CXCL12-mediated monocyte transmigration into brain perivascular space leads to neuroinflammation and memory deficits in neuropathic pain

Chun-Lin Mai[1$], Zhi Tan[1$], Ya-Nan Xu[1$], Jing-Jun Zhang[3$], Zhen-Hua Huang[4], Dong Wang[5], Hui Zhang[6], Wen-Shan Gui[1], Jun Zhang[1], Zhen-Jia Lin[1], Ying-Tong Meng[1], Xiao Wei[1], Ying-Tao Jie[1], Peter M. Grace[7], Long-Jun Wu[8], Li-Jun Zhou[1, 2]✉, Xian-Guo Liu[1, 2]

[1]Department of Physiology and Pain Research Center, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China

[2]Guangdong Province Key Laboratory of Brain Function and Disease, Guangzhou 510080, China

[3]Department of Anesthesiology and Pain Clinic, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, 510080, China

[4]Division of Emergency Medicine, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, 510080, China

[5]Department of Clinical Laboratory, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, China

[6]Department of Anesthesiology, Guangdong Second Provincial General Hospital, Guangzhou, 510317, China

[7]Department of Critical Care & Respiratory Care Research (PMG), University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

[8]Department of Neurology, Mayo Clinic, Rochester, MN 55905, USA

[$] These authors contributed equally to this work.

✉Corresponding author: Li-Jun Zhou, E-mail: zhoulij3@mail.sysu.edu.cn.

Supporting Figures

Figure S1

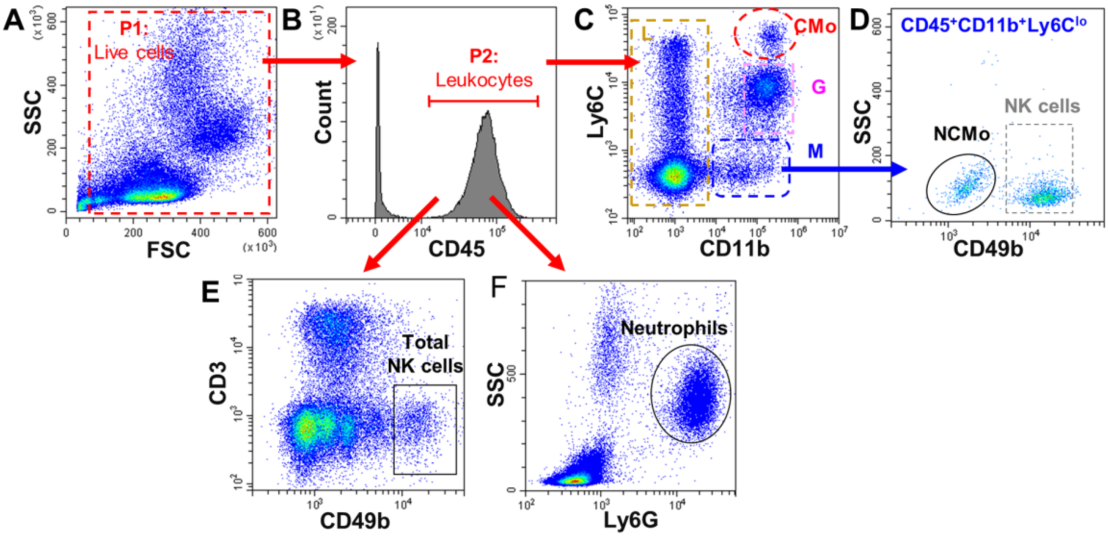

Figure S1. Gating strategy for circulating leukocyte subtypes. (**A, B**) After erythrocyte removal, blood live cells were first gated on forward and side scatter (FSC, SSC, A, P1), and then gated with CD45 antibody (B, P2) for isolating leukocytes. (**C, D**) The leukocytes were first subdivided into classical monocytes (CMo: CD45^{+}CD11b^{+}Ly6C^{high}, red box), granulocytes (G: CD45^{+}CD11b^{+}Ly6C^{med}, purple box) and lymphocytes (L: CD45^{+}CD11b^{-}, golden box) based on CD11b and Ly6C staining. CD45^{+}CD11b^{+}Ly6C^{low} (M, blue box) cells were further divided into NK cells (CD45^{+}CD11b^{+}Ly6C^{low}CD49b^{+}, grey box) and nonclassical monocytes (NCMo: CD45^{+}CD11b^{+}Ly6C^{low}CD49b^{-}, black circle) based on CD49b staining. (**E, F**) Representative plots pre-gated on CD45^{+} cells (B, P2) from which further subsets were divided into total NK cells (E, CD45^{+}CD3^{-} CD49b^{+}) based on CD3 and CD49b expression or neutrophils (F, CD45^{+}SSC^{med}Ly6G^{+}) based on SSC and Ly6G as indicated.

Figure S2

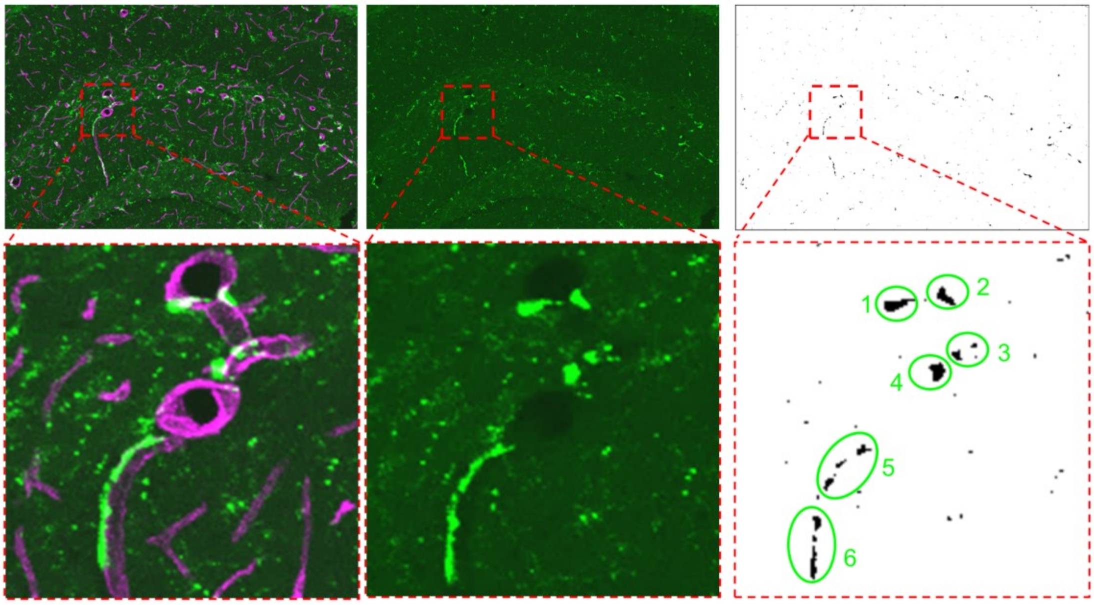

Figure S2. Fixed-intensity threshold for outlining CD68^{high} (green) cells around blood vessels (magenta, marked by PECAM-1) by Image J software. Quantification of immunofluorescence staining was analyzed by the number of CD68^{high} PVMs and fluorescent integrated intensity (IntDen) in 10× field micrographs.

Figure S3

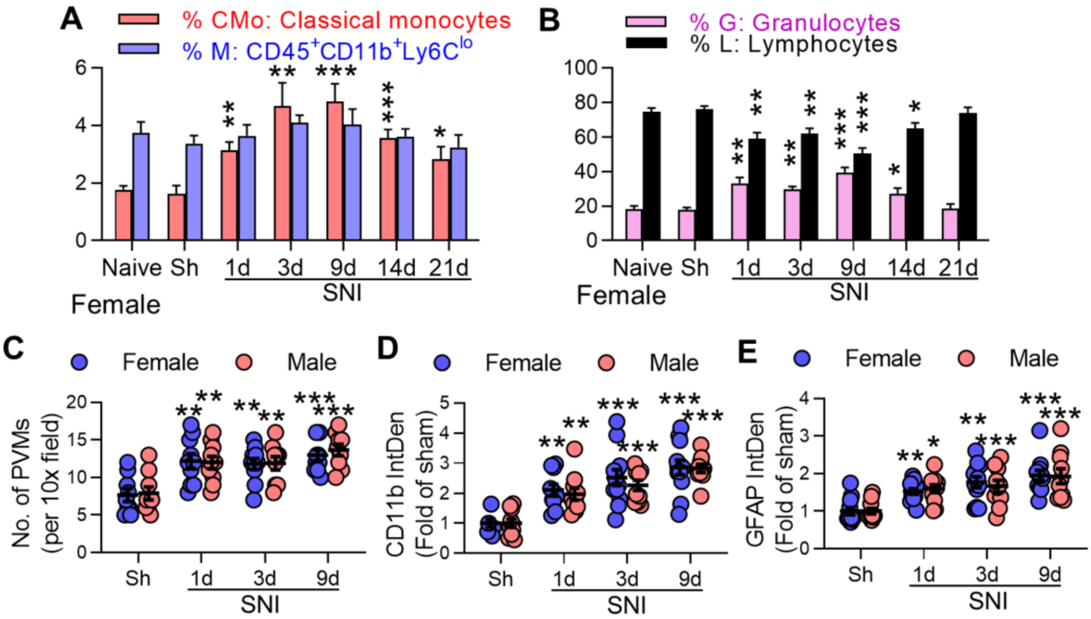

Figure S3. The changes in blood leukocytes, hippocampal PVMs, and gliosis in female mice induced by SNI are not different from those in male mice. (**A, B**) Time course of changes in circulating leukocyte subpopulations induced by SNI in female mice. n = 3-6 mice/group. (**C**) PVMs were increased in hippocampi of male and female SNI mice. (**D, E**) Fluorescent IntDen of CD11b (microglia marker) and GFAP astrocyte (marker) in hippocampi of male and female mice in sham and SNI mice. n = 3 mice/group, 3-4 sections/mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* sham group, two-way ANOVA with Bonferroni's post hoc test.

Figure S4

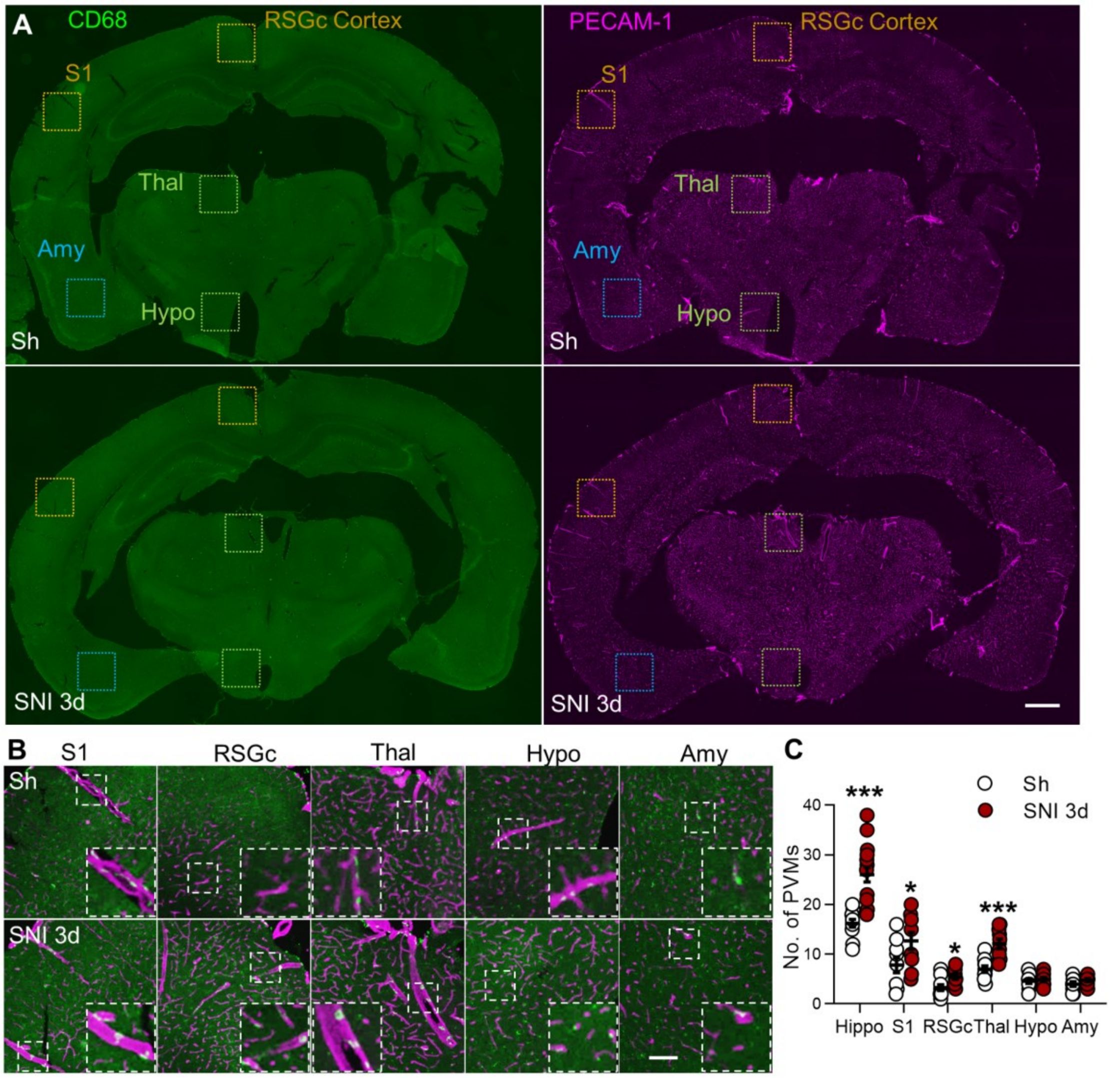

Figure S4. SNI increases PVMs in many brain regions, but most profoundly in the hippocampus. (**A-C**) Representative confocal images and quantitative data showing the expression of CD68 (green) and PECAM-1 (magenta) immunoreactivity in different brain regions in sham and SNI groups at 3 d after surgery (n = 3 mice/group, 3-4 images/mice). Small colored dotted boxes (in A) are magnified in B. Hippo: Hippocampus; S1: primary somatosensory cortex, RSGc: retrosplenial granular cortex, Thal: thalamus, Hypo: hypothalamus, Amy: amygdala. Scale bars, 500 μm (A), 25 μm (B). *$P < 0.05$, ***$P < 0.001$ vs. sham group, two-way ANOVA with Bonferroni's post hoc test.

Figure S5

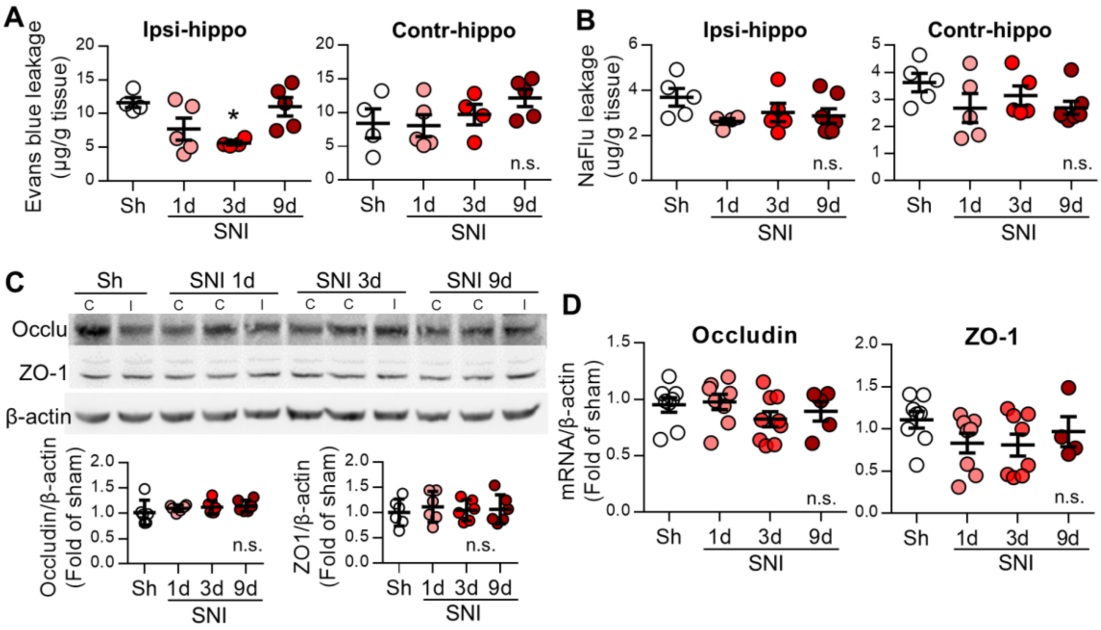

Figure S5. Hippocampal BBB permeability was not interrupted by SNI. (A, B) Quantification of Evans Blue (EB, A) or Fluorescein sodium (NaFlu, B) content in the bilateral hippocampi at different time points after SNI. n = 4-7 mice/group. (C) Western blot analysis for occludin-1 and ZO-1 in bilateral hippocampi. Western blot results are expressed by comparing the relative expression protein/β-actin from the sham group vs. SNI groups (n = 6 in each group). I: ipsilateral hippocampus, C: contralateral hippocampus. (D) qRT-PCR analysis for occludin-1, ZO-1 mRNA in bilateral hippocampi. n = 4-9 mice/group. Values are presented as means ± SEM. *$P < 0.05$ *vs.* sham group; n. s.: not significant; one-way ANOVA with Bonferroni's post hoc test.

Figure S6

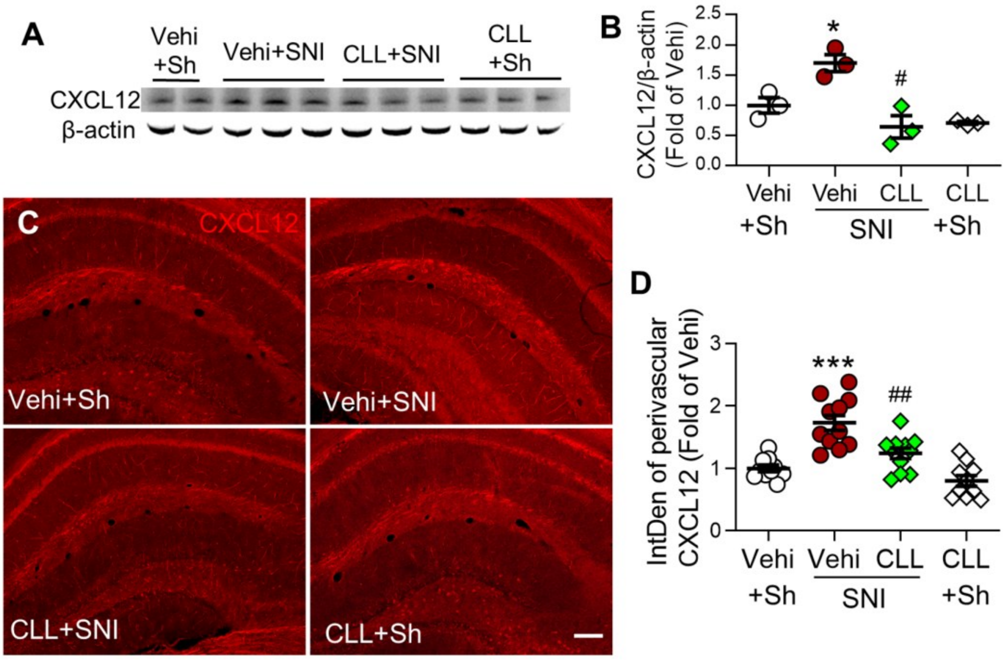

Figure S6. The upregulation of CXCL12 in hippocampal perivascular spaces induced by SNI is prevented by deletion of circulating monocytes with clodronate. (**A, B**) Western blots show hippocampal CXCL12 expression in various groups as indicated in sham (Sh) and SNI mice with or without clodronate at 9 days after surgery (n = 3 in each group). Vehi: vehicle. (**C, D**) Immunostaining revealed that injection of clodronate prevented the SNI-induced CXCL12 upregulation in the hippocampus but had no effect in sham mice (n = 3 mice/group, 3-4 images/mice). Scale bar, 100 μm. * $P < 0.05$, ***$P < 0.001$ *vs.* vehicle sham group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *vs.* SNI group, one-way ANOVA with Bonferroni's post hoc test.

Figure S7

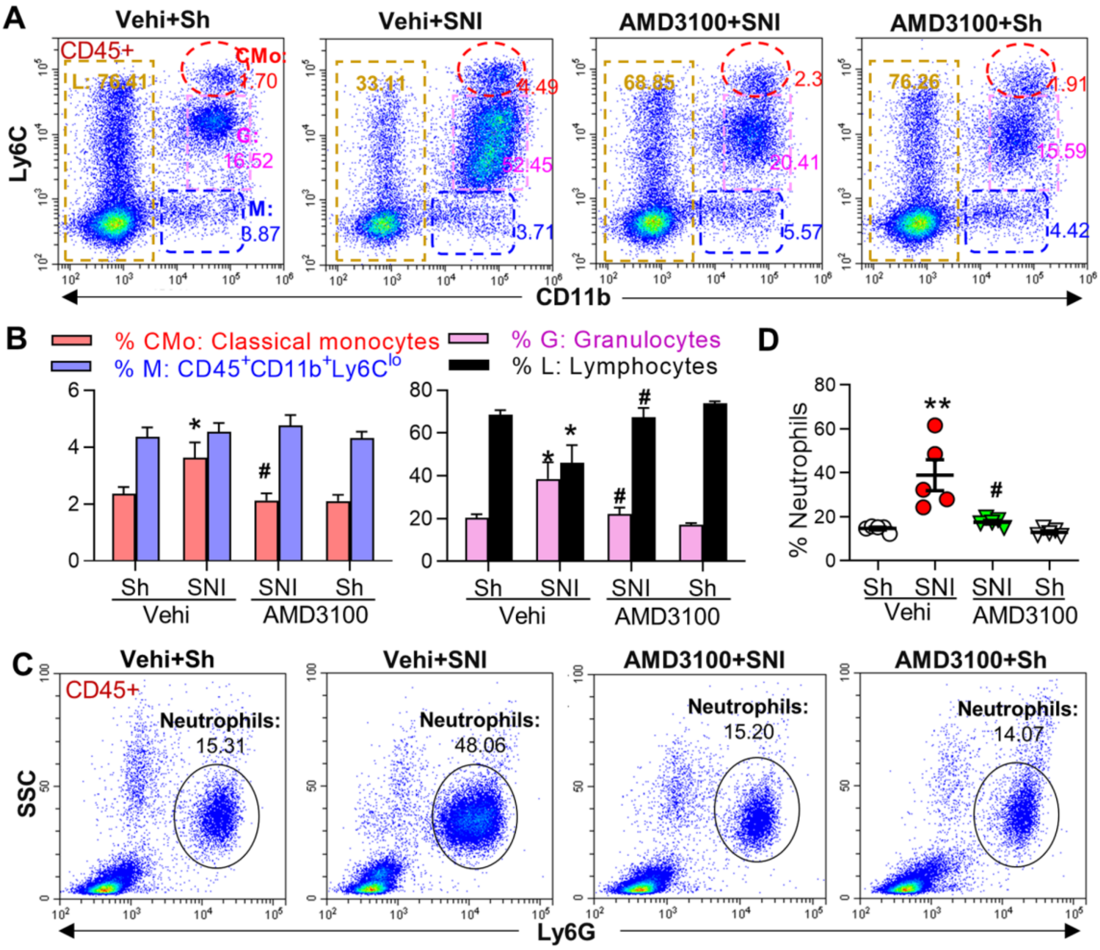

Figure S7. Blockade of CXCR4 prevents SNI-induced changes in circulating leukocytes. (**A-D**) Representative flow bivariate dot plots and statistical data show the changes in blood classical monocytes (CMo), $CD45^{+}CD11b^{+}L6C^{low}$ cells (M), granulocytes (G) and lymphocytes (L) and neutrophils in various groups (n = 5 mice/group). **P* < 0.05, ***P* < 0.01, *vs.* vehicle sham group, $^{\#}P < 0.05$, *vs.* vehicle SNI group, two-way (B) and one-way (D) ANOVA with Bonferroni's post hoc test.

Table S1. Antibodies used in this study.

Antibody	Application (Dilution)	Catalog number	Source
rat anti-CD68	IF (1:200)	Cat# 137006	Biolegend, San Diego, CA, USA
rabbit anti-CXCL12	IF (1:400) WB (1:1000)	Cat# ab18919	Abcam, Cambridge, UK
goat anti-PECAM-1	IF (1:100)	Cat# AF3628	R&D Systems, Minneapolis, MN, USA
goat anti-GFAP	IF (1:1000)	Cat# ab53554	Abcam, Cambridge, UK
rat anti-CD11b	IF (1:200)	Cat# 101202	Biolegend, San Diego, CA, USA
rabbit anti-P2Y12	IF (1:400)	Cat# AS-55042A	Anaspec, Fremont, CA, USA
goat anti-CD13	IF (1:200)	Cat# AF2335	Novus, Centennial, CO, USA
mouse anti-β-actin	WB (1:5000)	Cat# ab170325	Abcam, Cambridge, UK
mouse anti-occludin	WB (1:1000)	Cat# 331500	Invitrogen, Carlsbad, CA, USA
rabbit anti-ZO-1	WB (1:1000)	Cat# 617300	Invitrogen, Carlsbad, CA, USA
AF700 rat anti-mouse CD45	FC (1:400)	Cat# 103128	Biolegend, San Diego, CA, USA
APC rat anti-mouse/human CD11b	FC (1:400)	Cat# 101212	Biolegend, San Diego, CA, USA
BV510 rat anti-mouse Ly6C	FC (1:400)	Cat# 128033	Biolegend, San Diego, CA, USA
BV510 rat anti-mouse Ly6G	FC (1:400) IF (1:200)	Cat# 127633	Biolegend, San Diego, CA, USA
APC rat anti-mouse CD3	FC (1:400) IF (1:200)	Cat# 100235	Biolegend, San Diego, CA, USA
FITC hamster anti-mouse CD49b	FC (1:400) IF (1:200)	Cat# 103503	Biolegend, San Diego, CA, USA
AF488 donkey anti-rat IgG	IF (1:500)	Cat# A21208	Life Technologies, Carlsbad, CA, USA
AF555 donkey anti-rabbit IgG	IF (1:500)	Cat# A31572	Life Technologies, Carlsbad, CA, USA
AF647 donkey anti-goat IgG	IF (1:500)	Cat# A21447	Life Technologies, Carlsbad, CA, USA

AF555 donkey anti-goat IgG	IF (1:500)	Cat# A21432	Life Technologies, Carlsbad, CA, USA
FITC goat anti-hamster IgG	IF (1:500)	Cat# 405502	Biolegend, San Diego, CA, USA
Goat anti-mouse IgG (H&L) HRP	WB (1:10000)	Cat# ab136815	Abcam, Cambridge, UK
Donkey anti-rabbit IgG (H&L) HRP	WB (1:10000)	Cat# ab6802	Abcam, Cambridge, UK

Table S2. The demographic data and blood test results of chronic pain patients and healthy controls.

Note: Chronic pain patients (P), healthy controls (C), Postherpetic neuralgia (PHN), Trigeminal neuralgia (TN), Orofacial Pain (OFP), neuropathic pain (NP), chronic back pain (CBP), osteoarthritis (OA), complex regional pain syndrome (CRPS), Numeric Rating Scale (NRS), Montreal Cognitive Assessment (MoCA), leukocytes (Leu), granulocytes (G), neutrophil (Neu), lymphocyte (L), monocytes (Mo), eosinophils (Eos), basophil (Bas).

#P	Gender	Age	Years of education	Disease diagnosis	Years of diseases	NRS	MoCA	Leu ($\times 10^9$/L)	G (%Leu)	Neu (%Leu)	L (%Leu)	Mo (%Leu)	Eos (%Leu)	Bas (%Leu)	CXCL12 (pg/mL)
1	M	44	9	OA	2.00	5	25	4.19	56.10	52.70	37.70	6.20	2.40	1.00	58.33
2	F	81	9	CRPS	5.00	6	24	5.04	77.10	74.90	17.10	5.80	1.80	0.40	153.75
3	M	38	12	CBP	1.00	7	23	7.68	60.30	54.00	31.00	8.70	4.90	1.40	175.42
4	F	54	7	OA	2.00	6	23	4.25	53.90	51.10	37.60	8.50	2.10	0.70	177.92
5	M	80	9	PHN	0.25	9	22	6.43	71.40	69.10	22.70	5.90	2.00	0.30	179.17
6	F	75	12	PHN, OA	1.00	4	23	9.83	74.90	74.80	18.10	7.00	0.00	0.10	137.92
7	M	59	12	PHN	10.00	8	22	11.88	76.40	72.20	13.00	10.60	3.60	0.60	373.33
8	F	70	9	OA, CRPS	0.50	5	23	4.12	64.80	63.10	27.40	7.80	1.50	0.20	434.17
9	F	25	16	CBP	0.25	5	26	11.26	68.80	67.80	22.60	8.60	0.30	0.70	190.00
10	M	37	12	NP	6.00	6	26	6.63	65.90	64.40	27.80	6.30	0.90	0.60	87.92
11	F	51	6	CBP	20.00	4	22	5.42	55.60	53.90	38.70	5.70	1.30	0.40	145.83
12	M	49	7	CRPS	0.42	7	20	3.65	58.20	54.10	31.80	10.00	3.40	0.70	185.83
13	M	45	9	OFP, CRPS	0.50	6	22	5.92	76.80	76.00	18.60	4.60	0.30	0.50	586.25
14	M	34	16	NP	0.25	6	29	5.92	61.60	59.90	32.10	6.30	1.00	0.70	157.08
15	M	63	16	CBP, CRPS	0.25	6	25	5.17	67.00	64.10	25.10	7.90	2.50	0.40	426.67
16	F	58	6	PHN, CRPS	10.00	7	20	7.14	61.60	61.20	30.00	8.40	0.10	0.30	766.67
17	F	19	13	CRPS	1.00	7	30	7.88	62.30	59.70	26.00	11.70	2.20	0.40	125.00
18	F	64	12	NP	0.25	5	24	5.79	59.60	57.40	34.70	5.70	1.60	0.60	139.58
19	F	52	12	OA, CRPS	0.50	6	26	4.95	65.30	63.30	23.80	10.90	1.60	0.40	805.42
20	M	67	9	OA	1.00	5	22	5.96	70.40	66.90	22.80	6.80	2.90	0.60	19.17
21	F	67	9	CBP	0.25	5	25	9.01	65.50	63.60	28.60	5.90	1.60	0.30	104.58
22	F	55	7	CBP	0.50	5	21	8.17	73.00	67.50	21.40	5.60	4.00	1.50	104.58
23	F	63	6	OA	1.00	5	22	6.38	66.50	59.50	26.50	7.00	6.60	0.40	102.08
24	F	49	6	TN	2.00	5	23	7.28	76.00	74.50	16.30	7.70	1.20	0.30	85.83

25	M	83	6	CRPS	40.00	9	21	6.96	71.60	70.60	22.10	6.30	0.90	0.10	150.42
26	F	39	12	CBP	0.25	8	27	12.67	77.90	71.90	17.40	4.70	3.70	2.30	114.58
27	M	76	6	OA, CBP	2.00	5	22	5.33	58.40	53.50	33.20	8.40	4.50	0.40	141.67
28	M	52	9	CBP	1.00	6	28	7.48	68.40	66.70	25.20	6.40	1.60	0.10	170.83
29	F	42	6	OA, CBP	0.42	7	22	6.52	66.30	64.60	26.20	7.50	1.40	0.30	116.67
30	M	28	16	NP, CBP	1.00	5	26	6.01	65.00	63.80	28.00	7.00	1.20	0.00	186.67

#C	Gender	Age	Years of education	Disease diagnosis	Years of diseases	NRS	MoCA	Leu ($\times10^9$/L)	G (%Leu)	Neu (%Leu)	L (%Leu)	Mo (%Leu)	Eos (%Leu)	Bas (%Leu)	CXCL12 (pg/mL)
1	M	56	9	-	-	0	28	5.81	60.50	57.40	33.60	5.90	2.40	0.70	31.74
2	F	50	9	-	-	0	28	4.74	59.50	57.20	34.30	6.20	1.30	1.00	8.01
3	F	42	12	-	-	0	29	5.34	58.90	55.40	34.60	6.50	2.80	0.70	11.89
4	F	47	7	-	-	0	26	5.11	52.90	49.50	39.20	7.90	2.80	0.60	17.85
5	F	49	12	-	-	0	26	7.69	63.50	61.20	32.40	4.10	1.80	0.50	31.80
6	M	42	9	-	-	0	28	5.38	53.10	45.70	41.30	5.60	6.70	0.70	23.87
7	F	35	16	-	-	0	28	4.85	55.70	54.50	39.60	4.70	0.80	0.40	21.97
8	M	44	12	-	-	0	28	7.76	53.20	50.90	39.60	7.20	1.60	0.70	23.70
9	M	59	9	-	-	0	29	4.75	60.10	55.00	34.10	5.80	4.40	0.70	16.96
10	F	50	9	-	-	0	27	9.36	70.10	68.00	25.00	4.90	1.50	0.60	20.68
11	F	57	12	-	-	0	29	6.12	55.20	52.20	39.00	5.80	2.60	0.40	38.57
12	F	52	9	-	-	0	30	5.16	50.60	48.70	44.30	5.10	1.50	0.40	31.67
13	F	66	9	-	-	0	27	5.60	62.10	57.20	30.10	7.80	4.30	0.60	40.00
14	F	37	9	-	-	0	27	5.15	64.20	63.10	29.60	6.20	0.90	0.20	15.00
15	M	45	12	-	-	0	26	5.39	47.20	44.00	45.20	7.60	2.20	1.00	0.83
16	F	57	12	-	-	0	26	6.30	49.80	48.50	42.40	7.80	1.30	0.00	35.00
17	F	60	9	-	-	0	29	6.53	55.20	53.20	39.70	5.10	2.00	0.00	40.00
18	M	57	6	-	-	0	27	4.58	70.50	67.50	23.90	5.60	2.40	0.60	29.89
19	F	43	6	-	-	0	26	6.54	63.90	60.40	29.60	6.50	3.10	0.40	30.69
20	F	58	6	-	-	0	28	5.22	60.30	58.80	33.40	6.30	0.90	0.60	40.83
21	M	40	12	-	-	0	30	6.17	59.30	57.30	34.00	6.70	1.40	0.60	78.72
22	M	38	7	-	-	0	30	6.27	61.30	58.20	32.60	6.10	2.70	0.40	10.03
23	M	47	6	-	-	0	25	5.70	57.40	55.50	34.60	8.00	1.40	0.50	58.04
24	M	54	16	-	-	0	29	7.25	58.90	56.80	34.90	6.20	1.60	0.50	39.59
25	M	44	12	-	-	0	29	6.84	57.40	54.60	36.20	6.40	1.90	0.90	32.35
26	F	44	9	-	-	0	26	3.96	50.50	46.40	42.50	7.00	3.30	0.80	28.00
27	F	36	12	-	-	0	28	4.39	55.10	53.50	38.90	6.00	1.00	0.60	32.20
28	F	46	9	-	-	0	27	4.52	55.50	52.30	36.80	7.70	2.60	0.60	49.99
29	F	53	9	-	-	0	26	8.19	72.50	71.20	22.20	5.30	1.20	0.10	2.50
30	M	53	7	-	-	0	29	8.27	63.00	49.20	30.60	6.40	13.30	0.50	28.33
31	F	51	12	-	-	0	28	5.57	58.60	55.40	35.50	5.90	3.20	0.00	16.67
32	F	47	6	-	-	0	27	6.75	64.00	63.00	28.30	7.70	0.90	0.10	19.17
33	M	52	12	-	-	0	28	11.15	60.60	59.30	33.40	6.00	1.20	0.10	0.00
34	M	73	8	-	-	0	27	10.25	78.40	71.90	16.00	5.60	6.40	0.10	51.67
35	F	37	9	-	-	0	29	6.44	57.20	56.20	36.60	6.20	0.80	0.20	40.00

36	F	29	16	-	-	0	30	4.34	67.40	66.00	29.60	3.00	1.10	0.30	56.67
37	M	71	12	-	-	0	28	7.53	87.90	86.60	7.60	4.50	0.90	0.40	39.80
38	F	27	9	-	-	0	26	5.99	50.20	48.70	42.20	7.60	1.00	0.50	16.18
39	M	44	9	-	-	0	28	7.23	62.30	58.50	31.60	6.10	2.90	0.90	14.00
40	F	58	12	-	-	0	27	6.08	51.00	48.40	43.00	6.00	1.90	0.70	52.88