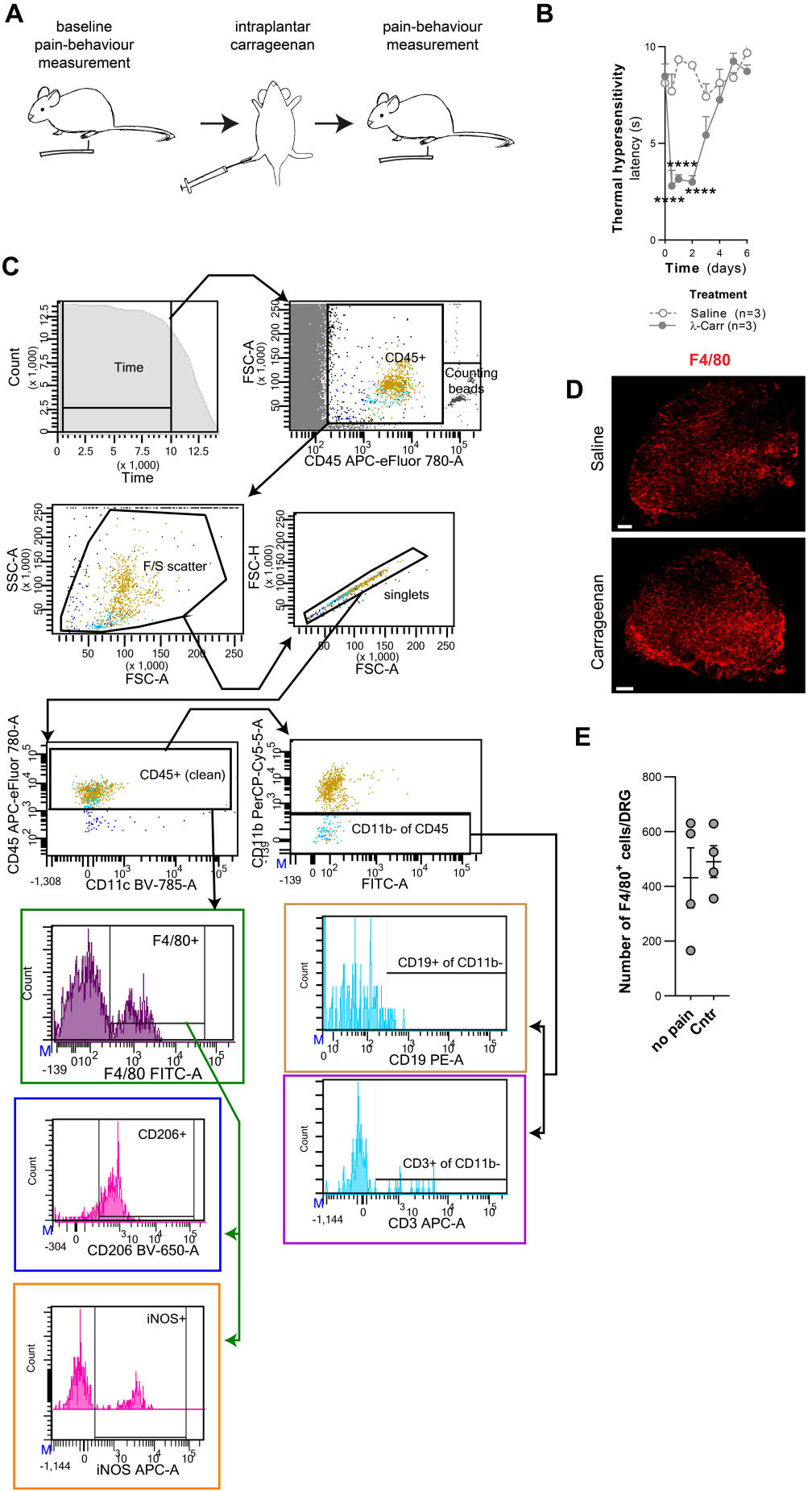
Raoof - Supplemental fig. 1



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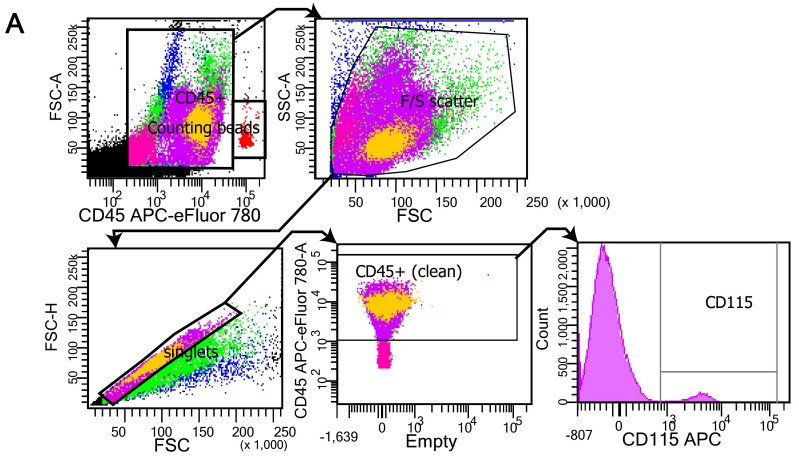

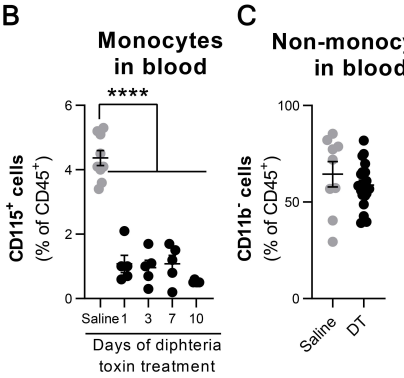

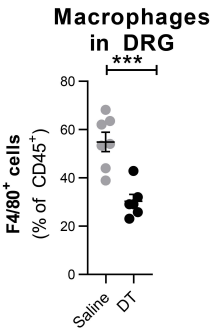

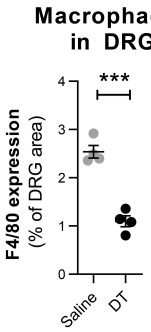

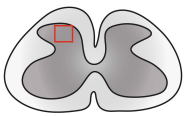

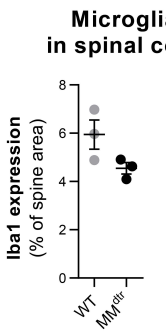

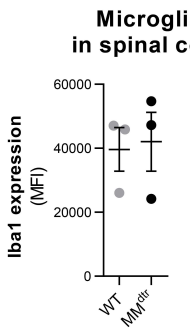

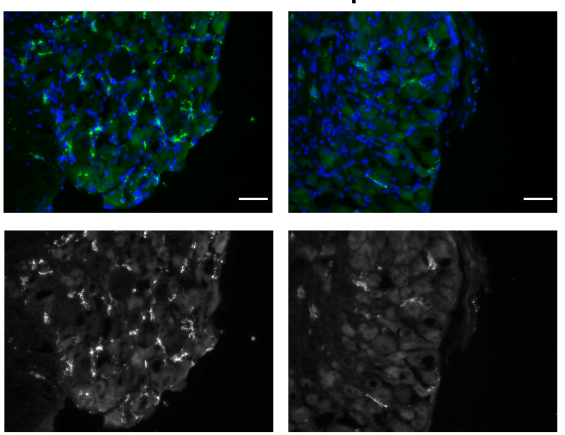

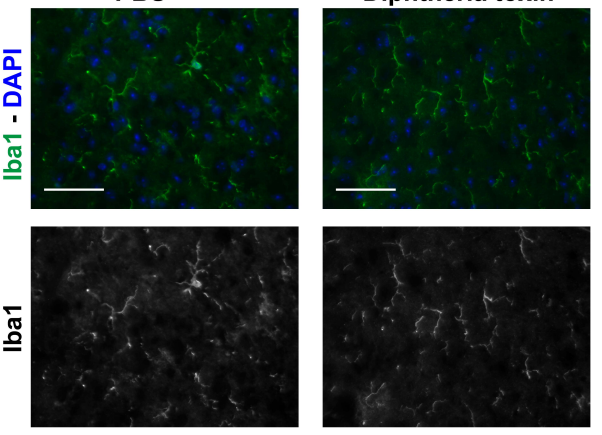

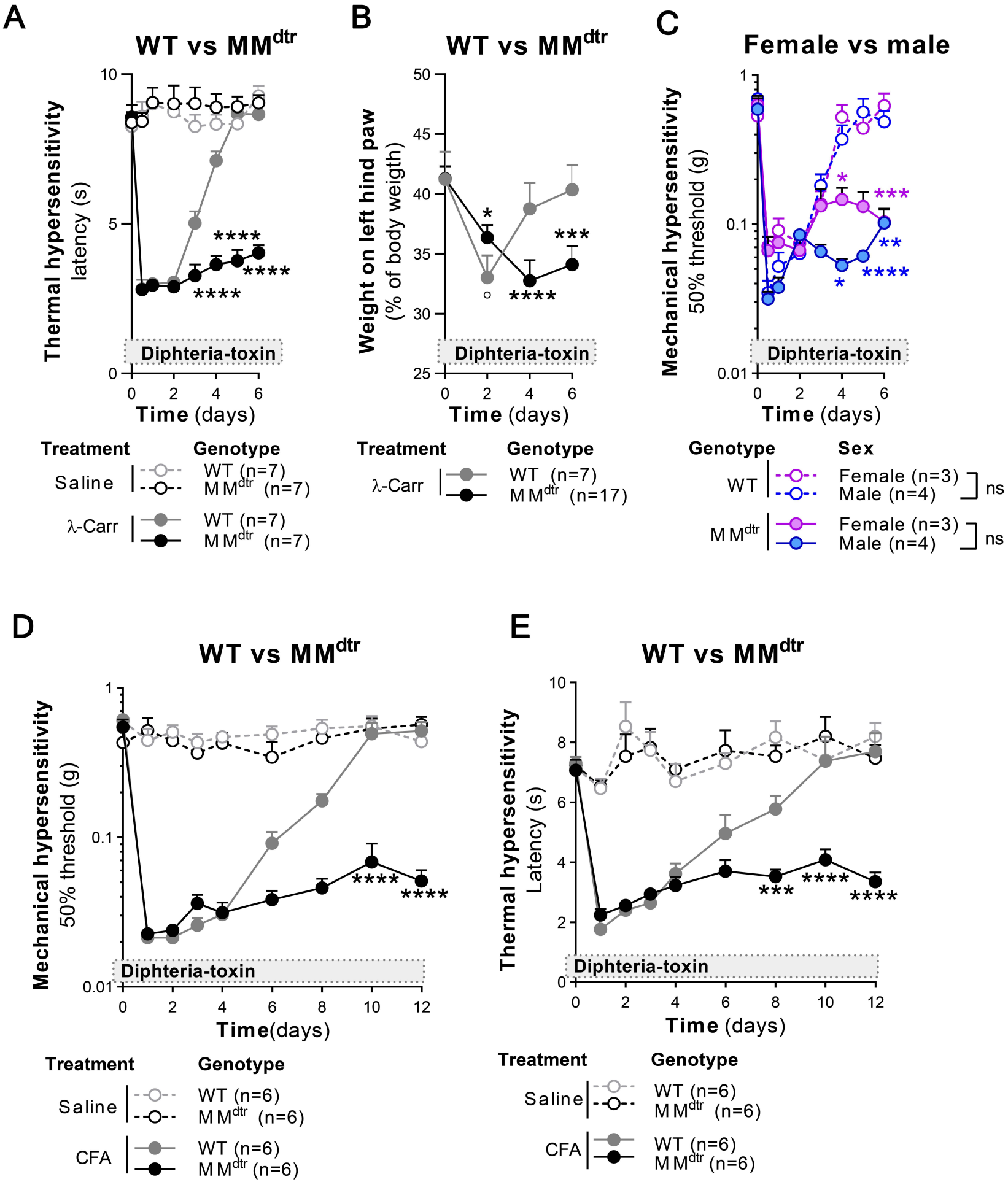
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WT vs MMdtr
Thermal hypersensitivity
latency (s)
Diphteria-toxin
Time (days)
Treatment
Genotype
Saline
WT (n=7)
MMdtr (n=7)
λ-Carr
WT (n=7)
MMdtr (n=7)
B
WT vs MMdtr
Weight on left hind paw
(% of body weigth)
Diphteria-toxin
Time (days)
Treatment
Genotype
λ-Carr
WT (n=7)
MMdtr (n=17)
C
Female vs male
Mechanical hypersensitivity
50% threshold (g)
Diphteria-toxin
Time (days)
Genotype
Sex
WT
Female (n=3)
Male (n=4)
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MMdtr
Female (n=3)
Male (n=4)
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WT vs MMdtr
Mechanical hypersensitivity
50% threshold (g)
Diphteria-toxin
Time(days)
Treatment
Genotype
Saline
WT (n=6)
MMdtr (n=6)
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E
WT vs MMdtr
Thermal hypersensitivity
Latency (s)
Diphteria-toxin
Time (days)
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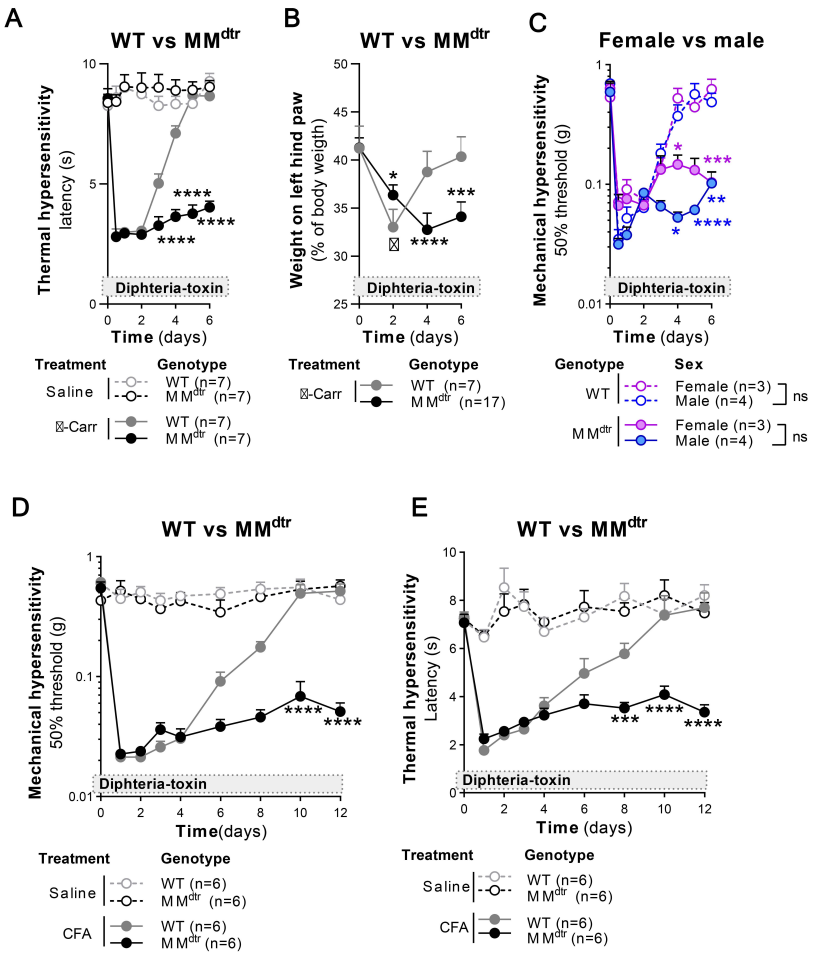
Raoof - Supplemental fig. 3



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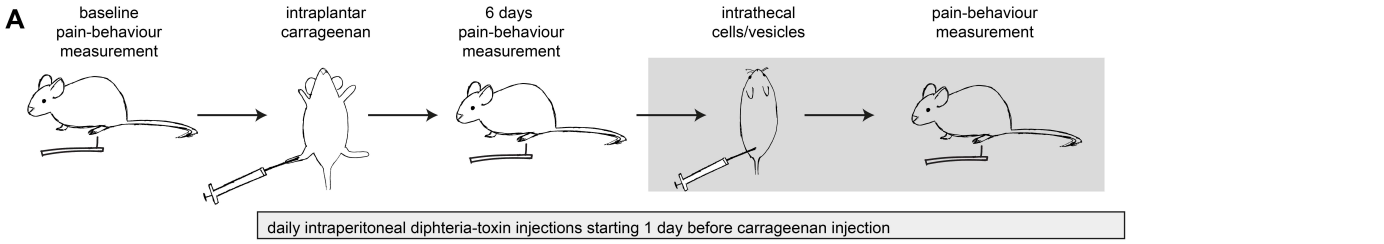

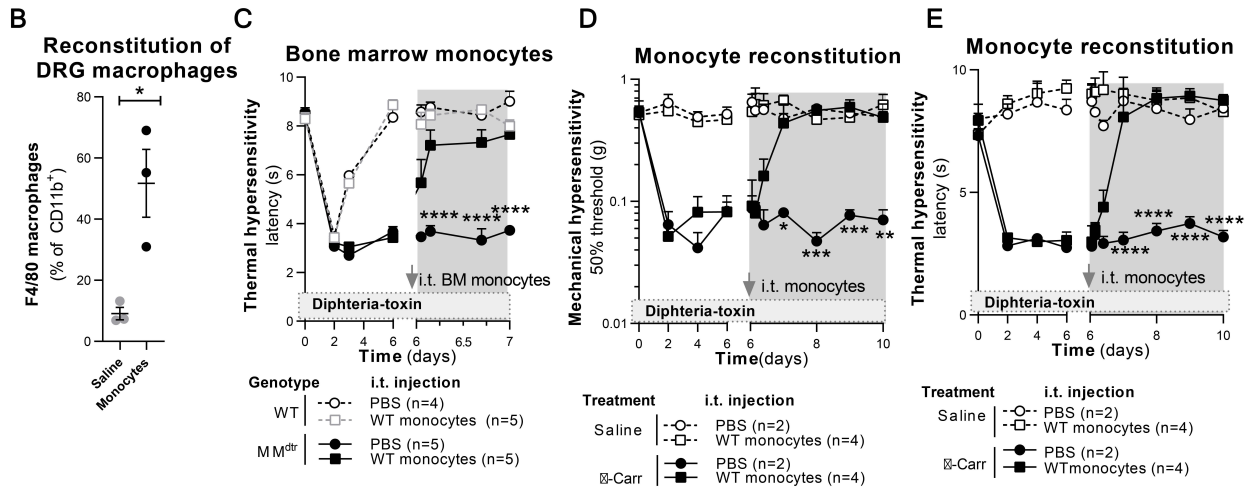

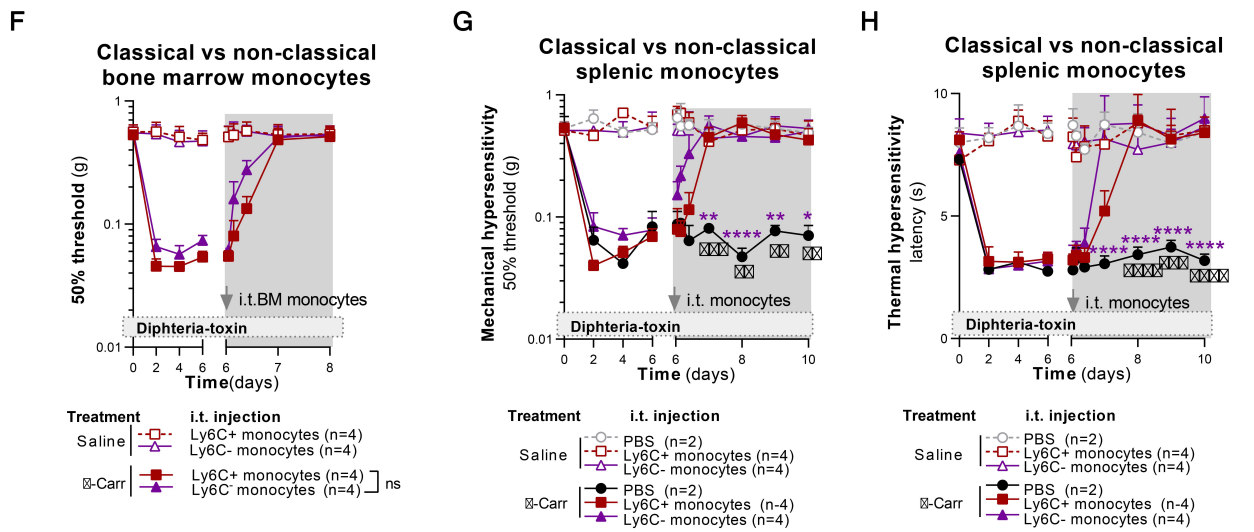

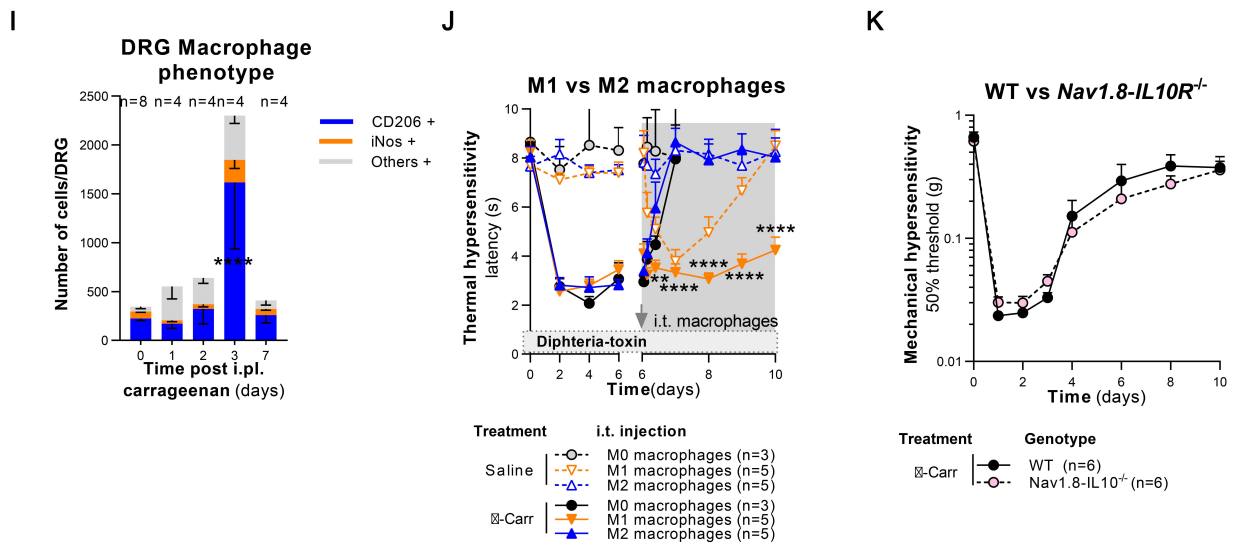

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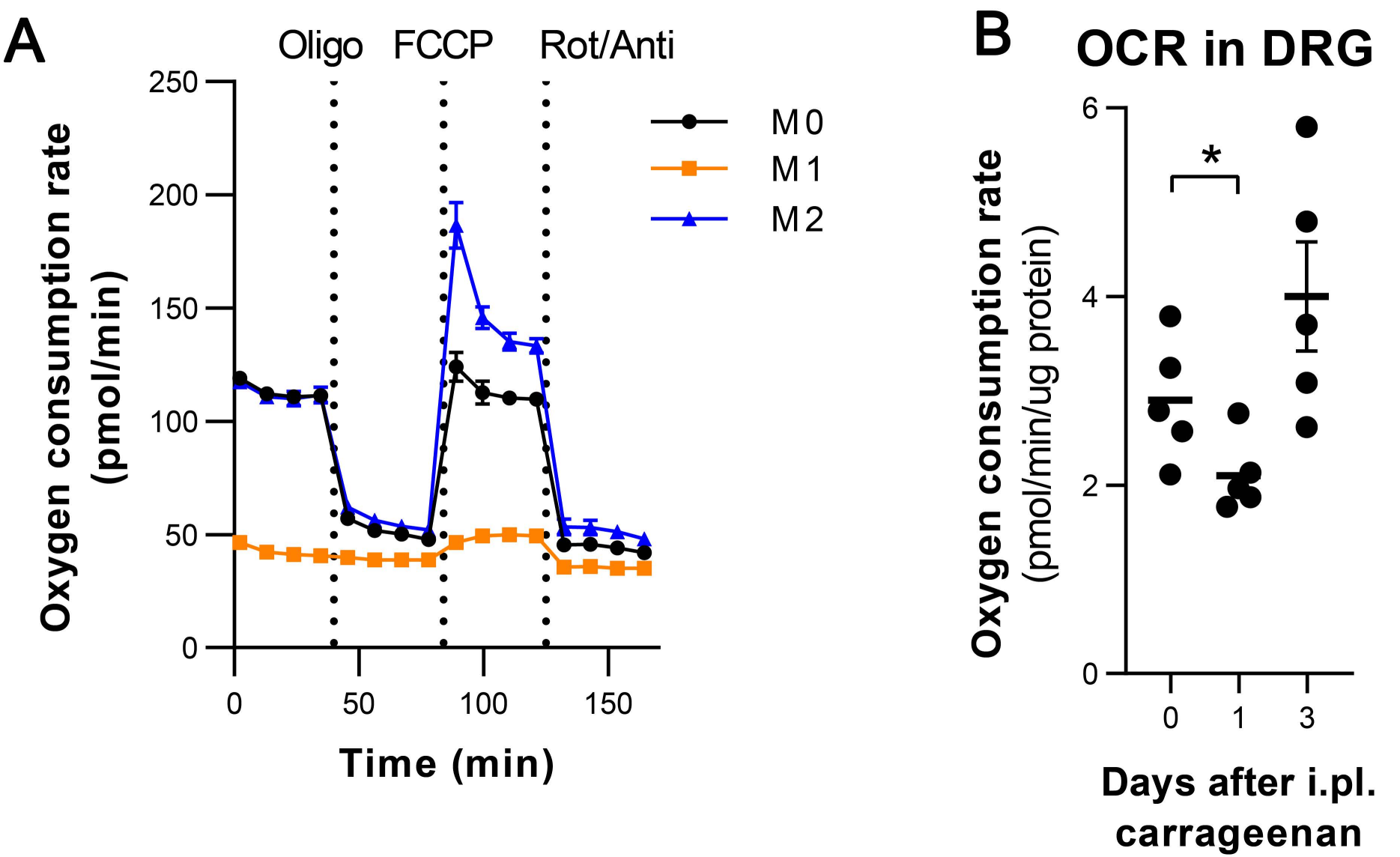

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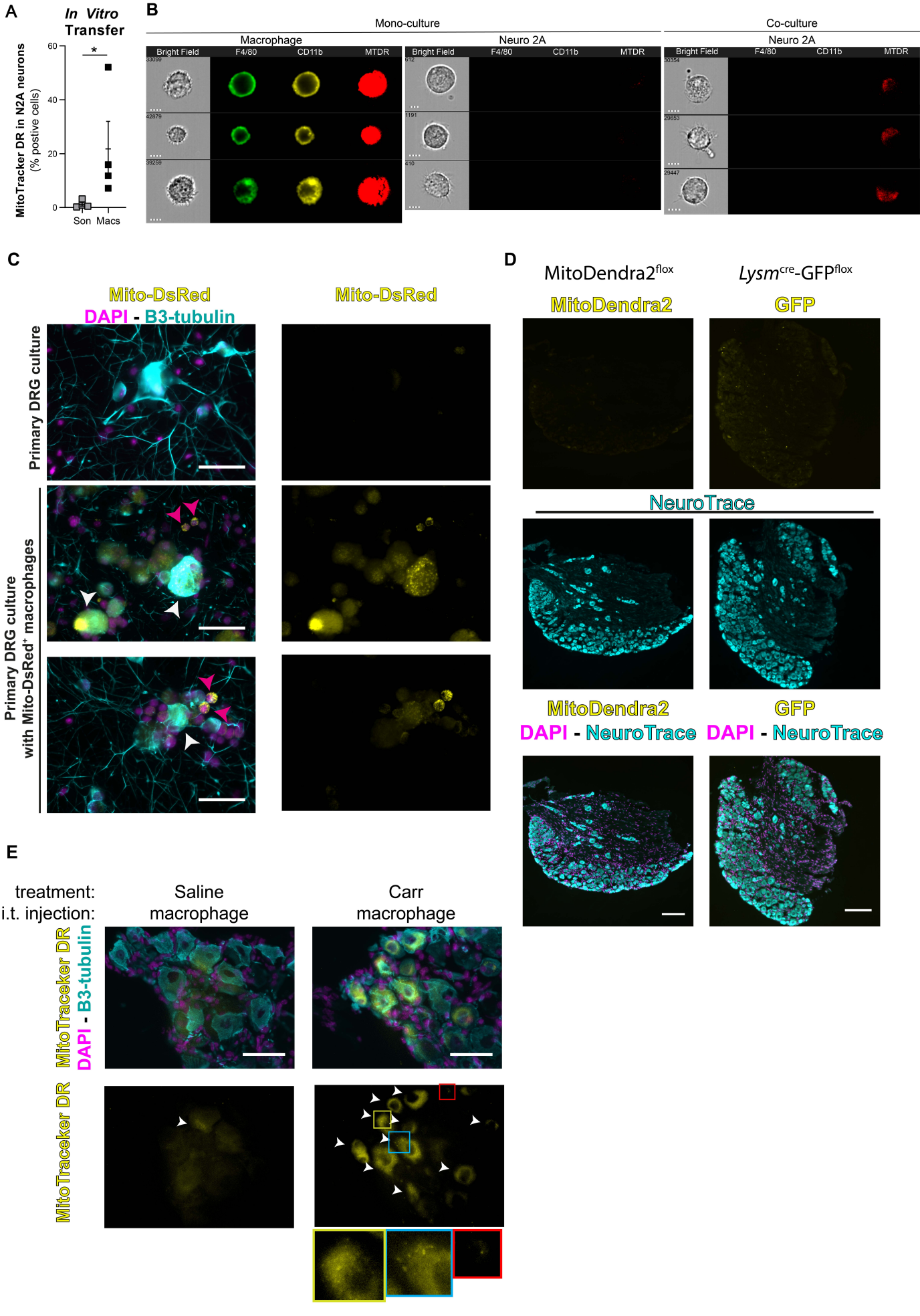

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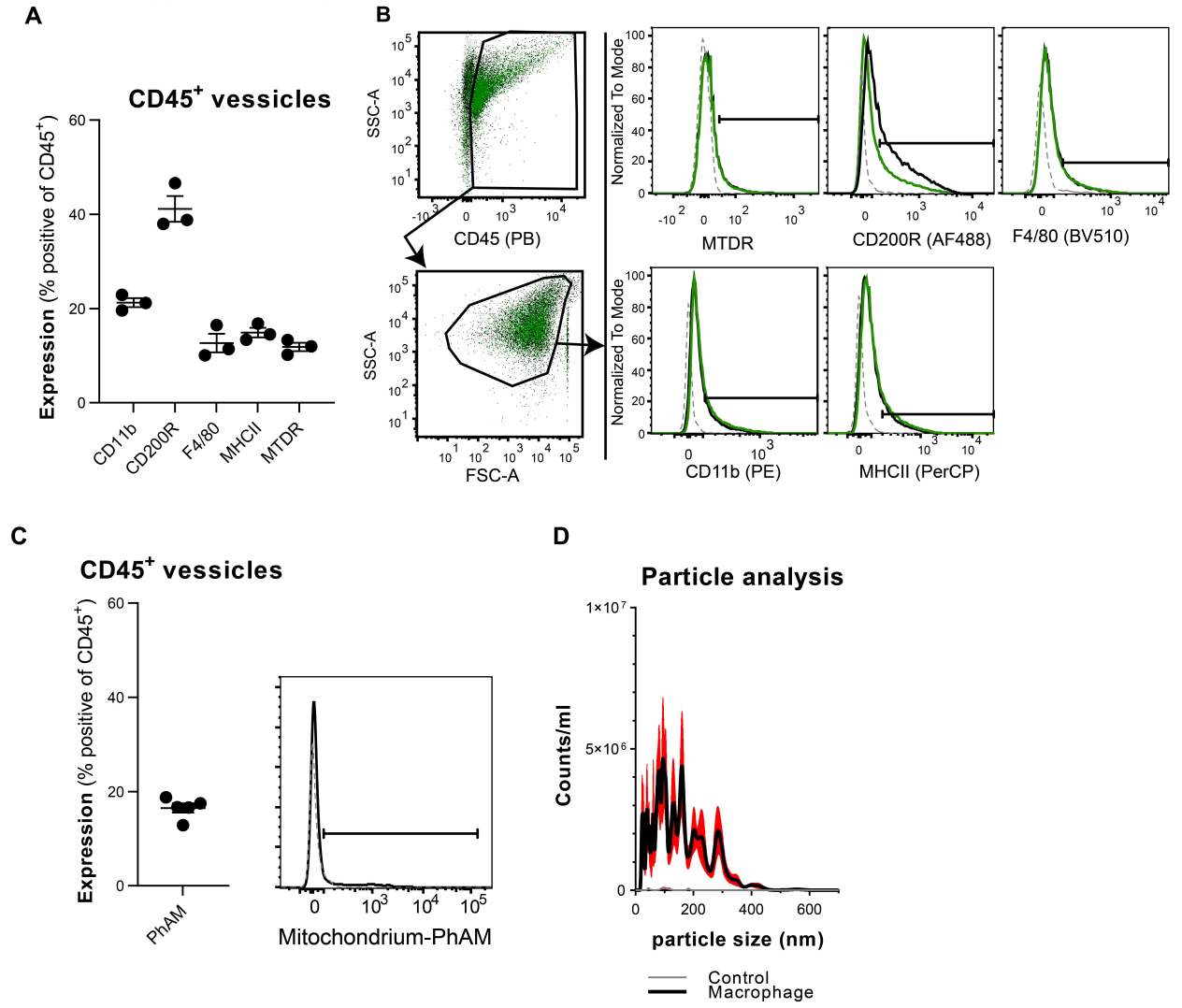

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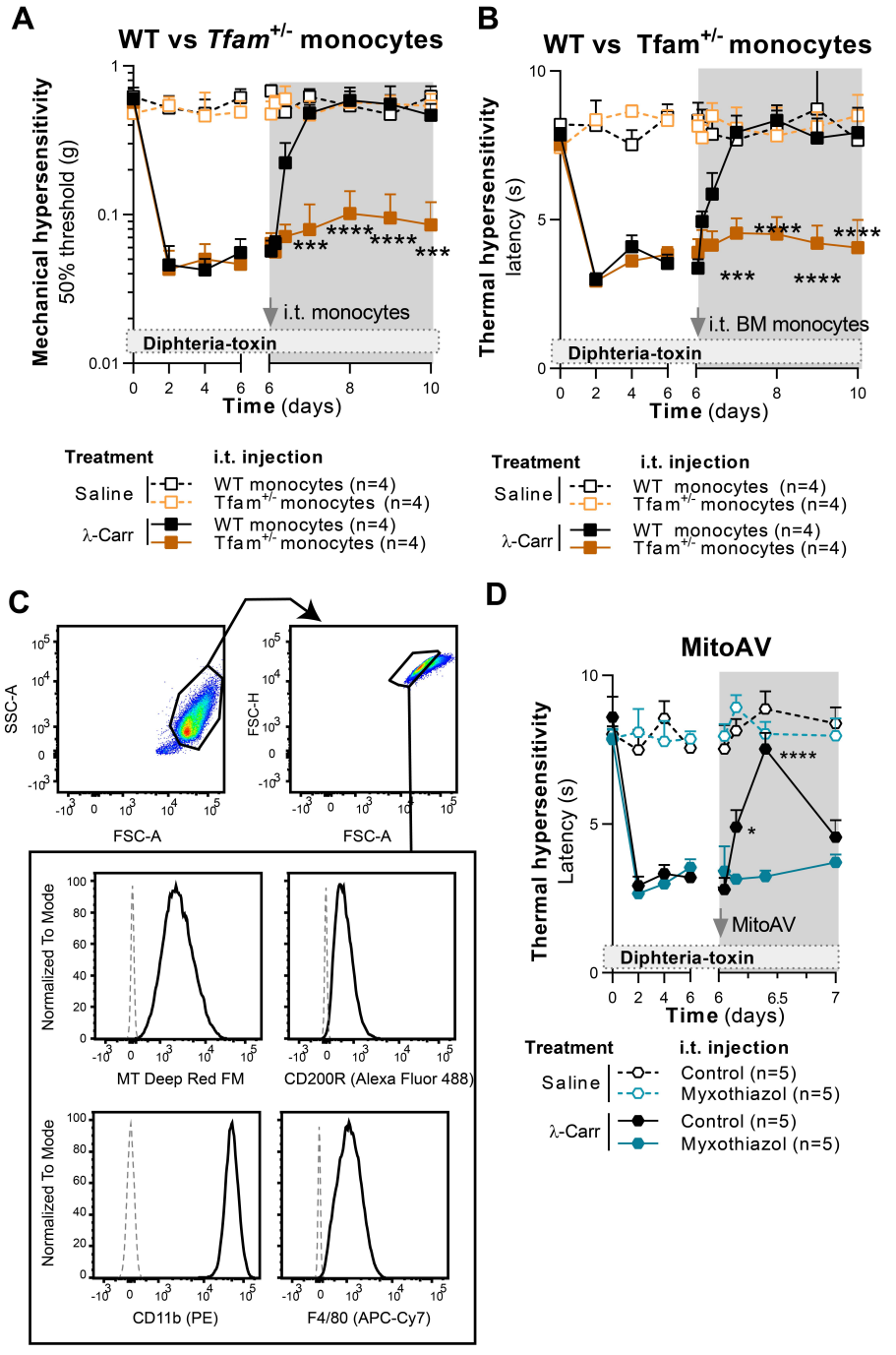

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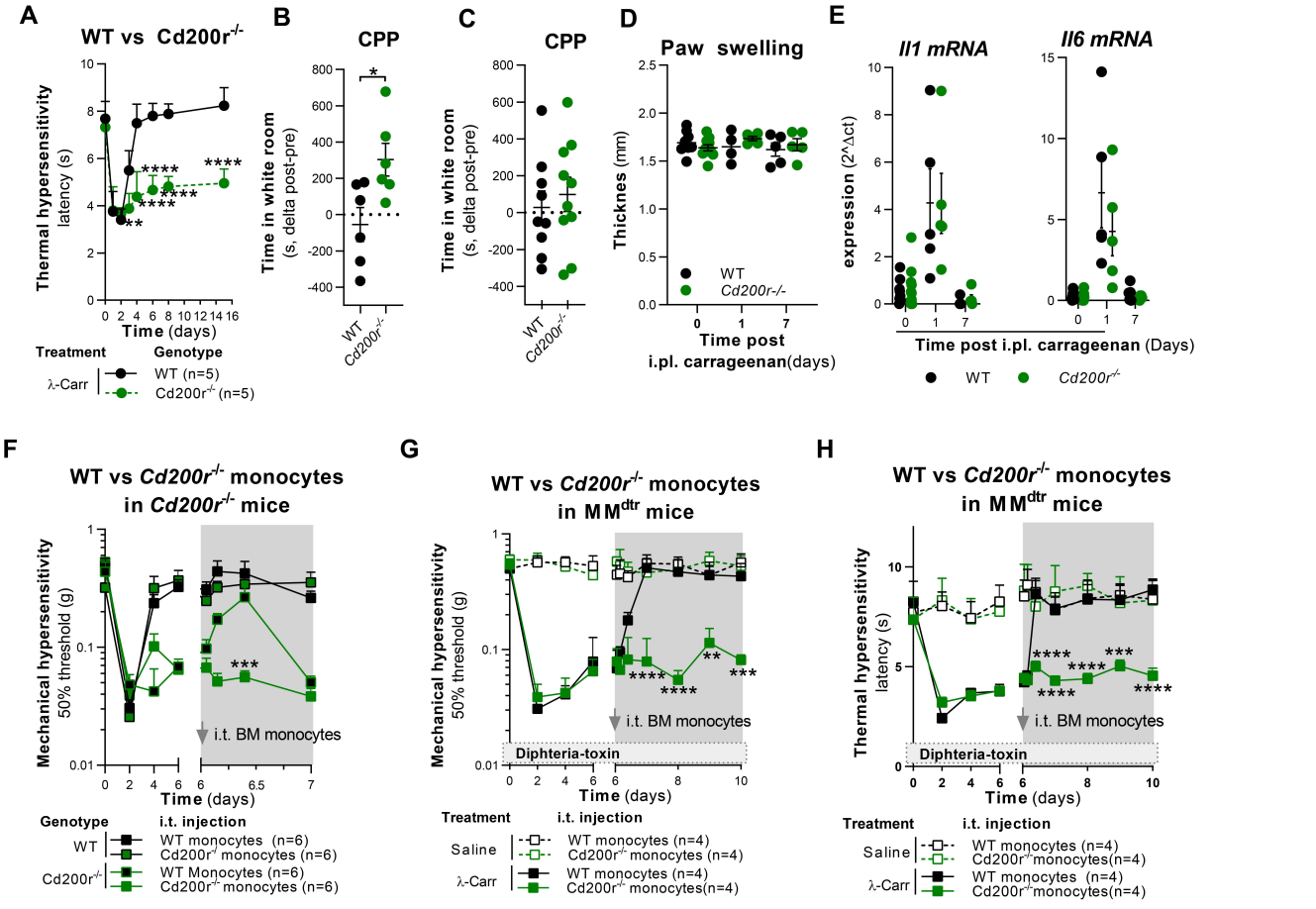

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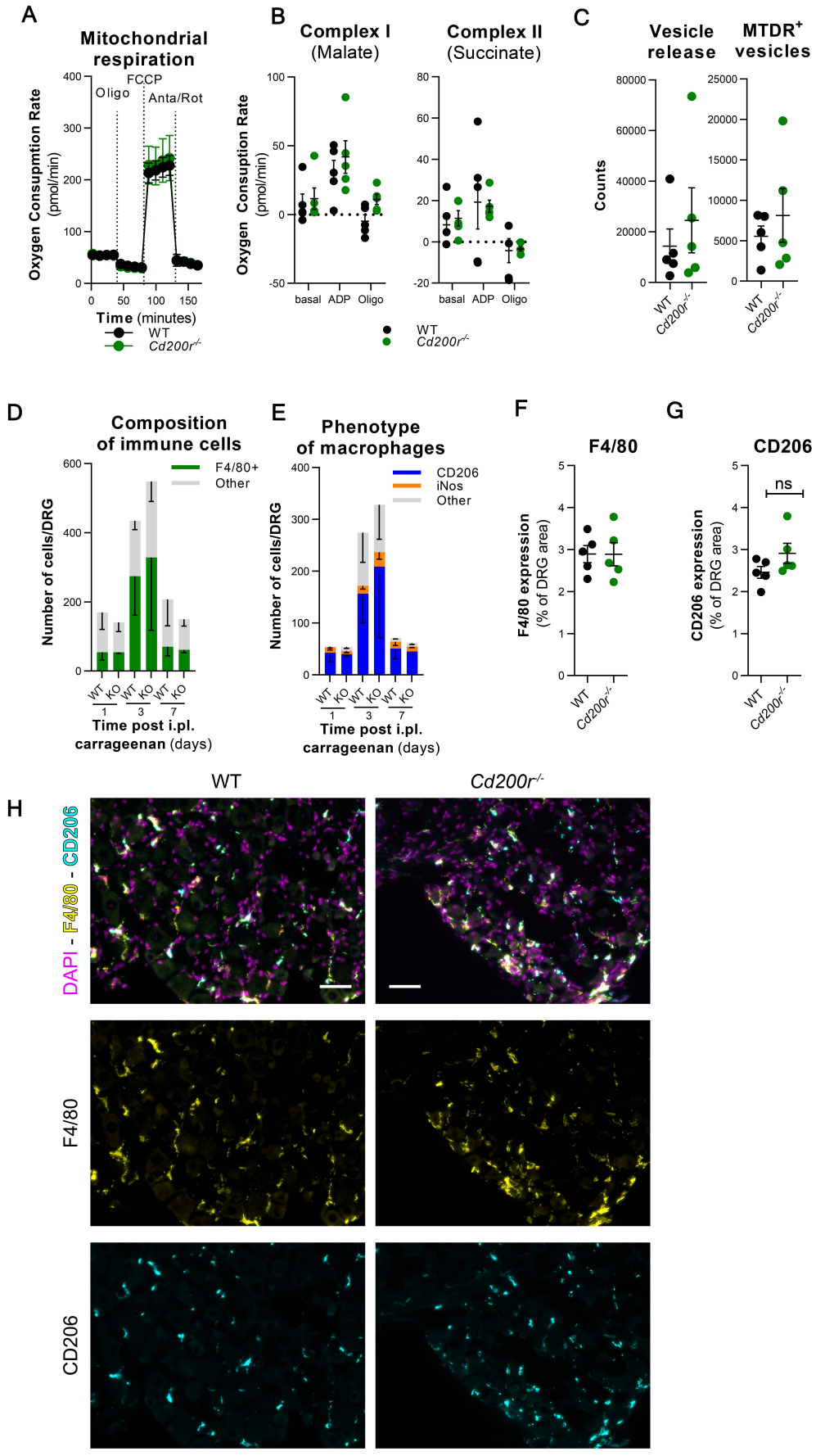

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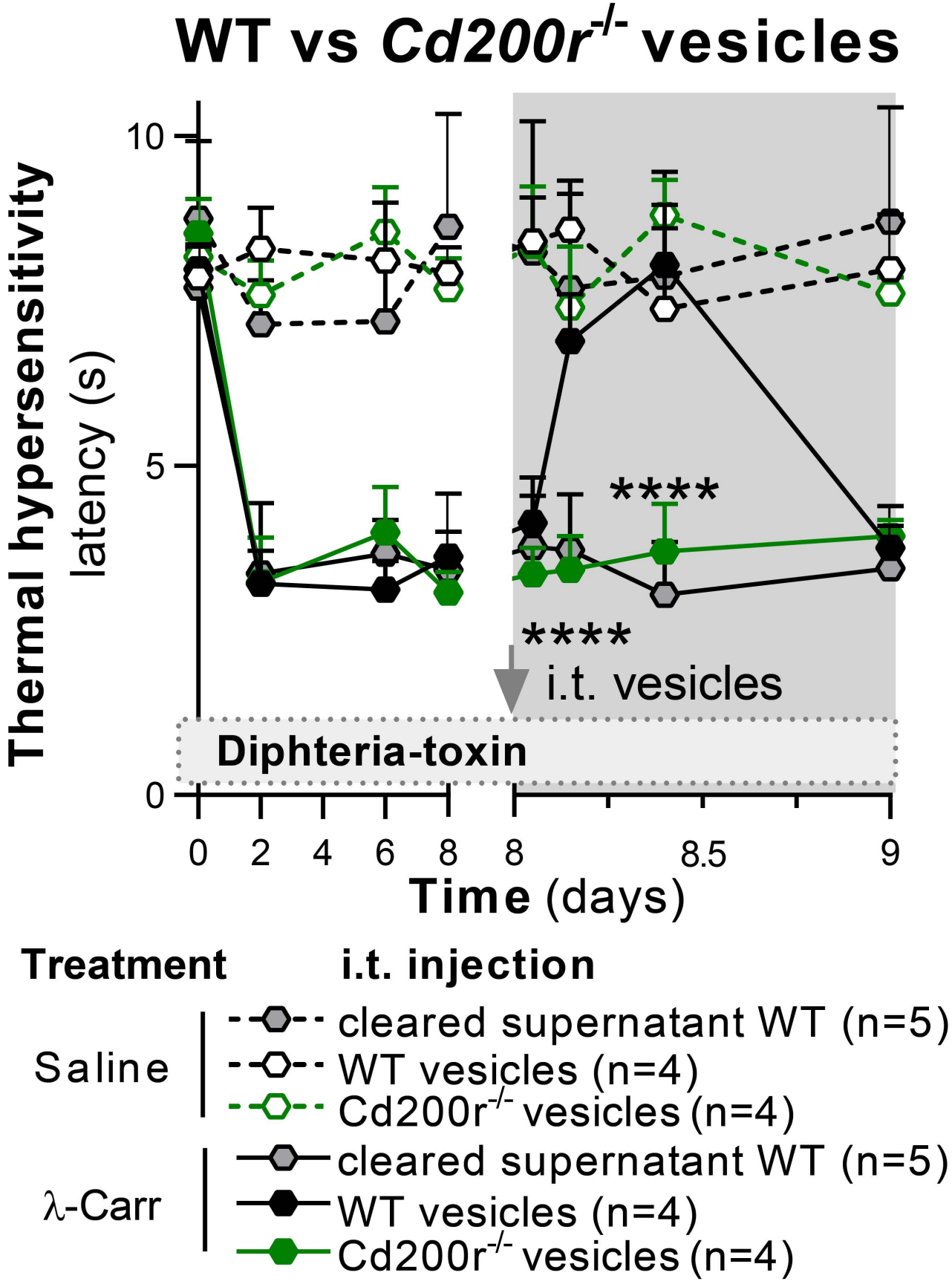

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