Supplementary Information

Nociceptive neurons regulate innate and adaptive immunity and neuropathic pain through MyD88 adapter

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Supplementary Methods

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Supplementary Methods

Reagents and intrathecal injection

We purchased paclitaxel (PTX), capsaicin, complete Freund's adjuvant (CFA) from Sigma-Aldrich. We also obtained monoclonal antibodies (mAbs) from BD Biosciences, collagenase, dispase and trypsin from Gibco BRL. T cells and CD8 antibody (53-6.7) were administered via intrathecal route. For intrathecal injection, spinal cord puncture was made with a 30 G needle between the lumbar L5 and L6 level to deliver reagents (10 μl) to the cerebral spinal fluid[1, 2].

Transgenic mice

$MyD88^{flox}$ mice were obtained from Jackson Labs. Upon arrival, mutant mice were bred with C57BL/6J inbred mice to establish the colony. To delete MyD88 in nociceptors neurons, we crossed mice carrying a conditional null allele of MyD88 (MGI: 108005; $MyD88^{f/f}$) with $Nav1.8^{cre}$ transgenic mice (kindly provided by Rohini Kuner, University of Heidelberg)[3], with the resulting homozygous conditional knockout mice ($MyD88^{f/f}$; $Nav1.8^{cre}$) referred to as CKO mice. The $MyD88^{f/f}$ littermates were referred as control mice. Breeding colonies were maintained by mating $MyD88^{f/f}$ with $Nav1.8^{cre}$-$MyD88^{f/f}$ mice. $Nav1.8^{cre}$ mice had been backcrossed for at least five generations on a C57BL/6 background. Mice were genotyped by PCR using genomic DNA isolated from ear according to standard protocols from the Jackson Labs. Animal experiments were

conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal models and behavioral tests

All the animal procedures were approved by the Institutional Animal Care & Use Committee (IACUC) of Duke University. Adult Mice (> 8 weeks) were group-housed and kept under a 12-hour light/dark cycle. Animals were habituated to the testing environment daily for at least two days before baseline testing. For producing chemotherapy neuropathy model, mice were given single injection (intraperitoneally, 6 mg/kg) of paclitaxel (PTX, Sigma-Aldrich) under isoflurane anesthesia. For inducing inflammatory pain, mice were given intraplantar injection of 20 μl of complete Freund 's adjuvant (CFA, Sigma)[2]. For testing mechanical sensitivity, we put a mouse in a box on elevated metal mesh floor and stimulated hindpaw with a series of von Frey hairs with logarithmically incrementing stiffness (0.02-2.56 grams, Stoelting, Wood Dale, IL), presented perpendicular to the plantar surface, and determined the 50% paw withdrawal threshold using up-down method [2, 4, 5]. The 50% paw withdrawal threshold (PWT) was determined using Dixon's up-down method. For testing mechanical allodynia, we also measured paw withdrawal frequency in response to a sub threshold von Frey hair (0.6 or 0.16 g, 10 times).

Immunohistochemistry

Animals were anesthetized with isoflurane and perfused through the ascending aorta with PBS, followed by 4% paraformaldehyde including 1% picric acid. The L4-L5 DRGs were removed and post-fixed overnight. DRGs were sectioned (10 μm) in a cryostat (CM 1950, Leica). To improve MyD88 immune staining, slides were first processed for antigen retrieval in citrate buffer (10 mM Aitric Acid, 0.05% Tween-20, pH6.0) at 95~100 °C for 30 min. The sections were blocked with 5% goat or donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature and were then incubated with anti-MyD88 antibody (rabbit, 1:1000; Abcam, Cambridge, MA) overnight at 4°C, followed by Cy3-conjugated secondary antibodies (1:400; Jackson ImmunoResearch). For the quantification of immunostaining, three sections from each DRG were selected and 5~6 animals were analyzed in each group. To determine the percentage of labeled neurons in DRGs, the number of positive neurons (3 times of background staining) was divided by the total number of neurons. Image-Pro Plus 5.0 software (Media Cybernetics, Silver Spring, EUA) was used to analyze the data.

Single-cell RT-PCR from dissociated DRG neurons

We aseptically removed DRGs from 4-5 week-old mice and digested the tissues with collagenase (1.25 mg/ml, Roche) and dispase-II (2.4 units/ml, Roche) for 90 min, followed by 0.25% trypsin for 8 min at 37 °C. We plated cells on cover slips and grew them in a neurobasal defined medium (with 2% B27 supplement) in the presence of 5 μM AraC, at 37°C, with 5% CO2/95% air for 24 h before experiments. Single-cell RT-PCR

was performed as previously described[2]. Briefly, a single cell was aspirated into a glass pipette with a tip diameter of about 25~40 μm, gently put into a reaction tube containing reverse transcription (RT) reagents. To avoid genomic DNA contaminations, a DNase I (40 min at 37 °C) digest was performed before reverse transcription. After heat inactivation, RT was processed for 1 hour at 50 °C (superscript

III, Invitrogen). The cDNA product was used in separate PCR. The sequences of the outer and inner primers used for single-cell PCR are included in the table below.

List of DNA primer sequences designed for single-cell RT-PCR.

Target gene (Product length)[a]	Outer primers	Inner primers	Genbank No.
MyD88 (364 bp, 210 bp)	GTATCCTGCGGTTCATCACTAT GAACTCTTCCACTCAGCTATCC	TCCCTGCCCTGAAGATGA GAGGAGGCATGTGTGTACTG	NM_010851
$Na_v1.8$ (316 bp, 203 bp)	CATGACAGAGGAGCAGAAGAAG CCAGCCGTTGGTGAAGTAATA	CTTTGAATAAGTACCAGGGCTTC GAACATCTTCATCACACACTCG	NM_001205321
NF200 (328 bp, 202 bp)	GGAAGGAGAAGAAGCAGAAGAG CTTCACTGTAGCTGGAGACTTG	CTGCAGAAGAGGCTGCAT GGCTCAGCTGGAGACTTG	NM_010904
GAPDH (367 bp, 313 bp)	AGCCTCGTCCCGTAGACAAAA TTTTGGCTCCACCCCTTCA	TGAAGGTCGGTGTGAACGAATT GCTTTCTCCATGGTGGTGAAGA	XM_001473623

[a] (n, n) indicates product size obtained from outer and inner primers, respectively.

The first round of PCR was performed in 50 μl of PCR buffer containing 0.2 mM dNTPs, 0.2 μM "outer" primers, 5 μl RT product and 0.2 μl platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). The protocol included a 5 min initial denaturation step at 95 °C followed by 40 cycles of 40 s denaturation at 95 °C, 40 s annealing at 55 °C, 40 s elongation at 72 °C. The reaction was completed with 7 min of final elongation. For the second round of amplification, the reaction buffer (20 μl) contained 0.2 mM dNTPs, 0.2 μM "inner" primers, 5 μl of the first round PCR products and 0.1 μl platinum Taq DNA

polymerase. The reaction procedure for these primers was the same as the first round. A negative control was obtained from pipettes that did not harvest any cell contents, but were submerged in the bath solution. The PCR products were displayed on ethidium bromide-stained 1.5% agarose gels.

Western Blot

DRG Tissues were homogenized in RIPA buffer (Boston BioProducts, Ashland, MA) containing protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF, Pierce, Rockford, IL) and phosphatase inhibitor cocktail (Sigma). Protein samples were prepared at concentration of 1 mg/ml, and 20 μg of proteins were loaded for each lane and separated by 10% SDS-PAGE gel. Blots were incubated overnight at 4°C with rabbit monoclonal anti-MyD88 antibody (1:1000; Cell Signaling Technology, Danvers, MA) or polyclonal antibodies against β-actin antibody (1:50000, mouse; Millipore). The blots were further incubated with HRP-conjugated secondary antibody (1:5000; R&D Systems) and developed in ECL solution (Pierce, Rockford, IL). Images of immune blots were captured with ChemiDocTMMP imaging system (Bio-Rad, Hercules, CA). The intensity of the interesting bands was analyzed using Image-Pro Plus 5.0 software (Media Cybernetics).

Cell Preparation and Flow Cytometry[6-10]

Mice were anesthetized under isoflurane and transcardially perfused with PBS (50 ml per

mouse). DRGs and spleens were harvested in HBSS containing 10 mM HEPES on ice. To obtain single cells, DRGs were incubated for 90 minutes at 37 °C with 5 mg/ml Collagenase and 1 mg/ml Dispase (Gibco BRL) in DMEM, and then were centrifuged at 1,000 g for 5 minutes. The pellets were digested in 0.25% Trypsin for 10 minutes before Trypsin being neutralized with DMEM media containing 10% FBS. After being gently aspirated and expirated using different sizes of pipette tips, the cells were filtered through 70 μm strainer. Spleens were ground against 100 μm strainer. Red blood cells in the splenocytes were lyzed with a lysis buffer from eBioscience (San Diego, CA). Cells were then washed with HBSS plus (2% FBS, 10 mM EGTA and 10 mM HEPES in HBSS), and blocked with the Fc receptors in staining buffer (1% anti-mouse-CD16/CD32, 2.4G2, 2% FBS, 5% NRS and 2% NMS in HBSS)) for 15 min. For cell surface markers, we stained cells in staining buffer on ice for 45 min with combinations of four to five fluorescent-labeled mAb obtained from BD Biosciences (San Diego, CA), unless specially listed. We tested the following mAbs: CD45 (30-F11), CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7), IA/IE (2G9), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418, eBioscience) and CD14 (rmC5-3). After being washed three time with HBSS plus or Perm/Wash buffer, the stained cells were finally resuspended in 400 μl HBSS plus and filtered through a 70 μm cell strainer for analysis. Up to 500,000 events from each sample were acquired using a BD FACSCanto II Flow Cytometer (BD Biosciences) and analyzed with Flowjo software (BD Biosciences). Nonstained DRG and spleen cells in the same preparation were also analyzed as the negative control and reference for data

analysis. We used CD45 as the gate[6], in which $CD45^+$ populations were identified as the infiltrating leukocytes. We then investigated immune cell sublets within $CD45^+$ population. As control, the positive cells were always recorded by ratio of total events.

Evans blue plasma extravasation

Under isoflurane anesthesia, Evans blue (50 mg/kg; Sigma-Aldrich) was injected into the jugular vein of mice. Ten minutes later, capsaicin (3 μg in 10 μl) was injected into the left hindpaw of a mouse, and the right paw was injected with vehicle. After 30 min the plantar skin of the paw was removed, dried of excess liquid, weighed and incubated in formamide for 24 h at 56 ^oC. Extravasated Evans blue was measured by X MarkTM microplate spectrophotometer (Bio-Rad, Hercules, CA) at 620 nm.

Adoptive Transfer of immune cells[8-11]

Single-cell suspensions of spleens were prepared from C57/B6 mice as above described. After being stained, $CD4^+CD25^+$ (Treg), $CD4^+CD25^-$ (Teff, control of Treg) and $CD8^+$ populations were sorted by negative and positive selection with BD FACSAria II cell sorter (BD Biosciences), respectively. Two donor mice were included to obtain 1×10^6 Treg cells, 1×10^7 Teff cells and 5×10^6 CTL cells. The recipient mice were anesthetized with isoflurane and given an intrathecal injection of 2.5×10^5 freshly sorted cells or 10 μl 3 days after PTX injection.

Statistical analyses

All data were expressed as mean ± S.E.M. Data were analyzed using student's t-test (two groups) and One-Way or Two-Way ANOVA followed by post-hoc Bonferroni test. The criterion for statistical significance was $P < 0.05$.

References

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Supplementary Table-1

Distribution of 14 populations of immune cells in DRGs and their fold changes after chemotherapy (PTX treatment) in WT and MyD88-CKO mice. Different populations of immune cells were revealed by FACS analysis and the data are expressed as fold of WT (first column) in WT + PTX, CKO, CKO + PTX groups. Note that MyD88 in nociceptor neurons is required for the initiation of both innate and adaptive immunity following chemotherapy. *P<0.05, vs. WT, $^{\#}$P<0.05, vs. WT-PTX, n=3-10 mice/group. Data are expressed as mean ±SEM.

	Markers	**Immune cells**	**WT** (% total cells)	**CKO** (Fold of WT)	**WT + PTX** (Fold of WT)	**CKO + PTX** (Fold of WT)
Gate	$CD45^+$	Leukocyte	0.89 ± 0.25	1.23 ± 0.13	2.95 ± 0.76 *	1.46 ± 0.14 #
Innate immunity						
	$CD11b^+$	Macrophage/Monocyte /Neutrophil	0.50 ± 0.05	1.25 ± 0.12	2.41 ± 0.31*	1.69 ± 0.22 #
	$CD11b^+CD14^+$	Monocyte	0.22 ± 0.01	1.13 ± 0.10	1.84 ± 0.13 *	1.48 ± 0.12 #
	$CD11b^+Gr\text{-}1^{high}$	Neutrophil	0.11 ± 0.02	1.70 ± 0.26	3.78 ± 0.84 *	1.79 ± 0.33 #
	IA/IE^+CD11c^+	Dendritic cell (DC)	0.06 ± 0.02	1.08 ± 0.22	1.50 ± 0.35	1.40 ± 0.31
	$NK1.1^+$	Natural killer cell (NK)	0.07 ± 0.02	0.57 ± 0.13	1.83 ± 0.19	1.57 ± 0.34
Adaptive immunity						
	IA/IE^+	Antigen presenting cell (APC)	0.37 ± 0.03	0.95 ± 0.19	2.03 ± 0.23 *	1.45 ± 0.16 #
	$CD3^+$	T lymphocyte cell	0.20 ± 0.01	1.01 ± 0.12	1.91 ± 0.12 *	1.40 ± 0.13 #
	$CD4^+$	Helper T cell	0.13 ± 0.01	1.04 ± 0.23	2.09 ± 0.33 *	1.37 ± 0.09 #
	$CD8^+$	Cytotoxic T lymphocyte cell (CTL)	0.08 ± 0.01	1.03 ± 0.15	2.23 ± 0.48 *	1.24 ± 0.11 #
	$CD3^+CD4^+IFN\text{-}\gamma^+$	T helper type 1 cell (TH1)	0.05 ± 0.02	1.05 ± 0.33	1.29 ± 0.28	1.14 ± 0.27 #
	B220	B lymphocyte cell	0.08 ± 0.01	0.96 ± 0.15	2.42 ± 0.30 *	1.71 ± 0.16 #
Negative regulator						
	$CD3^+CD4^+IL\text{-}2^+$	T helper type 2 cell (TH2)	0.04 ± 0.00	0.92 ± 0.08	0.68 ± 0.03 *	0.87 ± 0.06 #
	$CD4^+CD25^+Foxp3^+$	Regulatory T cell (Treg)	0.03 ± 0.01	1.27 ± 0.39	0.42 ± 0.06 *	0.62 ± 0.11

*, P < 0.05 compared with WT; #, P < 0.05 compared with WT + PTX

Supplementary Figure 1

MyD88 expression in DRG neurons of WT and MyD88-CKO mice (A-E) and populations of immune cells in DRG and spleen tissues of WT and CKO mice with and without PTX treatment (7 d, F-P).

(A,B) Immunohistochemistry shows MyD88 localization in DRG neurons of wild-type (WT, A) and MyD88-conditional knockout (CKO, B) mice. White arrows indicate MyD88-positive small DRG neurons in WT mice, and green arrows indicate MyD88-negative small DRG neurons in CKO mice. Scale, 50 μm.

(C) Size frequency (%) analysis of the profiles of $MyD88^+$ neurons in WT and CKO mice. Note a selective reduction of MyD88 in small-sized neurons.

(D,E) Single-cell PCR in 10 small-sized (< 25 μm) DRG neurons of WT (D) and MyD88-CKO mice (E). M, molecular weight; N, negative control. GAPDH is used as positive control. Note that MyD88 bands are lost in small-sized DRG neurons of CKO mice. These small-sized DRG neurons do not express NF-200, a marker for large-sized DRG neurons. Also note that Nav1.8 expression does not change in CKO mice.

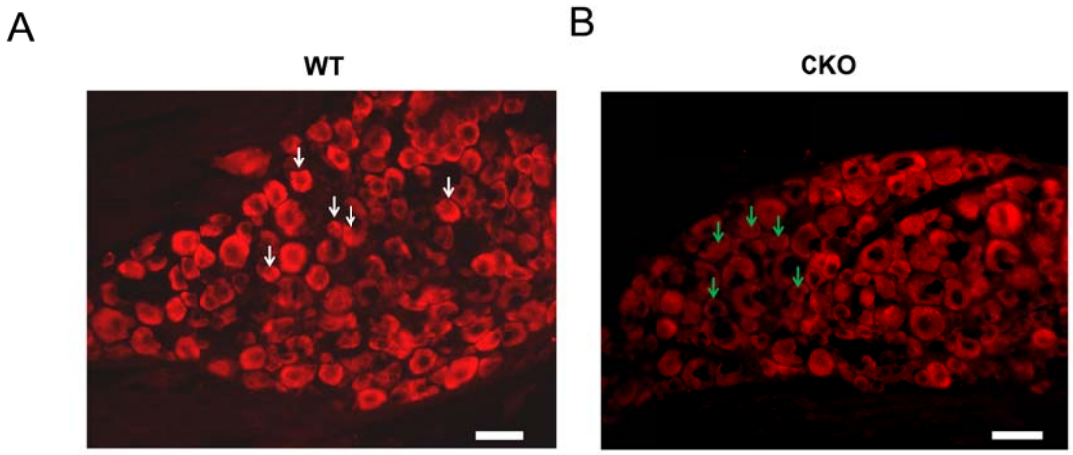
(F-H) MyD88 in nociceptive neurons is essential for chemotherapy (PTX)-induced innate immunity in DRGs. FACS analysis showing the populations of macrophages ($CD11b^+$, F), monocytes ($CD11b^+CD14^+$, G; $CD11b^+Gr\text{-}1^{medium}$, H), and neutrophils ($CD11b^+Gr\text{-}1^{high}$, I). Low panels are fold changes (vs. WT-control) $*P < 0.05$; n = 7-9 mice/group.

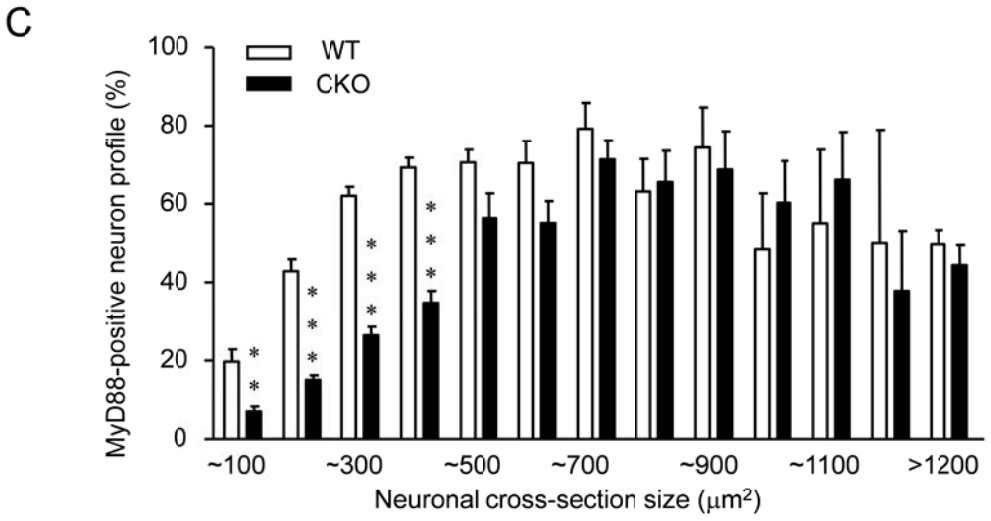
(I-L) MyD88 in nociceptive neurons is essential for chemotherapy-induced adaptive

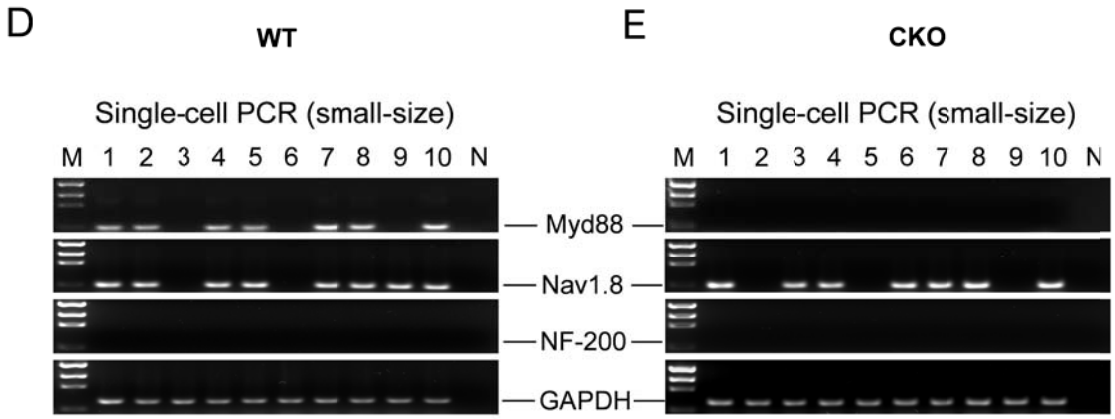
immunity in DRGs. FACS showing the populations of T lymphocytes ($CD3^+$, I), helper T cells ($CD4^+$, J) cytotoxic T cells ($CD8^+$, K) and antigen presenting cells (IA/IE^+, L) in DRGs of WT and CKO mice. Low panels are fold changes. *P < 0.05; n = 7-9 mice/group.

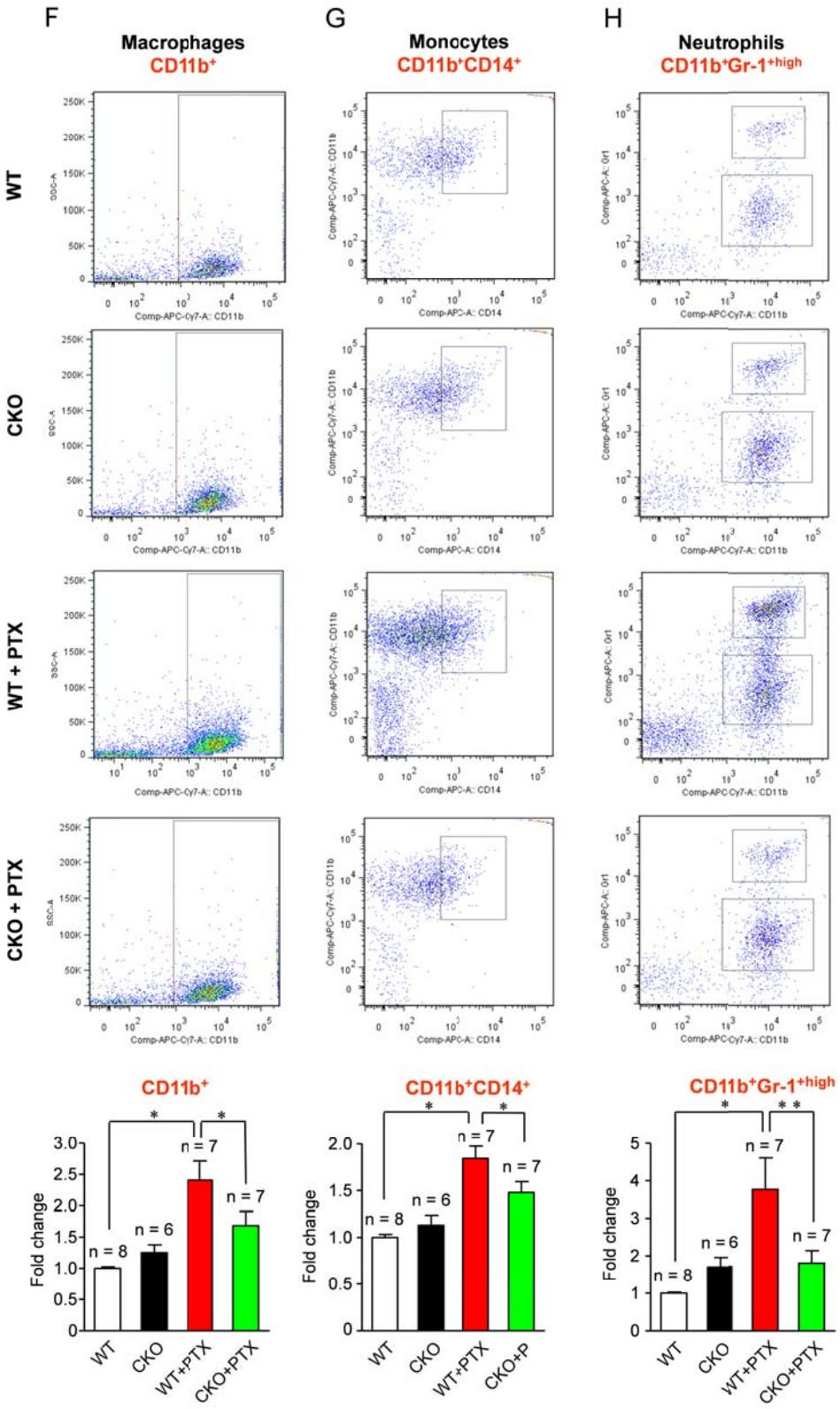
(M-O) MyD88 in nociceptive neurons is required for chemotherapy induced up-regulation of B cells and down-regulations of Th2 cells and Treg cells in DRGs. FACS showing the populations of B cells ($B220^+$, M), helper T type-2 cells ($CD3^+CD4^+IL\text{-}4^+$, N) and regulatory T cells ($CD4^+CD25^+FoxP3^+$, O) in DRGs of WT and CKO mice with and without PTX treatment (7 d). *P < 0.05; n = 3-9 mice/group; N.S., no significance.

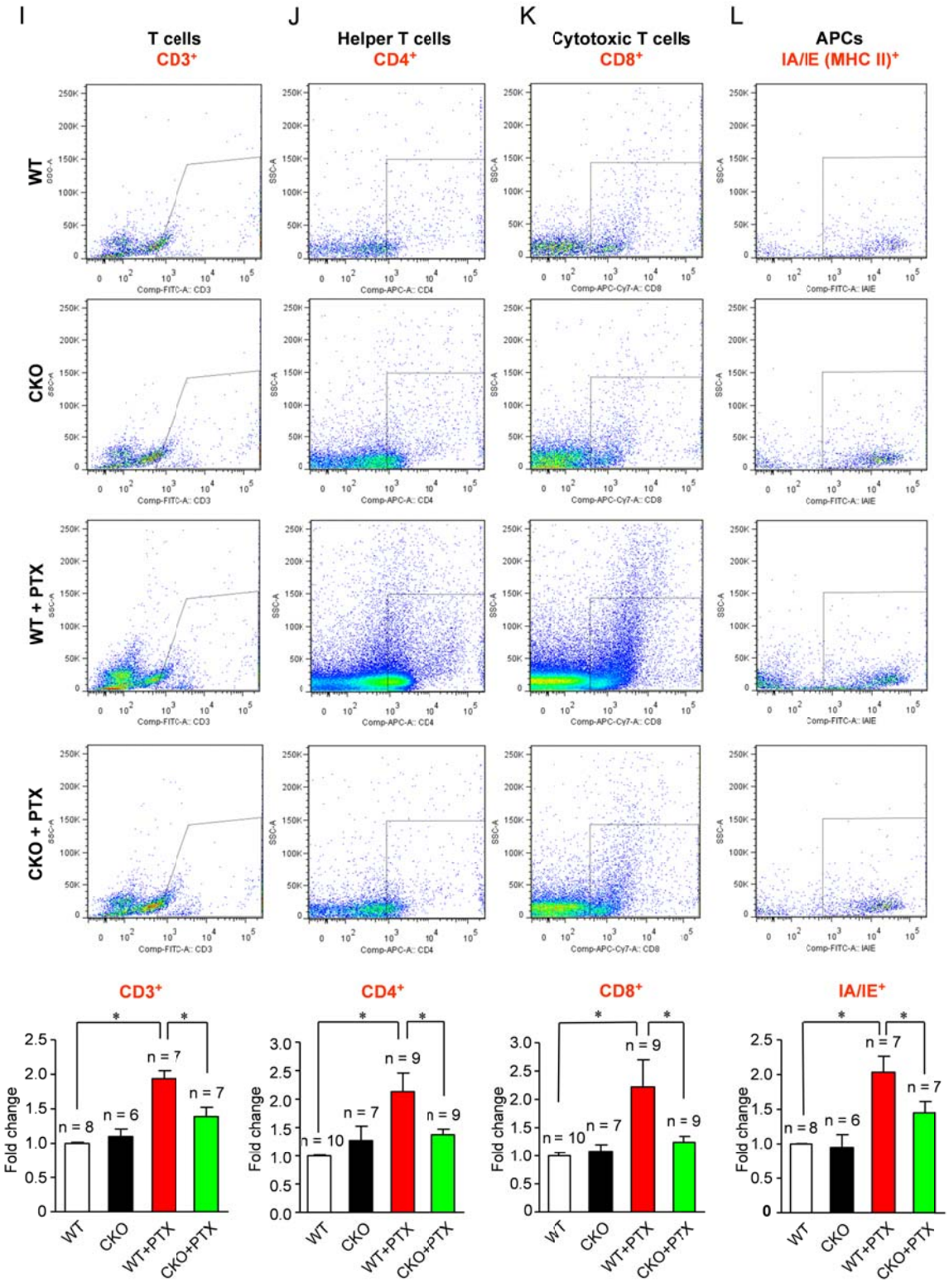
(P) Innate and adaptive immune cells in spleens after PTX-injection in WT and MyD88 CKO mice. These cells consist of leukocytes ($CD45^+$), innate immune cells including macrophages ($CD11b^+$), monocytes ($CD11b^+CD14^+$) and neutrophils ($CD11b^+Gr\text{-}1^{high}$; and adaptive immune cells including T cells ($CD3^+$), helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$) and antigen presenting cells ($IA/IE^{+;W}$). *P < 0.05; n = 5-10 mice/group.


A
WT
B
CKO


C
WT
CKO
MyD88-positive neuron profile (%)
100
80
60
40
20
0
~100
~300
~500
~700
~900
~1100
>1200
Neuronal cross-section size (μm²)


D
WT
E
CKO
Single-cell PCR (small-size)
Single-cell PCR (small-size)
M 1 2 3 4 5 6 7 8 9 10 N
M 1 2 3 4 5 6 7 8 9 10 N
Myd88
Nav1.8
NF-200
GAPDH


F
Macrophages
CD11b+
G
Monocytes
CD11b+CD14+
H
Neutrophils
CD11b+Gr-1+high
WT
CKO
WT + PTX
CKO + PTX
SSC-A
Comp-APC-Cy7-A: CD11b
Comp-APC-A: CD14
Comp-APC-A: Gr1
CD11b+
CD11b+CD14+
CD11b+Gr-1+high
Fold change
n = 8
n = 6
n = 7
n = 7
WT
CKO
WT+PTX
CKO+PTX
CKO+P


I
T cells
CD3+
J
Helper T cells
CD4+
K
Cytotoxic T cells
CD8+
L
APCs
IA/IE (MHC II)+
WT
CKO
WT + PTX
CKO + PTX
SSC-A
Comp-FITC-A: CD3
Comp-APC-A: CD4
Comp-APC-Cy7-A: CD8
Comp-FITC-A: IAIE
CD3+
CD4+
CD8+
IA/IE+
Fold change
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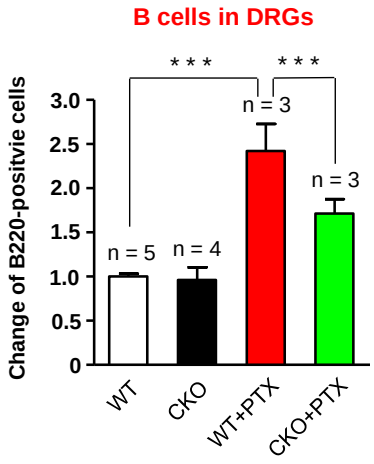
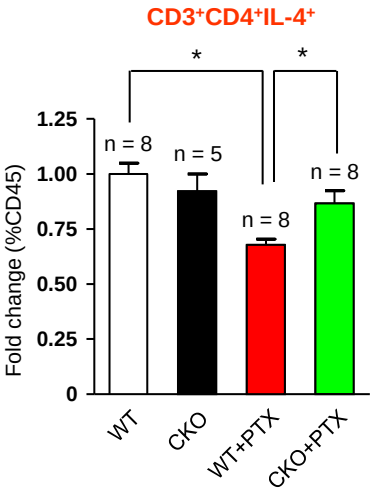
WT
CKO
WT+PTX
CKO+PTX

M


B cells in DRGs
Change of B220-positvie cells
3.0
2.5
2.0
1.5
1.0
0.5
0
* * *
* * *
n = 5
n = 4
n = 3
n = 3
WT
CKO
WT+PTX
CKO+PTX

N


CD3+CD4+IL-4+
Fold change (%CD45)
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0.75
0.50
0.25
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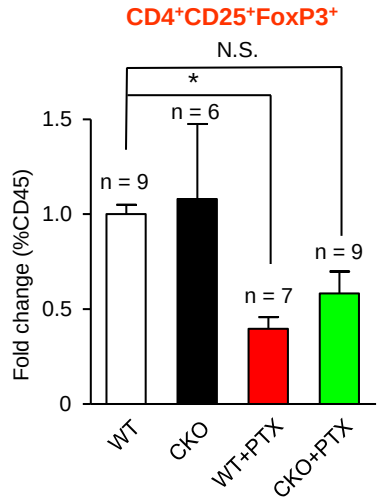
CKO
WT+PTX
CKO+PTX

O


CD4+CD25+FoxP3+
Fold change (%CD45)
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1.0
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N.S.
*
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n = 7
n = 9
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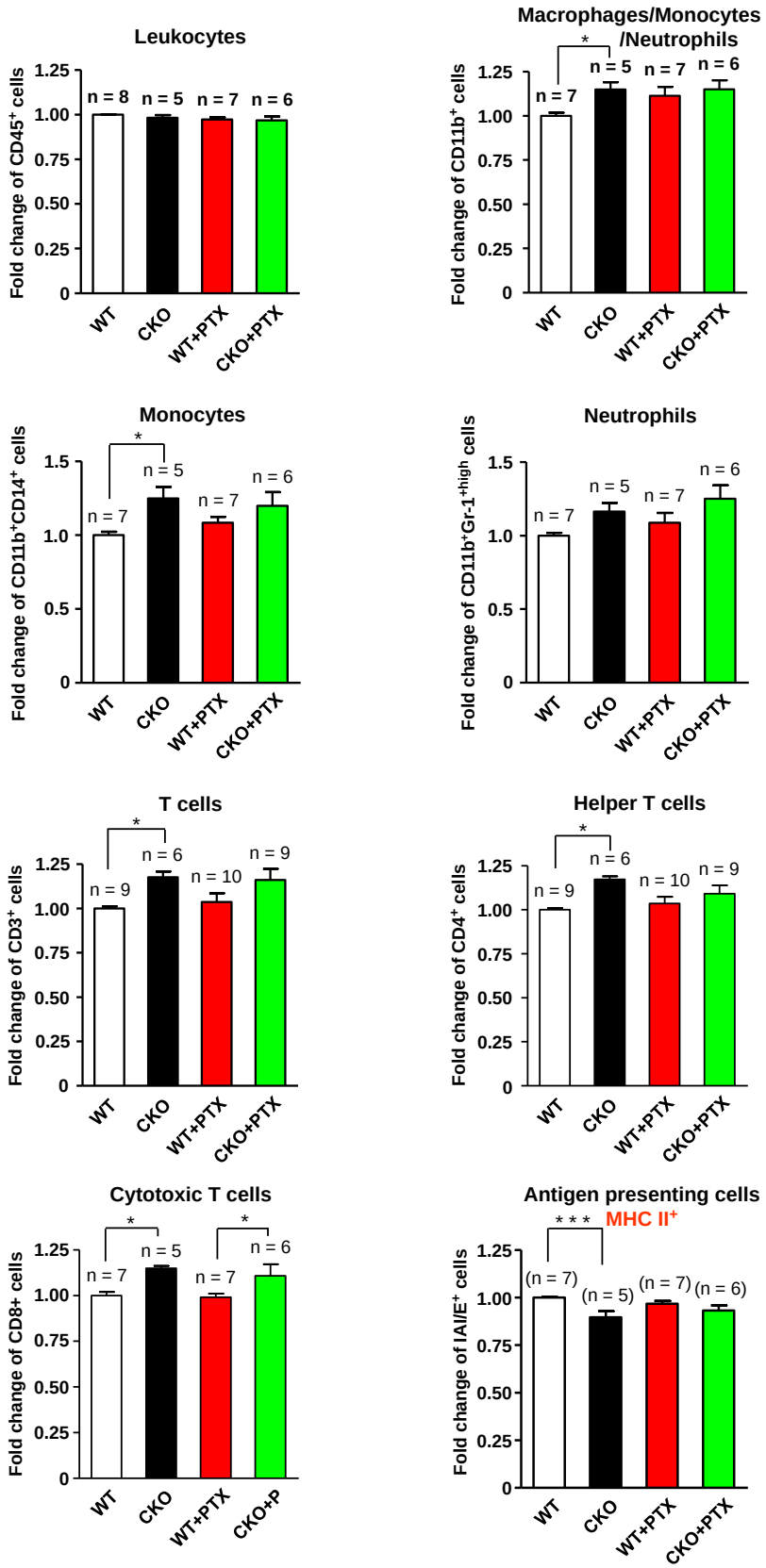
CKO
WT+PTX
CKO+PTX

P


Leukocytes
Fold change of CD45+ cells
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n = 5
n = 7
n = 6
Macrophages/Monocytes /Neutrophils
Fold change of CD11b+ cells
*
n = 7
n = 5
n = 7
n = 6
Monocytes
Fold change of CD11b+CD14+ cells
*
n = 7
n = 5
n = 7
n = 6
Neutrophils
Fold change of CD11b+Gr-1+high cells
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n = 5
n = 7
n = 6
T cells
Fold change of CD3+ cells
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n = 6
n = 10
n = 9
Helper T cells
Fold change of CD4+ cells
*
n = 9
n = 6
n = 10
n = 9
Cytotoxic T cells
Fold change of CD8+ cells
*
*
n = 7
n = 5
n = 7
n = 6
Antigen presenting cells
MHC II+
* * *
Fold change of IAI/E+ cells
(n = 7)
(n = 5)
(n = 7)
(n = 6)
WT
CKO
WT+PTX
CKO+PTX
CKO+P