**Supplementary Figures**

**C:\Users\Administrator\Desktop\s\图片1.tifFigure S1. The NOD-like receptor signaling pathway is enriched in the spinal cord of IMQ-treated mice.**

(A) the KEGG analysis of upregulated genes; (B) Gene set enrichment analysis (GSEA) of the NOD-like receptor signaling pathway (MMU04621). NES, normalized enrichment score; (C) Heatmap summaries of representative genes related to the NOD-like receptor signaling pathway, where the red genes indicate upregulated genes, and blue genes indicate downregulated genes; (D) Fpkm values of Pycard, Casp1, Nlrp3, Myd88, Tlr4, Tnf, Gsdmd, Nod1, and Irf9; (E) mRNA levels of *Gsdmd*, *Irf9, Myd88*, *Nod1, Tlr4*, and *Tnf* in the spinal cord were confirmed by qPCR. \**P* <0.05, \*\**P* <0.01, \*\*\**P* < 0.001, unpaired t test in D, one-way ANOVA followed by Dunnett’s test in E. Values are presented as mean ± SEM.

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**Figure S2.** Representative PCR-based genotyping gel images to confirm the targeting of *Il1b*(A)*, Grpr* (B)*, Casp1* (C), *eGFP* (D) in mice.

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**Figure S3. Microglia are the primary cellular source of IL-1β and ASC.**

(A) Representative plots (gating on live IL-1β+ or ASC+ cells) showing differentiation of two populations in the spinal cord based upon CD45 and CD11b expression: CD45dim CD11b+ microglia and CD45high CD11b+ monocytes; (B) The percentage of IL-1β+ microglia and monocytes are increased in spinal cord after three days IMQ treatment; (C) The percentage of ASC+ microglia and monocytes are increased in spinal cord after three days IMQ treatment; (D) Quantification of IL-1β or ASCcoexpressed on CD45dim CD11b+ microglia and CD45high CD11b+ monocytes. Data are presented as a percentage of all IL-1β+ CD11b+cells or ASC+ CD11b+cells; (E) Co-localization of IL-1β (green) and TMEM119 (red) after IMQ treatment compared to the control group; n = 10-15 sections from 3 mice; (F) Quantification of IL-1β expressed in TMEM119+ microglia; (G) A schematic of microglia inhibitor minocycline administration in IMQ-induced chronic itch; (H-J) *Il1b, Irf8,* and *Fos* mRNA levels were detected by qPCR in the spinal cord after minocycline treatment. \**P* <0.05, \*\**P* <0.01, \*\*\**P* < 0.001, unpaired t test in B, C, F, G and H. Values are presented as mean ± SEM.

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**Figure S4. Detection of neuronal activation after treatment with IMQ, VX-765 and MCC950.**

(A-B) Representative immunostaining images and quantification graphs showing the numbers of (A) c-Fos+ or (B) pERK+ cells that were increased in the spinal cords of IMQ-treated mice; (C and E) Representative immunostaining images and (D and F) quantification graphs showing the numbers of (C) c-Fos+ or (E) pERK+ cells that were decreased in the spinal cords after treatment with VX-765 (i.t. injection, 25 n mol) and MCC950 (i.t. injection, 10 n mol). \*\*\**P* < 0.001, unpaired t test in A and B. one-way ANOVA followed by Dunnett’s test in D and F. Values are presented as mean ± SEM.n = 10-15 sections from 3 mice. Scale bar, 50 µm.

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**Figure S5. IL-1β or microglia were localized close to activated neurons and GRPR+ neurons.**

(A, C) Double immunostaining of IL-1β with (A) c-Fos or (C) pERK in the spinal cord of chronic itch mice; (B, D) Double immunostaining of Iba1 with (B) c-Fos or (D) pERK in the spinal cord of IMQ-treated mice; (E-F) Double immunostaining of GRPR+ neurons with (E) IL-1β or (F) Iba1 in the spinal cord of chronic itch mice. Scale bar, 25 µm.

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**Figure S6. NLRP3 inflammasome activation in the spinal cords of multiple types of chronic itch.**

(A, C, E) Schematic experimental protocols of the house dust mite allergen Der f 2-induced itch model, SADBE-induced ACD model, and AEW model; (B, D, F) mRNA levels of *Nlrp3*, *Pycard*, *Casp1, Il1b, Il18, Tlr4*,and *Myd88* in spinal cords of the (B) Der f 2-induced itch model, (D) SADBE-induced ACD model, and (F) the AEW model were detected by qPCR; (G-I) Representative immunostaining images and quantification graphs showing the numbers of (G, H) IL-1β+, (G, I) ASC+ cells that were increased in the spinal cords of the Der f 2, SADBE, and AEW models. \**P* <0.05, \*\**P* <0.01, \*\*\**P* < 0.001, unpaired t test in B, D, F, H and I. Values are presented as mean ± SEM.n = 10-15 sections from 3 mice. Scale bar, 50 µm in G.

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**Figure S7. Spinal microglia contribute to Der f 2-, SADBE-, and AEW-induced chronic itch.**

(A-F) Representative immunostaining images and quantification graphs showed that (A-B) Der f 2, (C-D) SADBE, and (E-F) AEW treatment increased the protein expression of Iba1 in the spinal cords. \*\*\**P* < 0.001, unpaired t test in B, D and F. Values are presented as mean ± SEM.n = 10-15 sections from 3 mice. Scale bar, 50 µm in A, C and E.

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**Figure S8. cFos and pERK expression in spinal cord associated with multiple types of chronic itch.**

Representative immunostaining images and quantification graphs showing the numbers of (A, C, E) c-Fos+ or (B, D, F) pERK+ cells that were increased in the spinal cords of Der f 2-, SADBE-, and AEW-treated mice. \*\*\**P* < 0.001, unpaired t test in G and H. Values are presented as mean ± SEM.n = 10-15 sections from 3 mice. Scale bar, 50 µm in A-F.

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**Figure S9. Blockade of the Caspase-1-IL-1β axis attenuated SADBE-, Der f 2-, and AEW-induced chronic itch.**

(A-C) SADBE-, Der f 2-, and AEW-induced chronic itch was attenuated significantly in *Casp1*-/- mice; (D-E) The number of scratches was decreased in in *Il1b*-/- mice after SADBE and AEW treatment; (F-H) SADBE-, Der f 2-, and AEW-induced chronic itch was impaired after IL-1β mAb treatment. \**P* <0.05, \*\**P* <0.01, unpaired t test in A-H. Values are presented as mean ± SEM..

**Materials and Methods**

**Drugs and reagents**

GRP18-27 (Cat. #H-3120) was purchased from Bachem (King of Prussia, PA). Murine IL-1β was purchased from PeproTech (Cat. #AF-211-11B). Blank-saporin (Cat. #IT-21-25) and bombesin-saporin (BB-sap; Cat. #IT-40-25) were purchased from Advanced Targeting Systems (San Diego, CA). rmIL-1Ra (Cat. #480-RM-010) and mIL-1β-neutralizing antibody (Cat. #AF-401-SP) were purchased from R&D. Minocycline was purchased from Sigma (Cat. #M9511, St. Louis, MO). MCC950 Sodium (Cat. #S7809) and VX-765 (Cat. #S2228) were purchased from Selleck (Danvers, MA).

**Mouse model of psoriasis**

To induce psoriasis, 50 mg of imiquimod cream (IMQ) or vehicle cream was applied on the shaved dorsal skin of the neck once a day for 7 consecutive days. The experimental animals were sacrificed, and cervical spinal cord tissues were collected for immunostaining, ELISA, and Western blot analysis after imiquimod treatment for 3 days and RNA-seq analysis and RNAScope in situ hybridization (ISH) assay after IMQ treatment for 2 days.

**Mouse model of allergic contact dermatitis**

Mice were sensitized by topical application of 20 μL of 1% SADBE (dissolved in acetone, SADBE, Cat. #339792, Sigma-Aldrich) to shaved abdominal skin once daily for three consecutive days. After 5 days, the SADBE-treated group was challenged by topical application of 20 μL of 1% SADBE on the pre-shaved dorsal skin of the neck once a day for 3 consecutive days, while the control group was challenged with 25 μL of acetone alone. After the last SADBE treatment, spinal cord tissues were collected for immunostaining and qPCR analysis as shown in the results.

**House dust mite allergen Der f 2-induced itch model**

For allergen-associated itch, we first sensitized animals by intraperitoneal (i.p.) injection 50 μg of house dust mite allergen Der f 2 in saline with Imject Alum Adjuvant (PI77161, Thermo Scientific, Asheville, NC). Ten days later, animals were re-injected (i.p. injection) with 50 μg of Der f 2 and Imject Alum Adjuvant to induce allergic sensitivity. After one week, sensitized animals were challenged with 25 μg of Der f 2 to induce allergic itch. The experimental animals were sacrificed, and cervical spinal cord tissues were collected for immunostaining and qPCR analysis after Der f 2 treatment for three weeks.

House dust mite allergen Der f 2 was expressed in *Escherichia coli* BL21. The endotoxin was removed by Endotoxin Removal Spin Columns (Cat. #88277, Thermo Fisher Scientific). The concentration of LPS was detected by the endotoxin quantification assay (A39552S, Thermo Fisher Scientific).

**AEW model**

After shaving the skin on the neck of the mice, we applied a mixture of acetone and ether (1:1) on the neck skin for 15 seconds, followed by painting with distilled water on the same area for 30 seconds. The repetitive cutaneous treatment was applied twice daily for seven consecutive days to induce dry skin itch. The mice in the control group were only exposed to water for 45 seconds.

**Scratching behavior tests**

Itch behavior were performed as previously described (ref). Briefly, mice were given 30 minutes to acclimate to the test chamber (10 × 10.5 × 15 cm) before recording. The scratching behaviors were observed for 60 minutes before IMQ/SADBE/AEW treatment or after being challenged with Der f 2. The footage was manually counted to assess the number of scratches in a 60-minute period. A scratch is defined as lifting the hind limb toward the body and then returning the limb back to the ground, no matter how many scratching motions occur between the two movements. All applicable behavioral tests were performed and analyzed under the condition that the experimenter kept the genotype or grouping confidential.

**Drug treatment**

Blank-saporin (blank-sap, 400 ng/mouse) or bombesin-saporin (BB-sap, 400 ng/mouse) was intrathecally injected 14 days before IMQ treatment. The mice received intrathecal injection with anti-IL-1β mAb or IgG isotype (1 μg per mouse), interleukin 1 receptor antagonist IL-1ra (100 ng per mouse), MCC950 (10 n mol per mouse), VX-765 (25 n mol per mouse) and intraperitoneal injection with MCC950 (50 mg/kg, 10 mg/kg), VX-765 (100 mg/kg), or minocycline (33 mg/kg), followed by the recording of their behavior and sacrifice.

For the activated GRPR+ neuron staining in the cervical dorsal horn of mice, IL-1β (10 ng/mouse), GRP (0.03 nmol/mouse), or IL-1β combined with GRP were intrathecally administered, and the mice were perfused with saline rapidly followed by 4% paraformaldehyde.

**RNA extraction and Quantitative PCR (qPCR) analysis**

To extract whole tissue RNA, samples were homogenized in TRIzol (Cat. #15596018, Invitrogen) using a homogenizer. RNA-to-cDNA conversion was performed using RevertAid™ Master Mix (Cat. #M16325, Thermo Scientific). Real-time quantitative PCR (qPCR) was performed using LightCycer 480 SYBR Green Master (Cat. #4887352001, Roche). The specificity of the qPCR amplification was assessed by melting curve analysis. The primer sequences are presented in Table S1.

**RNA sequencing (RNA-seq) analysis**

Total RNA was isolated from mice spinal cords with TRIzol reagent as mentioned above. RNA sequencing (RNA-seq) analysis was performed by Novogene (Beijing, China). The differential expression analysis of two groups was performed using the DESeq2 R package (1.16.1). KEGG Pathway Analysis, GO Term Enrichment, Reactome pathway analysis, and gene set enrichment analysis (GSEA) were performed for pathway enrichment analysis.

**Histology and immunostaining of the skin or spinal cord tissue**

The skin sections were placed on a slide drier at 65 °C for 30 minutes until the paraffin on the slides was completely dissolved. The skin sections were soaked in TO transparent agent for 5 minutes twice, absolute ethanol for 5 minutes twice, 95% ethanol for 5 minutes, 80% ethanol for 5 minutes, 70% ethanol for 5 minutes, and distilled water for 5 minutes. After baking, dewaxing, and rehydration, hematoxylin–eosin staining could be performed. A hematoxylin staining solution was added dropwise to cover the tissue and stain for 10-15 min. The slides were rinsed and differentiated by the dropwise addition of 1% hydrochloric acid ethanol for 30 s. After washing, eosin staining solution was added dropwise to the slices for 1-2 min. After rinsing, dehydration, and transparency, the slides were sealed and observed under a microscope. The epidermal thickness was measured using ImageJ software.

For the immunostaining of the spinal cord tissues, mice were anesthetized and fixed by cardiac perfusion. The spinal cords were dissected and post-fixed in 4% paraformaldehyde for 48 hours. Then tissues were transferred to a 20% sucrose solution for dehydration for 24 hours. After dehydration, the spinal cords were embedded in OCT and cut into 4 μm frozen sections. Spinal cord tissues were washed 3 times with PBST, blocked with 3% goat serum, followed by incubation with primary antibody overnight. The following primary antibodies were used: GFP (1:500; Cat No. ab13970, Abcam), c-Fos (1:1000; Cat No. 226 003, Synaptic Systems), Phospho-p44/42 MAPK (p-ERK, 1:500; Cat No. 4370, CST), mouse anti-NeuN antibody (1:500; Cat No. MAB377, Merck Millipore, Temecula, CA), mouse anti-GFAP antibody (1:2000; Cat No. MAB360, Merck Millipore), mouse anti-Iba1 antibody (1:500; Cat No. MABN92, Merck), IL-1β (3A6) Mouse mAb(1:300; Cat No. 12242, CST), NLRP3 (D4D8T) Rabbit mAb(1:200; Cat No. 15101, CST), ASC (D2W8U) Rabbit mAb (1:300; Cat No. 67824, CST), Ki-67 (D3B5) Rabbit mAb (1:500; Cat No. 12202, CST), and TMEM119 (1:400; Cat No. ab209064, Abcam). The sections were incubated with the secondary antibody for one and a half hours at room temperature. After washing 3 times with PBST, the sections were adhered to the glass slide to dry until the tissues were translucent. The mounting medium was added dropwise to cover the slide. Fluorescent imaging was performed on a confocal laser-scanning microscope (Zeiss LSM880).

**RNAScope in situ hybridization (ISH) assay**

In situ hybridization (ISH) was performed using the RNAScope Multiplex Fluorescent Assay V2 (323100; Advanced Cell Diagnostics). The spinal cord tissue slices were placed on a baking tablet at 60 ℃ for half an hour and then incubated with dropwise hydrogen peroxide for 10 minutes to remove endogenous peroxidase. Then, the slices were transferred to the target retrieval in a beaker, and the temperature was maintained at 98–102 °C with a hotplate for 10 minutes. After rinsing with water, the slices were soaked in absolute ethanol for three minutes, taken out, and dried. Tissue was used to carefully dry the glass surrounding the sample slices, and a hydrophobic barrier pen was used to draw around the section. Then 2-4 drops of Protease Ⅲ were added to cover each tissue section, incubated at 40 °C for 30 minutes, and washed with distilled water twice. The following probes purchased from Advanced Cell Diagnostics were used: *Grpr* (Cat No. 317871-C2, target region bases 463-1596; accession No. NM\_008177.2), *Vglut2* (*Slc17a6*, Cat No. 319171-C2, target region bases 1986 - 2998; accession No. NM\_080853.3), *Vgat* (*Slc32a1*, Cat No. 319191-C2, target region bases 894 - 2037; accession No. NM\_009508.2), *Nlrp3* (Cat No. 439571-C2, target region bases 2 - 1098; accession No. NM\_145827.3), *Il1r1* (Cat No. 413211 -C1, target region bases 301 - 1407; accession No. NM\_001123382.1), *Casp1* (Cat No. 404551-C1, target region bases 429 - 1440; accession No. NM\_009807.2), *Rbfox3* (Cat No. 313311-C3, target region bases 1827 - 3068; accession No. NM\_001039167.1), *Gfap* (Cat No. 313211-C3, target region bases 2 - 1761; accession No. NM\_001131020.1), *Itgam* (Cd11b, Cat No. 311491-C3, target region bases 538 - 1528; accession No. NM\_001082960.1). Then probe hybridization was carried out according to the requirements of the kit and AMP, and probes of each channel and fluorescent dye were incubated in sequence. The tissue slices were washed twice with washing buffer after each incubation. After the above steps were completed, the mounting medium was added dropwise to seal the film for observation and analysis. For the coexpression studies, two punctuated dots associated with a single DAPI-stained nuclei were counted as copositive.

**Flow cytometry analysis**

After perfusion with PBS, the dorsal horn of the spinal cord was dissected out and incubated in 1ml RPMI 1640 medium containing 30 μL papain (Cat. No. LS003126, Worthington) at 37°C for 20 min. The cell suspension was filtered through a 70-μm cell strainer. Add 1 µg anti-mouse CD16/CD32 (2.4G2 mAb; BD Biosciences, Cat. No. 553142) to 100 µL of the cells to block the Fc receptors. Incubate 10-15 min on ice, protected from light. Dead cells were excluded using Fixable Viability Stain 510 (BD Bioscience, Cat. No. 564406). Cells were surface stained for 20 min with anti-mouse CD11b (M1/70)- PerCP/Cy5.5 (BioLegend, Cat. No. 101228), anti-mouse CD45 (30-F11)-FITC (BioLegend, Cat. No. 103108). Intracellular staining for IL-1β (3A6) Mouse mAb (1:300; Cat No. 12242, CST) and ASC (3A6) Rabbit mAb (1:400; Cat No. 67824, CST) was performed for 60 min, after treatment with permeabilization buffer (eBioscience). Goat anti-rabbit antibody conjugated to Alexa Fluor 647 (Cat No. A-21244, Thermo Fisher Scientific) and goat anti-mouse antibody conjugated to Alexa Fluor 555 (Cat No. A-21422, Thermo Fisher Scientific) were used for staining for 30 min. Data were analysed with FlowJo software (Tree Star Inc., Ashland, OR).

**Western blot analysis**

Tissues were lysed in ice-cold Tissue Extraction Reagent I (FNN0071, ThermoFisher, USA) containing protease inhibitor cocktail (Cat. #11873580001, Roche, Branford, CT). The protein samples with loading buffer and heat were mixed in a water bath at 100 °C for 8 minutes. Protein samples (20 μg) were separated by 12% SDS-PAGE gels and then transferred onto PVDF membranes. After electrophoresis, the membranes were blocked with 3% BSA for 60 minutes at room temperature, followed by incubation with primary antibody at 4 °C overnight. The following primary antibodies and secondary antibodies purchased from Cell Signaling Technology were used: NLRP3 (Cat# 15101), Caspase1 (Cat# 2225), ASC (Cat# 67824), IL-1β (Cat# 12242), β-Tubulin (Cat# 2146) and GAPDH (Cat# 2118). After removing the primary antibody diluent and washing three times with PBST, the membranes were incubated with the secondary antibody for 90 minutes at room temperature. A chemiluminescence reagent (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific) was added to the membranes. The chemiluminescence detection system was used to record the bands.

**Table S1.** Primers were used for real-time quantitative PCR.

|  |  |  |
| --- | --- | --- |
| **Target** | **Forward primer (’5 to 3’)** | **Reverse primer (’5 to 3’)** |
| *Gapdh* | CCCAGCAAGGACACTGAGCAA | TTATGGGGGTCTGGGATGGAAA |
| *Actb* | TGTTACCAACTGGGACGACA | GGGGTGTTGAAGGTCTCAAA |
| *Il-1β* | CACTCATTGTGGCTGTGGAGAAG | TTGTTCATCTCGGAGCCTGTAG |
| *Tnf* | AGAGGCACTCCCCCAAAAGAT | GGGTCTGGGCCATAGAACTGAT |
| *Nlrp3* | TCCCAGACACTCATGTTGCC | GTCCAGTTCAGTGAGGCTCC |
| *Casp1* | GCTACCTGGCAGGAATTCTGGA | TCAGTCCTGGAAATGTGCCATC |
| *Pycard* | CCATATGTGGCCCAGTGGTAG | TGGGGAGCCAGGAATCATTTG |
| *Il1r1* | CTTGAGGAGGCAGTTTTCGT | AGCCCCAGTAGCACTTTCAT |
| *Myd88* | ACTGATGCGGAGCCAGATTC | TGGGAGGAAAGGCAGTCCTA |
| *Gsdmd* | CCTGAGTTCCGCTCTTGGTC | CGATGGCATGGTCCTCGATT |
| *Irf9* | GACAGCAACAGCAACTGCAA | CGGCCACCATAGATGAAGGT |
| *Tlr4* | ACTGTTCTTCTCCTGCCTGAC | GGGACTTTGCTGAGTTTCTGA |
| *Il18* | TCAGACAACTTTGGCCGACT | GGTCACAGCCAGTCCTCTTAC |
| *Fos* | CCCGAGCTGGTGCATTACA | GAGGCCAGATGTGGATGCTT |
| *Aif1* | GGACAGACTGCCAGCCTAAG | GACGGCAGATCCTCATCATT |
|  |  |  |

**Table S2.** Primers were used for PCR-based genotyping.

|  |  |  |
| --- | --- | --- |
| Strains | Primer name | **Sequence (’5 to 3’)** |
| *Grpr-/-* | *Neo* | GATCTCTCGTGGGATCATTG |
|  | *2* | AGCCAGGTACTTGCTGGCAT |
|  | *3* | CATCAACAAACTGAGCTAGAGT |
| *Casp1-/-* | *F1* | AATCTGTATTCACGCCCTGTTG |
|  | *R1* | GATCACCTTGGGCTTGTCTTTC |
|  | *R2* | TGCTCCACATTTGTCCTTCAAC |
| *Il1b1-/-* | *F1* | GCTCTGCTGTTGCTTCCTTCC |
|  | *R1* | ACGTTGACAGCTAGGTTCTGTTC |
|  | F2-WT | GGAAACAACAGTGGTCAGGAC |
|  | R2-WT | ACGTTGACAGCTAGGTTCTGTTC |
| GRPR-eGFP | *eGFP*-Fwd | GCGACGTAAACGGCCACAAG |
| *eGFP*-Rev | AGCTCGATGCGGTTCACCAG |