Supplementary Material

Materials and Methods

Materials

The production and characterization of the TNFR2 agonist EHD2-sc-mTNF$_{R2}$ was described previously **(1)**. The *InVivo*Mab anti-mouse CD25 antibody (PC-61) and the control IgG were purchased from BioXCell (West Lebanon, NH). Fluorescence-labeled antibodies against CD4, CD8, CD11b, CD11c, CD115, CD120b (TNFR2), CD25, CD45R (B220), CD49c, Ly6G, Ly6C, iNos, MHC class II, and FoxP3 were from Miltenyi Biotech (Bergisch-Gladbach, Germany), against Arg1 from eBioscience (San Diego, CA) and against CD45 from Biolegend (San Diego, CA). Other antibodies included anti-NF200 (clone N52) from Sigma Aldrich (Burlington, MA), anti-GAP43 and anti-proBDNF from Novus Biologicals (Centennial, CO), anti-BDNF from Abcam (Cambridge, MA), anti-CD68 (clone: E-11) from Santa Cruz (Dallas, TX), and anti-β-tubulin, anti-CGRP from Cell Signaling Technology (Danvers, MA). All other chemicals were of analytical grade.

Animals

Male and female mice were used for this study. Results show the combined data of male and female mice. Equal number of male and female mice were used for the biochemical and immunological studies. TNFR2 null mice, carrying the Tnfrsf1b^{tm1Mwm} mutation (TNFR2$^{-/-}$) and wild type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a virus/antigen free facility with a 12-hour light/dark cycle and unlimited access to water and food. All animal experiments were performed according to guidelines of the Institutional Animal Care and Use Committee (IACUC) of Drexel University under protocol numbers 20295/ 20628. All studies were conducted in accordance with the United States Public Health Service's

Policy on Humane Care and Use of Laboratory Animals. Data show combined results obtained in male and female mice.

Chronic constriction injury

Chronic constriction injury was performed as previously described **(2)**. Briefly, mice were anesthetized via intraperitoneal (i.p.) injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail. Then, the right sciatic nerve was exposed at the level of the mid-thigh and 3 loose silk sutures (MV-711, Oasis, Mettawa, IL) were tied around the sciatic nerve with a 1.0-1.5 mm interval between each ligature. The wound was closed with 6-0 Ethilon monofilament nylon. Sham operated animals underwent a similar procedure with exposure of the sciatic nerve without ligation. Mice then were randomly divided into treatment groups. Treatments with control IgG or PC-61 (500 µg per injection) and/or saline and EHD2-sc-mTNF$_{R2}$ (10 mg/kg per injection) were performed according to the description in the figure legends. Upon sacrifice, an incision was applied at the level of the right thigh and the right sciatic nerve was exposed to verify that the ligatures were still intact. All behavioral analyses were performed by experimenters blinded to the surgery and the genotype or treatment. Mice were handled daily for at least 1 week prior to any behavioral analysis.

Mechanical allodynia

Mechanical allodynia was determined using the Von Frey test as described previously with standardized Von Frey filaments ranging from 0.02 to 2 g **(2)**. To evaluate tactile sensitivity each mouse was placed individually on a wire mesh beneath a transparent plastic jar and habituated for 30 min before starting the behavioral test. Von Frey filaments (Touch-Test Sensory Evaluator) were applied to the plantar surface of each hind paw in a series of ascending forces (ranging between 0.02 g and 2 g) starting from the 0.4 g filament. A single trial of stimuli consisted of 5 applications of Von Frey filaments every minute perpendicularly to the plantar surface of the paw

for about 4-5 s. To qualify a response to tactile stimulation as being painful, the mouse had to show cognitive awareness of pain. Therefore, at least one of the following cognitive responses had to coincide with a withdrawal response to a “painful” stimulation before it was considered a painful response and not a reflexive reaction: 1) avoidance behavior, 2) protecting the injured paw and 3) licking and/or looking at the injured paw. Depending on the positive or negative response, subsequent filaments were applied in order of descending or ascending intensity, respectively. If paw withdrawal occurred in response to the minimum 0.02 g filament, the next trial was performed with the same 0.02 g, cut-off filament. In case the maximum 2 g filament was reached with no response, the next trial was performed with the same 2 g, cut-off filament. Each test consisted of 6 trials. Paw movements associated with locomotion or weight-shifting were not counted as a response. The withdrawal threshold was expressed as the tolerance level in grams.

Thermal Hyperalgesia

Thermal sensitivity was assessed using a plantar test apparatus (UGOBASILE) as described previously **(2)**. Each animal was placed beneath an inverted transparent plastic cage on a thin glass platform and habituated for at least 30 min before starting the test. The hind paws were in contact with a 1/4-inch-thick glass maintained at room temperature. An infrared (IR) thermal radiant stimulus was applied to the plantar surface of the hind paws and the latency of the paw withdrawal response was measured automatically with a photoelectric-sensitive device. The IR intensity was set 30, which produced a baseline paw withdrawal latency of about 16 s in naïve animals. The mean latency of withdrawal response of each hind paw was determined by the average of 6 measurements per paw at each time point. A cut off of 20 s was used. The paw withdrawal latency was determined alternating between the left and right hind paw, with a 5-min inter-trial interval. Only quick hind paw withdrawal movements from the heat light were considered as a response.

Cold allodynia

Cold allodynia was assessed using the acetone test. Briefly, mouse was placed individually on a wire mesh beneath a transparent plastic jar and habituated for 30 min before starting the behavioral test. Acetone (50 µl) was gently applied onto the plantar surface of the hind paw. The brisk foot withdrawal response after the acetone spray was considered as a positive response. Responses were graded to a four-point scale: 0, no response; 1, brisk withdrawal or flick of the paw; 2, repeated flicking of the paw; 3, repeated flicking of the hind paw and licking of the paw according to a previous description **(3)**. The acetone spray was applied at least 4 times with an interval of 5 min between each application.

Flow cytometry Spleen

Mice were sacrificed at the times indicated in the figure legends, spleens were extracted, dissociated through a 40 µm cell strainer and collected in 10 ml MACS buffer (PBS, 0.5% BSA, 2 mM EDTA). Splenocytes were harvested by centrifugation (300 *g*, 5 min) and washed once with 10 ml MACS buffer. Then, expression of different immune cell markers was investigated by flow cytometry. Therefore, approximately 10^6 splenocytes per staining were incubated for 20 min with fluorescence-labeled antibodies against surface antigens. Then cells were washed with MACS buffer by centrifugation (300 *g*, 5 min) and incubated for 30 minutes in fixation/permeabilization buffer (Miltenyi Biotech, Bergisch-Gladbach). Cells were washed with MACS buffer by centrifugation (300 *g*, 5 min), followed by another washing step with permeabilization buffer (Miltenyi Biotech, 300 *g*, 5 min) and incubation with fluorescence-labeled antibodies against intracellular antigens for 30 minutes. Cells were washed in permeabilization buffer (300 *g*, 5 min) and collected in MACS buffer. Data were acquired using a BD FACS Canto (BD Biosciences, San Jose, CA) and analyzed with FlowJo (TreeStar, Ashland, OR). Gating strategy is shown in **Fig S15**.

Flow cytometry Spinal cord

Mice were sacrificed at the times indicated in the figure legends, lumbar spinal cords were extracted, dissociated through a 70 µm cell strainer and collected in 10 ml MACS buffer (PBS, 0.5% BSA, 2 mM EDTA). Cells were harvested by centrifugation (300 *g*, 10 min) and incubated together with Myelin Removal Beads II (Miltenyi Biotech) for 15 minutes at 4°C. Cell/bead mixture was then washed with MACS buffer by centrifugation (300 *g*, 10 min) and myelin was removed using magnetic separation with LD columns (Miltenyi Biotech). Eluted cells were washed once in MACS buffer and surface antigens were stained using fluorescence-labeled antibodies (Miltenyi Biotech) for 20 minutes. Then cells were washed with MACS buffer by centrifugation (300 *g*, 5 min) and incubated for 30 minutes in fixation/permeabilization buffer (Miltenyi Biotech, Bergisch-Gladbach). Cells were washed with MACS buffer by centrifugation (300 *g*, 5 min), followed by another washing step with permeabilization buffer (Miltenyi Biotech, 300 *g*, 5 min) and incubation with fluorescence-labeled antibodies against intracellular antigens for 30 minutes. Cells were washed in permeabilization buffer (300 *g*, 5 min) and collected in MACS buffer. Data were acquired using a BD FACS Canto (BD Biosciences, San Jose, CA) and analyzed with FlowJo (TreeStar, Ashland, OR).

Western Blot

Mice were sacrificed at the times indicated in the figure legends and perfused with PBS. The lumbar spinal cord and hippocampus were dissected and homogenized in RIPA lysis buffer (0.01 M sodium Phosphate pH 7.2, 0.15 M NaCl, 1% Nonidet-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) with protease and phosphatase inhibitors. Lysates were incubated for 30 min on a rocker at 4°C, centrifuged at 16,000 *g* for 15 min at 4°C and protein concentration of supernatants was determined using the colorimetric DC Protein Assay (Biorad, Hercules, CA). For each sample, 12.5 µg total protein were denatured in Laemmli buffer and resolved by SDS-PAGE. Then, proteins were transferred onto nitrocellulose membranes (Trans-Blot Turbo Blotting

System, Bio-Rad) and nonspecific protein binding was blocked with 5% skim milk powder solution in PBS/0.1% Tween 20 (TBST) for 60 min at RT. Membranes were incubated overnight at 4°C using specific antibodies. After incubation with HRP-conjugated secondary antibodies for 60 min at RT, the signals were detected by enhanced chemiluminescence (West Pico PLUS; Thermo Scientific). Signals were quantified using scanned densitometric analysis and normalized to a housekeeping gene.

RNA Isolation, cDNA Synthesis

Mice were sacrificed two weeks after CCI, perfused with PBS and the lumbar spinal cord was dissected. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions (Thermo Fisher Scientific). The A260/A280 ratio of the RNA was determined using a UV spectrophotometer (Nanodrop, Thermo Fisher Scientific). Equal concentrations of RNA samples with an A260/A280 ratio ranging from 1.8 to 2.0 were used for cDNA synthesis. RNA was retro-transcribed into cDNA with the AffinityScript QPCR cDNA Synthesis Kit and oligo(dT) primers (Agilent, Santa Clara, CA). The cDNA was preserved at -80°C until further use.

Quantitative real-time PCR

Gene expression was quantified by real-time PCR (qPCR; Rotor-Gene Q, Qiagen) using specific primers (ThermoFisher, **Table 1**) and the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent). To determine the expression of distinct genes ΔΔCt values were determined by correlating the obtained values to the house-keeping gene GAPDH. Data are presented relative to saline controls as normalized fold expression.

Immunofluorescence analysis

Mice were transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The sciatic nerves were extracted and post-fixed in 4% PFA overnight followed by two days of 20% sucrose solution in 0.1 M phosphate buffer, embedded in Tissue-Tek O.C.T compund (Sakure Finetek, Torrance, CA) and stored at -80°C until further investigation. 10 µm thick cryosections were obtained using a cryostat (Leica Biosystems, Wetzlar, Germany). Sections were permeabilized with PBS/0.3% Triton X-100 for 10 min at RT and unspecific binding sites were blocked with PBS/4% BSA for 30 min. Then sections were incubated with antibodies against CGRP (Cell Signaling Technologies), CD3 (BD) or FoxP3 (eBioscience), followed by incubation with fluorescence-labeled secondary antibodies for 1 h. After staining the nuclei with Hoechst, the sections were mounted with Fluoro Gel (Electron Microscopy Science). The stained sections were analyzed by wide-field fluorescence microscopy with a 20x objective (AxioObserver Carl Zeiss). All image acquisition and analyses was performed blind to sex and experimental condition. Area fraction (CGRP) and number of CD3 or FoxP3 positive cells was quantified using ImageJ Fiji **(4, 5)**. For all analysis, immunostained cells in each section from injured nerves were counted at the site of injury, distal and proximal to it (about 3 mm from the injury site). For each group, four serial sections were counted and averaged.

EHD2-sc-mTNF$_{R2}$-specific ELISA

ELISA plates (Nunc MaxiSorp) were coated with polyclonal goat anti-Human IgE (Novus Biologicals, NB7456) at 1 µg/ml in PBS and incubated at 4°C overnight. Residual binding sites were blocked with 2% skim milk powder in PBS at RT for 2 hours. Tissue homogenates were diluted in 2% skim milk powder in PBS and incubated for 1 hour at RT. Purified EHD2-sc-mTNF$_{R2}$ was diluted in 2% skim milk powder and used as a standard. Bound EHD2-sc-mTNF$_{R2}$ was detected with rabbit polyclonal antibodies to mouse TNF (HP8001; 1 µg/ml; incubation for 1 hour at RT) and HRP-conjugated anti-rabbit IgG antibodies (diluted 1:10000; incubation for 1 hour at RT), followed by incubation with TMB substrate solution. Reaction was stopped by addition of 1

M H_2SO_4 and the absorbance at 450 nm was determined with an absorbance reader (Multiskan FC, Thermo Scientific, Karlsruhe, Germany) and data were analyzed using the software Microsoft Excel and GraphPad Prism (GraphPad, La Jolla, CA). Between each step, non-bound proteins were removed by washing 4 times with 0,005% Tween-20 in PBS.

Statistics

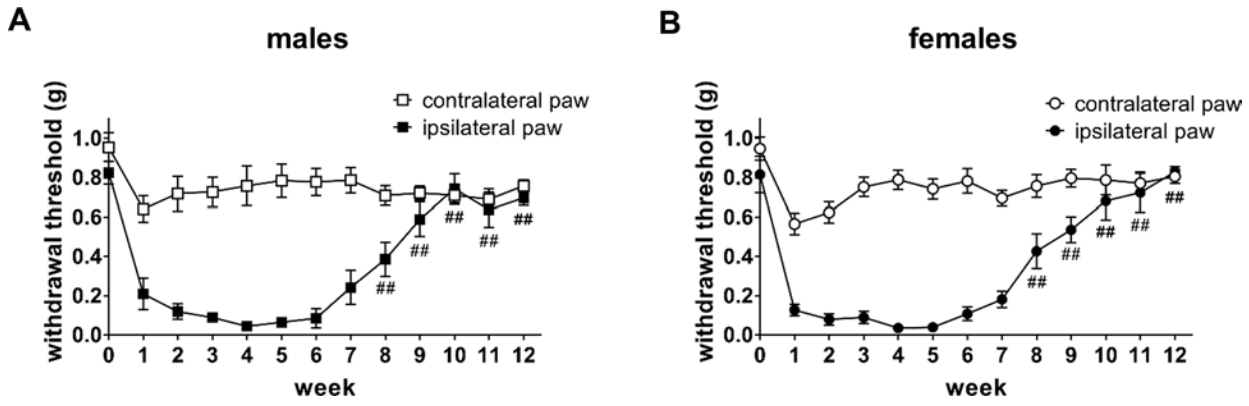
Data are presented as mean ± standard error of the mean (SEM) of *n* animals. Normal distribution was analyzed by Shapiro-Wilk normality test. Statistical analyses were performed by Student's t-test or analysis of variance, followed by a post hoc Tukey's range test. Correlation analysis was performed using the Pearson test. For correlations between allodynia and Treg percentage, the individual data of each mouse were used. A value of $p < 0.05$ was considered statistically significant.

Supplementary Data

Supplementary Table 1

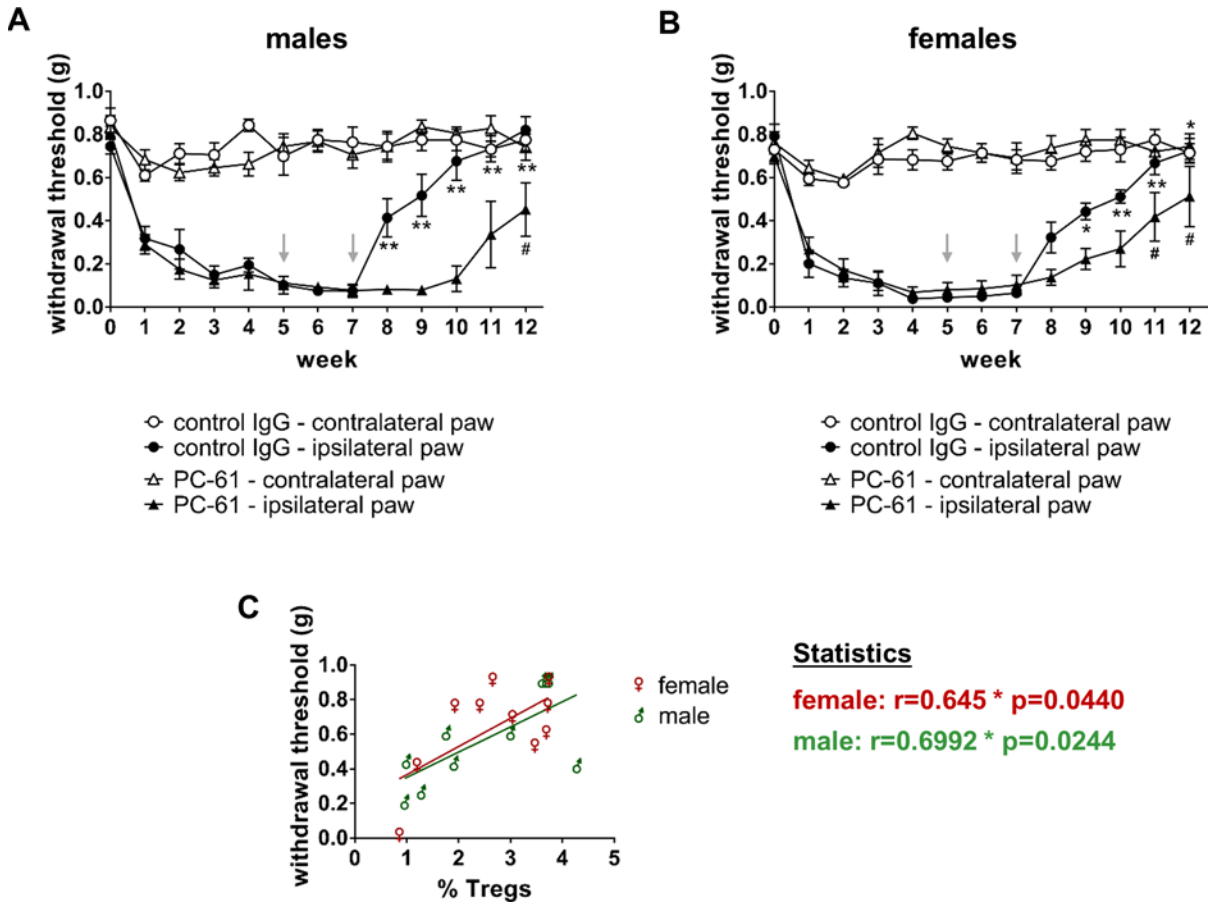
Gene	forward	reverse
FOXP3	5’-GCCATGGCAATAGTTCCTTC-3’	5’-CGAACATGCGAGTAAACCAA-3’
CD4	5’-CTGCTTCAGGGTCAGTCTCA-3’	5’-GGCAACCTGACTCTGACTCT-3’
CD8A	5’-GCCCCAGAGACCAGAAGATT-3’	5’-ATCAAGGACAGCAGAAGGGC-3’
NOS2	5’-GGAAGAAATGCAGGAGATGG-3’	5’-TGCAGGATGTCCTGAACGTA-3’
TNF	5’-CACTTGGTGGTTTGCTACGA-3’	5’-CCCCAAAGGGATGAGAAGTT-3’
ARG1	5’-TCTCTTCCATCACCTTGCCA-3’	5’-GGTGACTCCCTGCATATCTG-3’
MRC1	5’-TTGTGGAGCAGATGGAAGGT-3’	5’-TCGTAGTCAGTGGTGGTTCC-3’
IL10	5’-AGAGAGCTCCATCATGCCTG-3’	5’-TGGGAAGTGGGTGCAGTTAT-3’
CXCL2	5’-AGACTCCAGCCACACTTCAG-3’	5’-GCATTGACAGCGCAGTTCA-3’
CCL2	5’-CCCAATGAGTAGGCTGGAGA-3’	5’-TCTGGACCCATTCCTTCTTG-3’
CXCL1	5’-CTTGACCCTGAAGCTCCCTT-3’	5’-GTTGTCAGAAGCCAGCGTTC-3’
IL6	5’-ACAAAGAAATGATGGATGCTACC-3’	5’-TTCATGTACTCCAGGTAGCTATG-3’
IL1A	5’-GGGCTGGTCTTCTCCTTGAG-3’	5’-ATGAAGCTCGTCAGGCAGAA-3’
CCL3	5’-CTGCCTCCAAGACTCTCAGG-3’	5’-AGATTCCACGCCAATTCATC-3’
PTGS2	5’-CGTGGGGAATGTATGAGCAC-3’	5’-GTAGTGCACTGTGTTTGGGG-3’
ICAM1	5’-CACGCTACCTCTGCTCCTG-3’	5’-AAGGCTTCTCTGGGATGGAT-3’
IFNG	5’-GCGTCATTGAATCACACCTG-3’	5’-CTGGACCTGTGGGTTGTTG-3’
CCL7	5’-ATCTCTGCCACGCTTCTGT-3’	5’-TAACAGCTTCCCAGGGACAC-3’
MPO	5’-GCCCGCATTCCTTGTTTTCT-3’	5’-GACCATGGCCCCTACAATCT-3’
BDNF	5’-GGCCCAACGAAGAAAACCAT-3’	5’-GTTTGCGGCATCCAGGTAAT-3’
NGF	5’-CCTGAAGCCCACTGGACTAA-3’	5’-TACAGTGATGTTGCGGGTCT-3’
CSF1	5’-GGGTGTTGTCTTTAAAGCGC-3’	5’-GACTTCATGCCAGATTGCCT-3’
IL4	5’-GGACTCATTCATGGTGCAGC-3’	5’-GTCTGTAGGGCTTCCAAGGT-3’
CXCL2	5’-AGACTCCAGCCACACTTCAG-3’	5’-GCATTGACAGCGCAGTTCA-3’
IL1B	5’-GGAGCTCCTTAACATGCCCT-3’	5’-CCTCACAAGCAGAGCACAAG-3’

Supplementary Figure S1



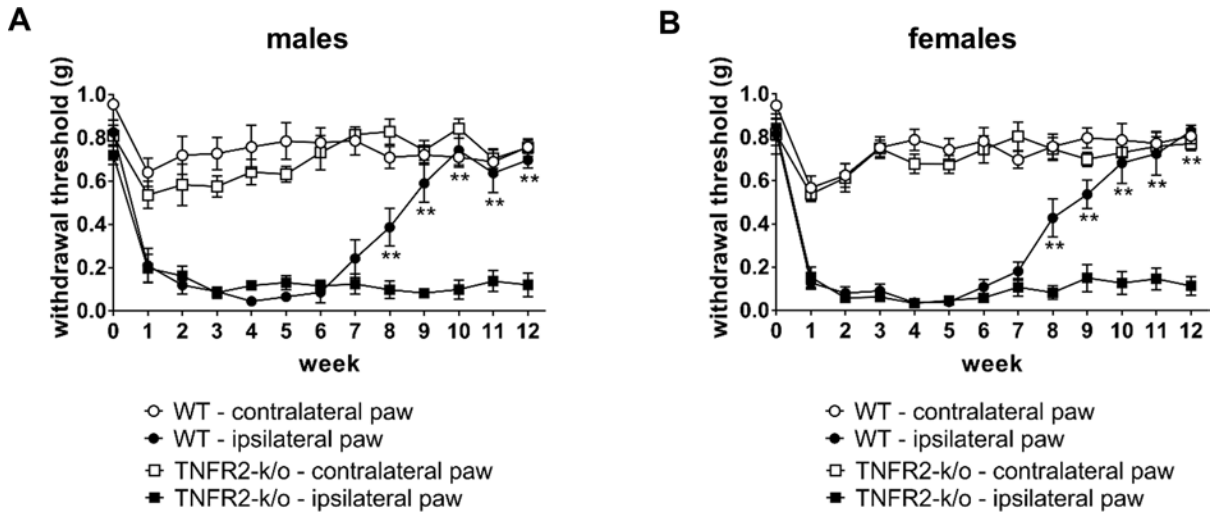
Supplementary Figure 1. (A) Male and (B) female wild type (WT) mice underwent CCI at week 0. Mechanical allodynia was determined over a period of 12 weeks after injury (n=6 ±SEM per group/sex). ## p<0.01 week 6–10 vs week 5 ipsilateral paw

Supplementary Figure S2



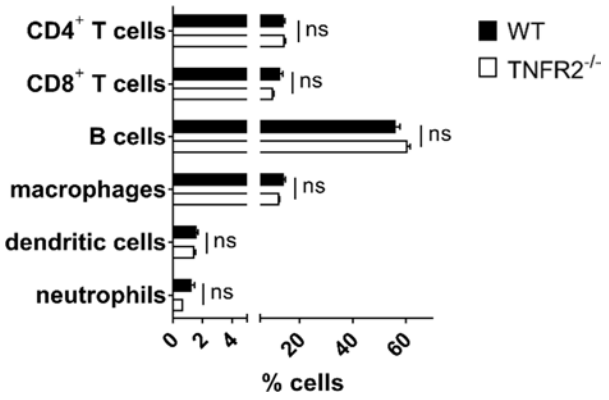
Supplementary Figure 2. (A) Male and (B) female wild type mice underwent CCI at week 0. Mechanical allodynia was determined using Von Frey test over a period of 12 weeks after injury. 5 weeks and 7 weeks after injury mice were injected with control IgG or PC-61 (500 µg per injection each, i.p., gray arrows) to deplete Tregs (n=5 each group/sex). * $p<0.05$, ** $p<0.01$ control IgG vs PC-61 ipsilateral paw, # $p<0.05$ week 6–10 vs week 5 PC-61 ipsilateral paw. (C) Correlation of the percentage of splenic Tregs with the withdrawal threshold at 12 weeks after injury separated for male and female mice from both treatment groups.

Supplementary Figure S3



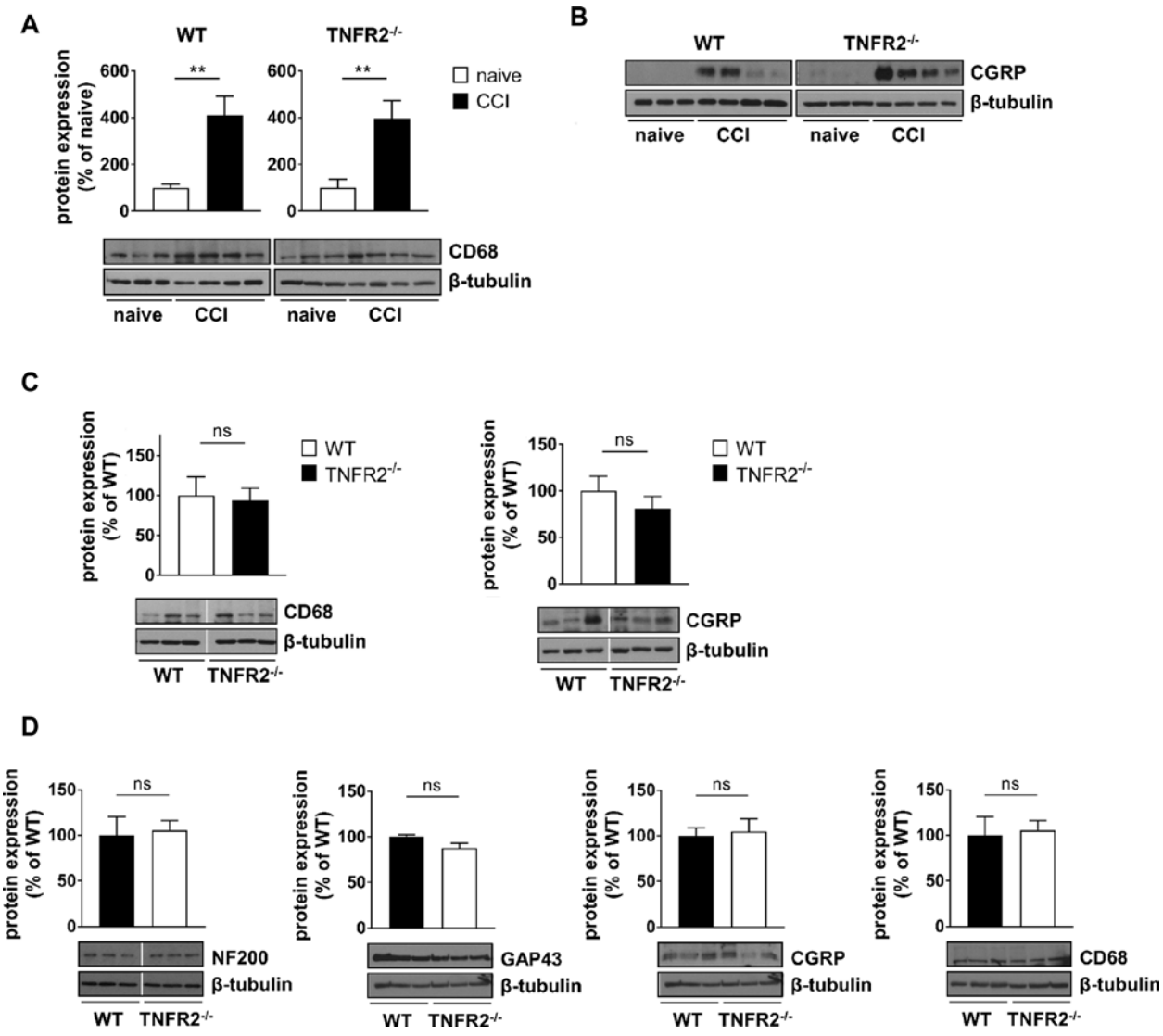
Supplementary Figure 3. (A) Male and (B) female wild type (WT) and TNFR2$^{-/-}$ mice underwent CCI at week 0. Mechanical allodynia was determined using Von Frey test over a period of 12 weeks after injury (n=6 each group/sex) ** $p<0.01$ WT vs TNFR2$^{-/-}$ ipsilateral paw

Supplementary Figure S4



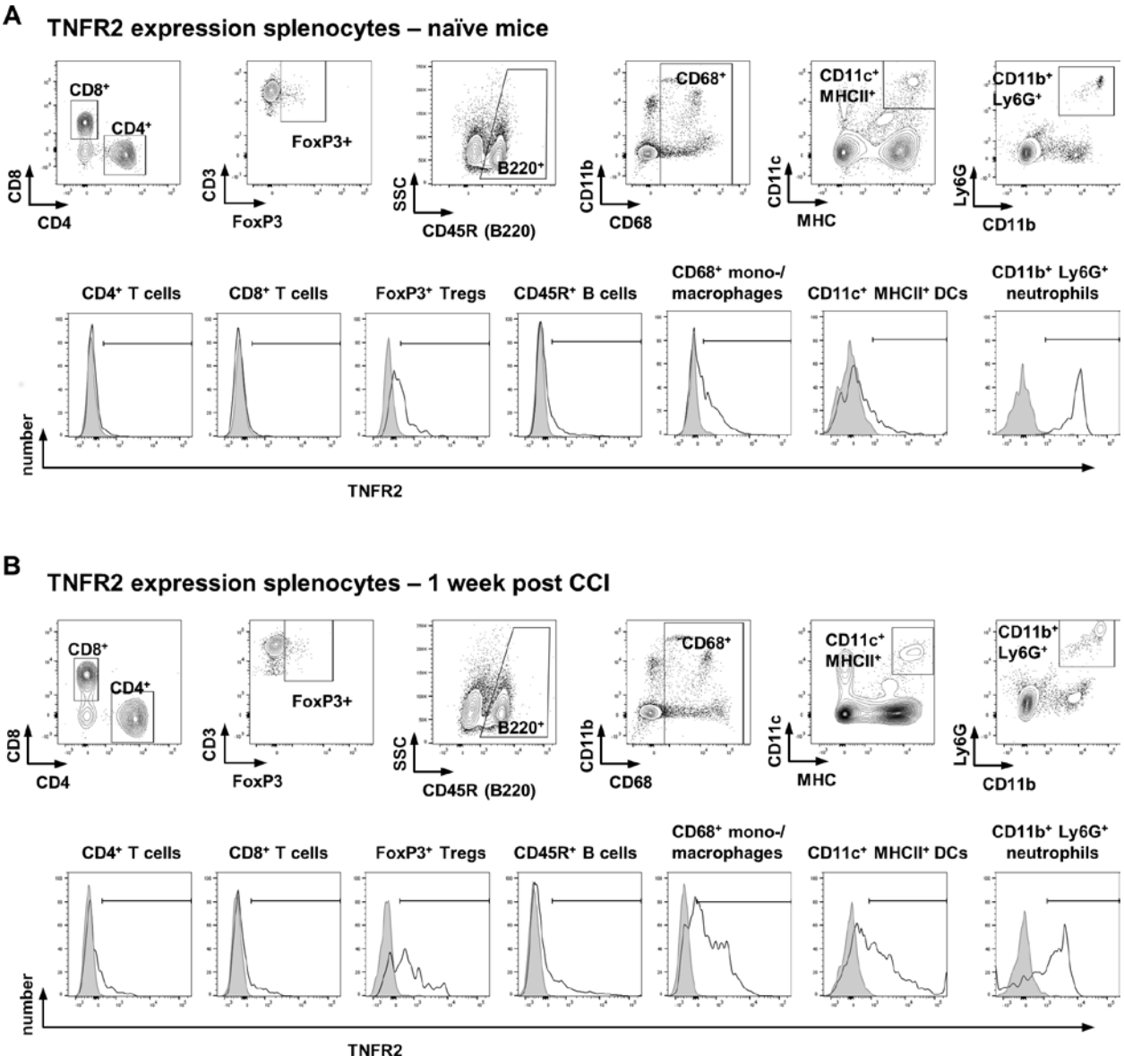
Supplementary Figure 4. Percentage of splenic immune cell subsets in naïve uninjured wild type (WT) and TNFR2$^{-/-}$ mice was quantified by flow cytometry (mean ±SEM, WT: n=16, TNFR2$^{-/-}$: n=6). ns = not significant

Supplementary Figure S5



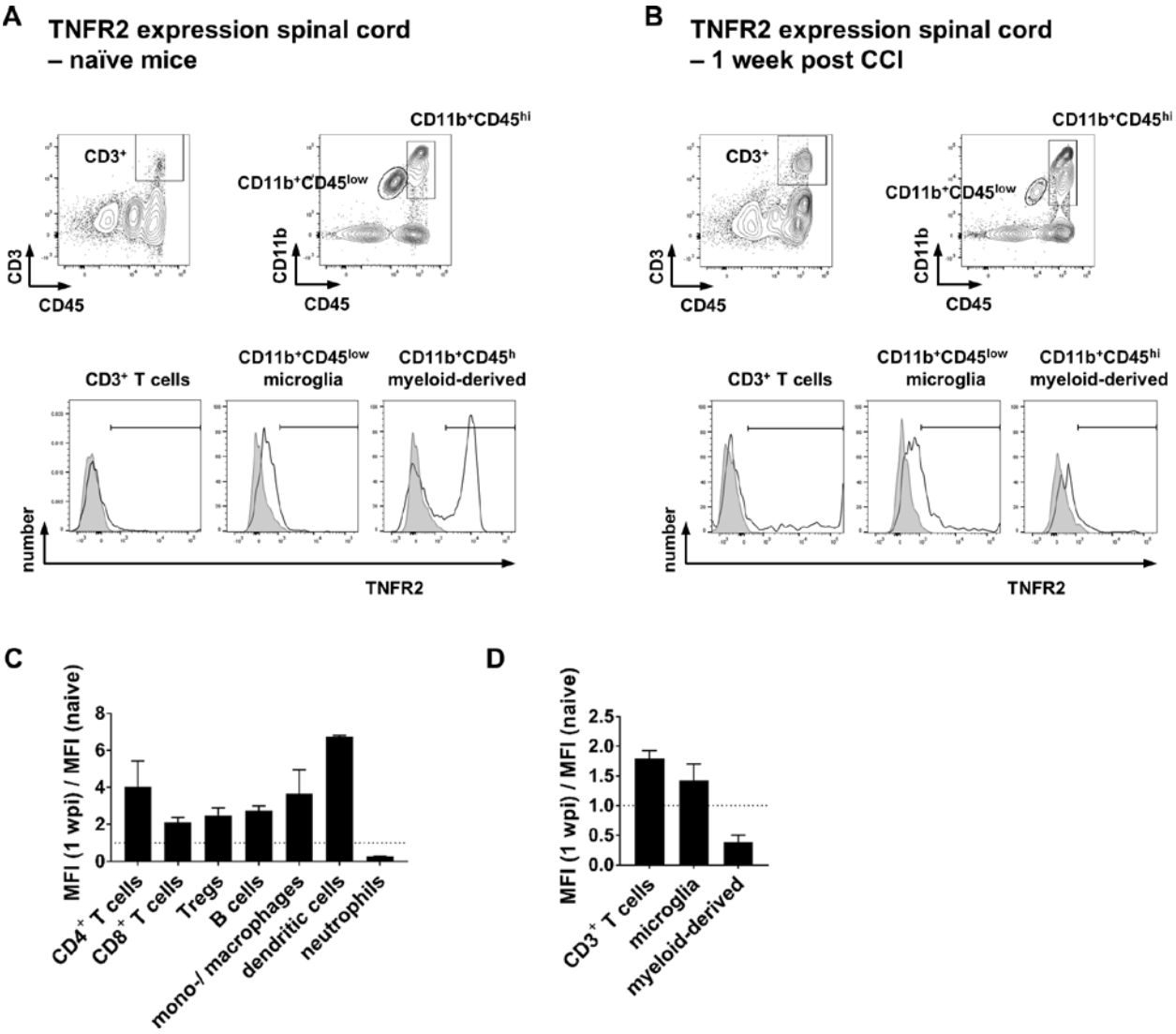
Supplementary Figure 5. Protein expression in the spinal cord of (A-C) wild type (WT) and TNFR2$^{-/-}$ mice was quantified by Western Blot. (A,B) Shown is the comparison of samples from naïve mice vs mice 12 weeks after injury (CCI), (C) the comparison of WT vs TNFR2$^{-/-}$ mice 12 weeks post-CCI or (D) the comparison of naïve uninjured WT vs TNFR2$^{-/-}$ mice. Shown are representative blots and the mean ±SEM of optical density of each protein normalized to β-tubulin, percentage of protein expression of (A,B) naive and (C,D) WT (A,B: n=6 naïve, n=8 CCI; C: n=8 WT, n=10 TNFR2$^{-/-}$, D: n=6/group). ** p<0.01, ns = not significant

Supplementary Figure S6



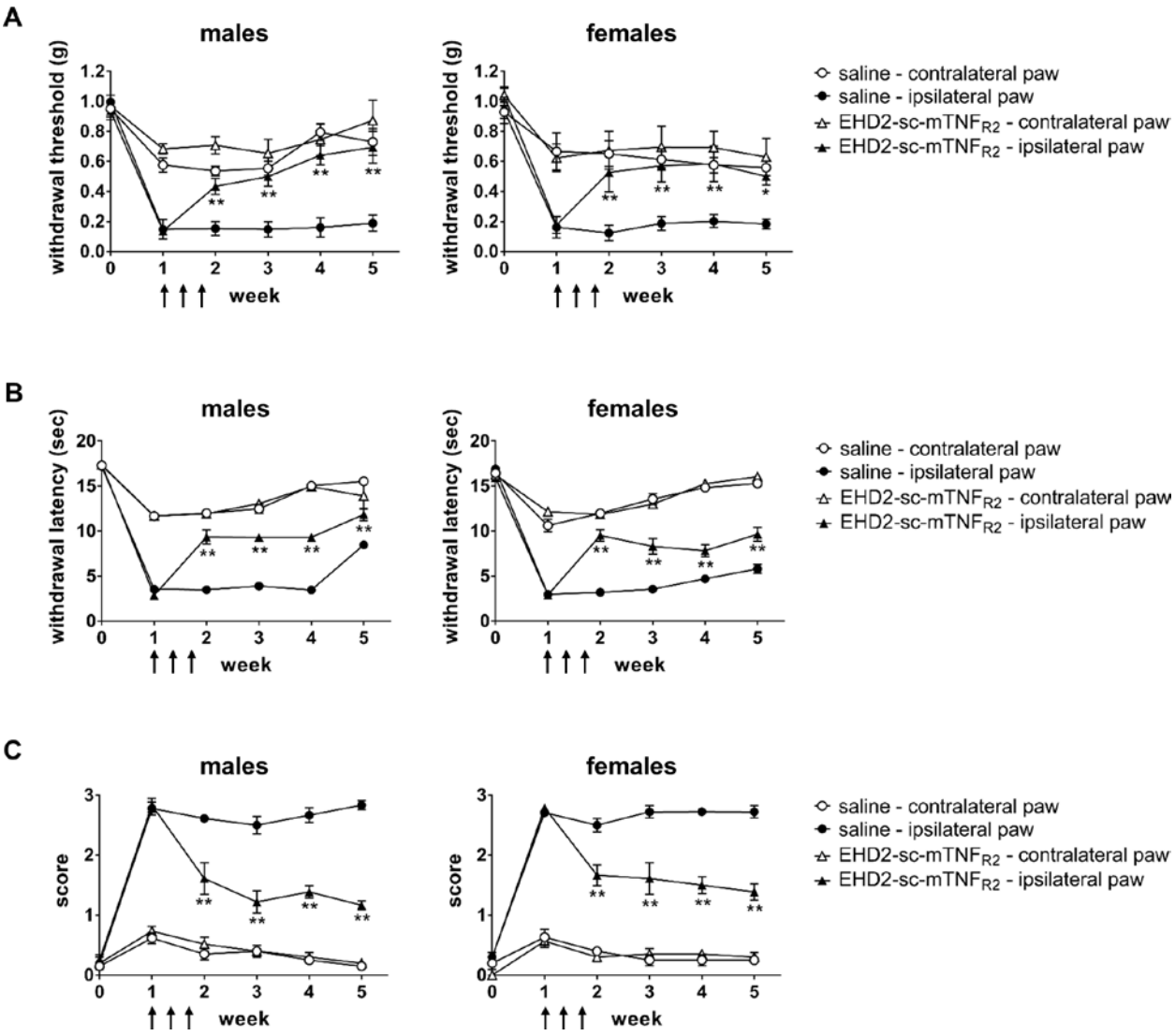
Supplementary Figure 6 TNFR2 expression on different splenocytes was quantified by flow cytometry. Shown is the comparison between (A) naïve mice and (B) mice 1 week after CCI. Top panel: Gating strategy for identification of different immune cell subsets; Bottom panel: Histograms showing TNFR2 expression (grey histogram: isotype control, black histogram: αTNFR2 staining). Shown are representative data from n=3 mice/sex/group

Supplementary Figure S7



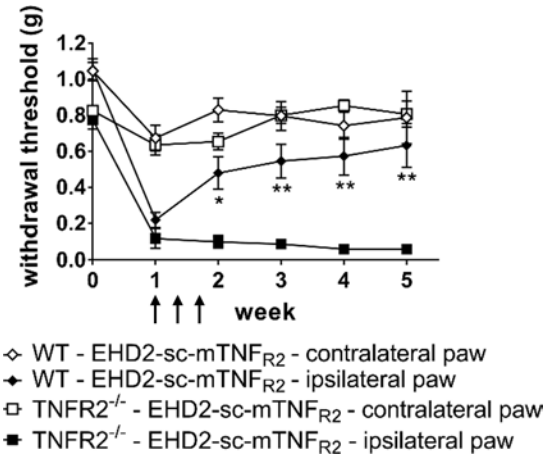
Supplementary Figure 7 TNFR2 expression on different immune cells from the spinal cord was quantified by flow cytometry. Shown is the comparison between (A) naïve mice and (B) mice 1 week after CCI. Top panel: Gating strategy for identification of different immune cell subsets; Bottom panel: Histograms showing TNFR2 expression (grey histogram: isotype control, black histogram: αTNFR2 staining). (C,D) To investigate regulation of TNFR2 expression after injury, we compared the median fluorescence intensity (MFI) of mice 1 week post injury (wpi) to the MFI of naïve mice. Shown are representative data from n=3 mice/sex/group

Supplementary Figure S8



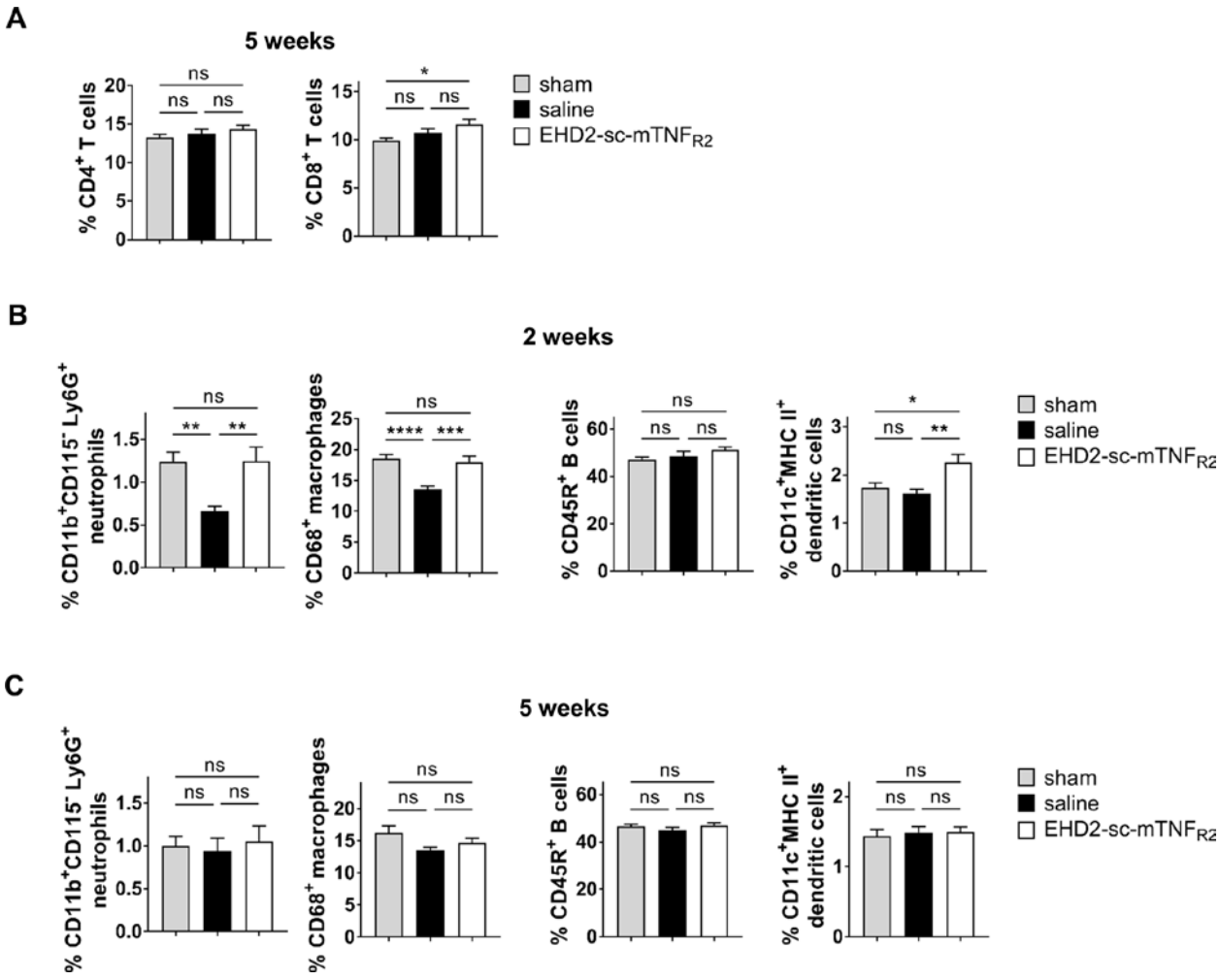
Supplementary Figure 8. Male or female WT mice underwent CCI at week 0. (A) Mechanical allodynia, (B) thermal hyperalgesia and (C) cold allodynia were determined over a period of 5 weeks after injury. On day 7, 10 and 13, mice were treated with saline or EHD2-sc-mTNF$_{R2}$ (10 mg/kg, black arrows) via i.p. injection (mechanical allodynia: n=12 males/group and n=7 females/group, thermal hyperalgesia/cold allodynia: n=6 each group/sex). * $p<0.05$ ** $p<0.01$ saline vs EHD2-sc-mTNF$_{R2}$ ipsilateral paw.

Supplementary Figure S9



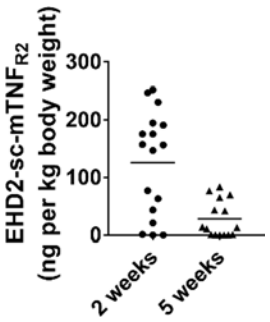
Supplementary Figure 9. Male and female WT and TNFR2$^{-/-}$ mice underwent CCI at week 0. Mechanical allodynia was determined over a period of 5 weeks after injury. On day 7, 10 and 13, mice were treated with EHD2-sc-mTNF$_{R2}$ (10 mg/kg) via i.p. injection (black arrows). Shown are the combined data for males and females (WT: n=4 males/4 females, TNFR2$^{-/-}$: n=2 males/2 females). * $p<0.05$, ** $p<0.01$ WT vs TNFR2$^{-/-}$ ipsilateral paw.

Supplementary Figure S10



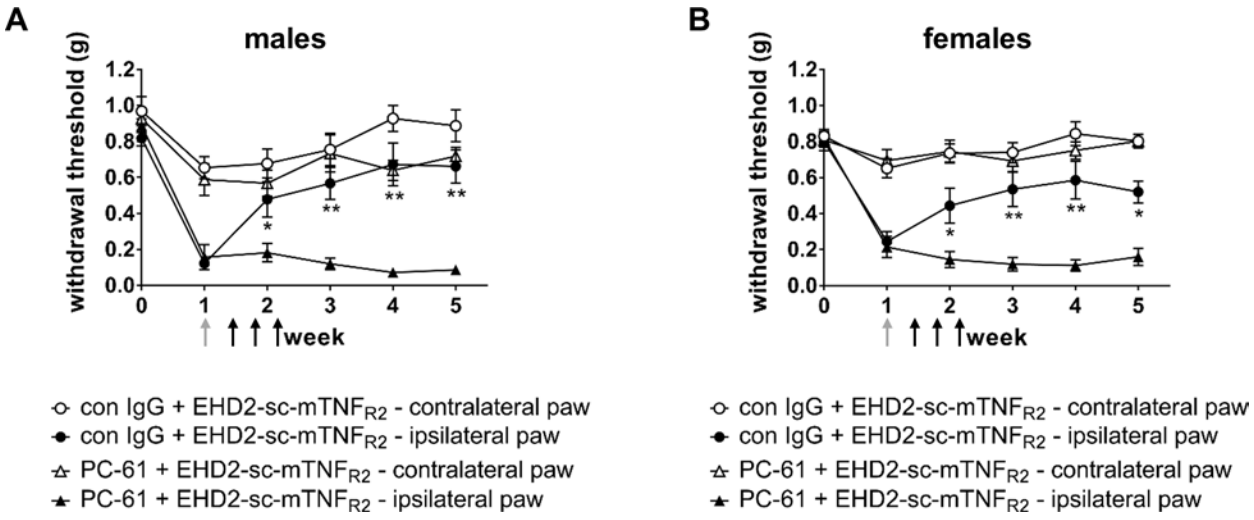
Supplementary Figure 10 CCI mice were treated with saline or EHD2-sc-mTNF$_{R2}$ (10 mg/kg, i.p.) 7, 10 and 13 days after injury. Mice were sacrificed 2 or 5 weeks after injury. Percentage of splenic (A) CD4$^+$ and CD8$^+$ T cells, (B,C) CD11b$^+$CD115$^-$Ly6G$^+$ neutrophils, CD68$^+$ macrophages, CD45R$^+$ B cells or CD11c$^+$MHC II$^+$ dendritic cells was quantified by flow cytometry. Quantification shows the mean ±SEM (2 weeks: n=16 each group, 5 weeks: sham/EHD2-sc-mTNF$_{R2}$: n=16 and saline: n=18). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns = not significant

Supplementary Figure S11



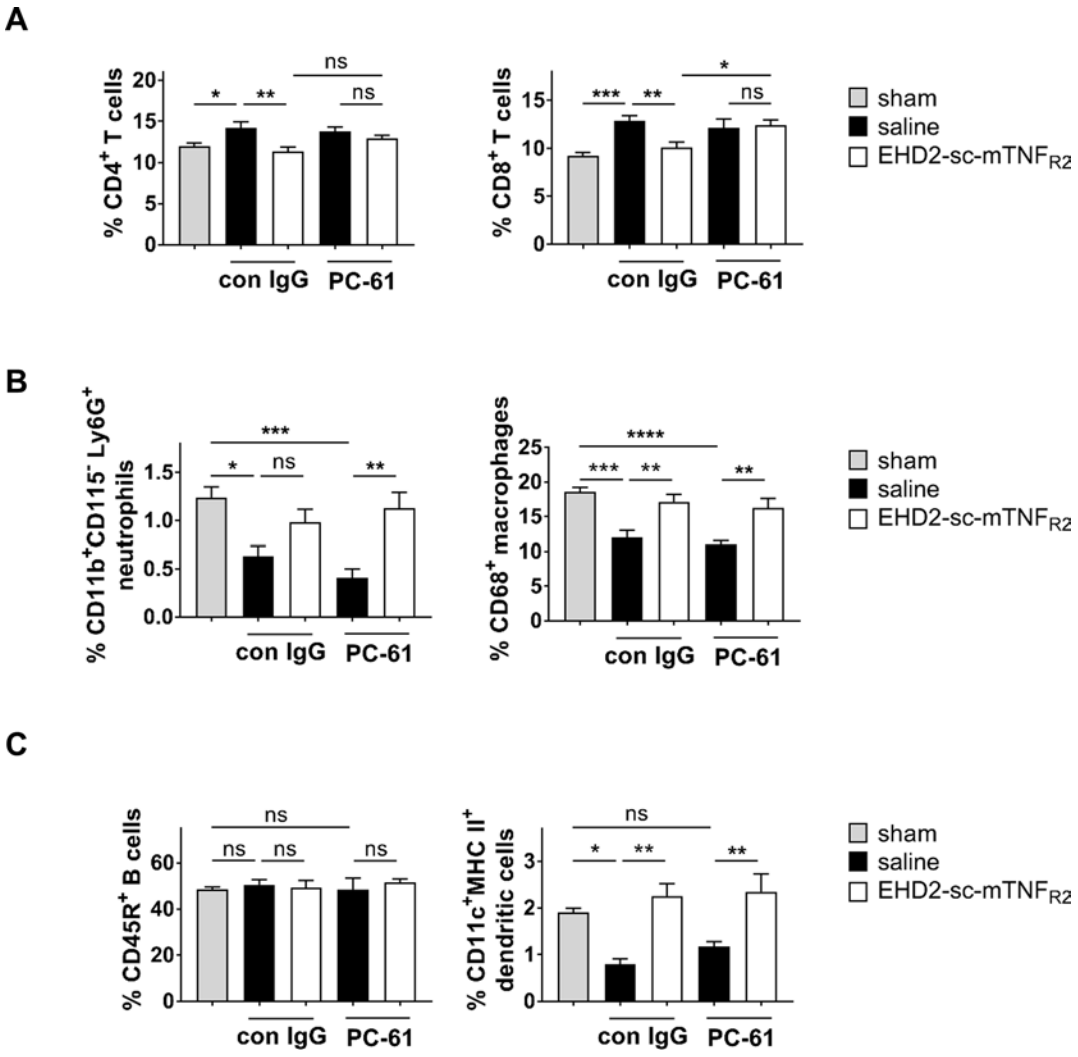
Supplementary Figure 11 CCI mice were treated with saline or EHD2-sc-mTNF$_{R2}$ (10 mg/kg, i.p.) 7, 10 and 13 days after injury. Mice were sacrificed 2 or 5 weeks after injury. Concentration of EHD2-sc-mTNFR2 was determined by ELISA. Quantification shows the results for individual mice (2 weeks: n=9 males/8 females; 5 weeks: n=8 males/7 females

Supplementary Figure S12



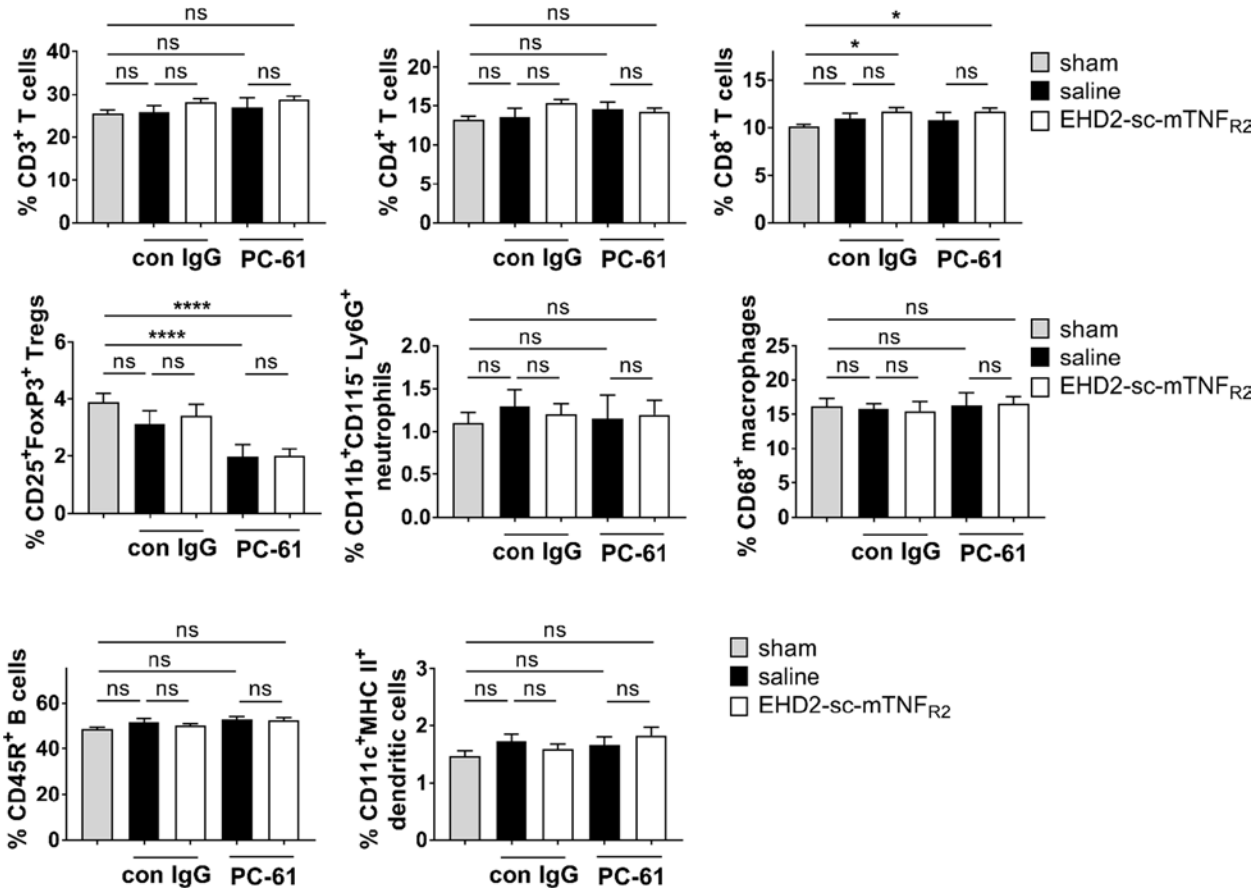
Supplementary Figure 12. (A) Male and (B) female WT mice underwent CCI at week 0. Mechanical allodynia was determined using Von Frey test over a period of 5 weeks after injury. Mice were treated with control IgG (con IgG) or PC-61 (500 µg each, i.p., gray arrow) after 7 days. On day 9, 12 and 15 mice received i.p. injections with saline or EHD2-sc-mTNF$_{R2}$ (10 mg/kg, black arrows) (n=8 per group/sex) * $p<0.05$, ** $p<0.01$ con IgG + EHD2-sc-mTNF$_{R2}$ vs PC-61 + EHD2-sc-mTNF$_{R2}$ injured paw.

Supplementary Figure S13



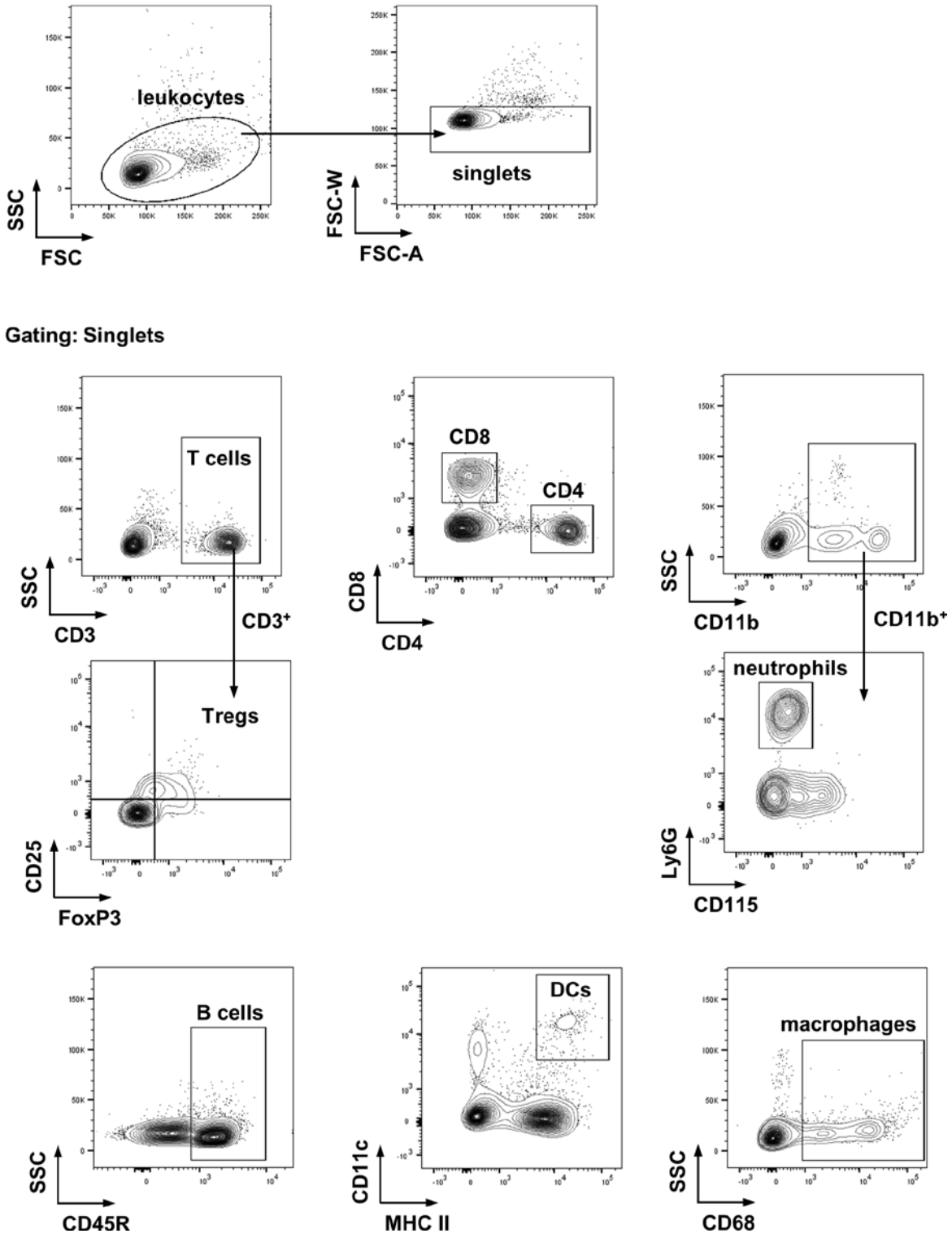
Supplementary Figure 13. CCI mice were treated with control IgG (con IgG), PC-61, saline and/or EHD2-sc-mTNF$_{R2}$ (10 mg/kg, i.p.). Mice were sacrificed 2 weeks after injury. Percentage of splenic (A) CD4$^+$ and CD8$^+$ T cells, (B) CD11b$^+$CD115$^-$Ly6G$^+$ neutrophils and CD68$^+$ macrophages and (C) CD45R$^+$ B cells and CD11c$^+$MHC II$^+$ dendritic cells was quantified by flow cytometry. Quantification shows the mean ±SEM. (n=8-14 mice each group). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns = not significant

Supplementary Figure S14



Supplementary Figure 14. CCI mice were treated with control IgG (con IgG), PC-61, saline and/or EHD2-sc-mTNF$_{R2}$ (10 mg/kg, i.p.). Mice were sacrificed 5 weeks after injury. Percentage of splenic CD3$^+$, CD4$^+$ and CD8$^+$ T cells, CD25$^+$FoxP3$^+$ Tregs, CD11b$^+$CD115$^-$Ly6G$^+$ neutrophils, CD68$^+$ macrophages, CD45R$^+$ B cells and CD11c$^+$MHC II$^+$ dendritic cells was quantified by flow cytometry. Quantification shows the mean ±SEM. (n=8-14 mice each group). * $p<0.05$, **** $p<0.0001$, ns = not significant

Supplementary Figure S15



Supplementary Figure 15. Gating strategy to identify splenic T cells, neutrophils, B cells, dendritic cells (DCs) and macrophages

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