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ORIGINAL ARTICLE

Microglia–neuron interactions promote chronic itch via the NLRP3-IL-1β-GRPR axis

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Abstract

Background: Spinal astrocytes contribute to chronic itch via sensitization of itch-specific neurons expressing gastrin-releasing peptide receptor (GRPR). However, whether microglia–neuron interactions contribute to itch remains unclear. In this study, we aimed to explore how microglia interact with $GRPR^+$ neurons and promote chronic itch.

Methods: RNA sequencing, quantitative real-time PCR, western blot, immunohistochemistry, RNAscope ISH, pharmacologic and genetic approaches were performed to examine the roles of spinal NLRP3 (The NOD-like receptor family, pyrin-containing domain 3) inflammasome activation and IL-1β-IL1R1 signaling in chronic itch. Grpr-eGFP and Grpr KO mice were used to investigate microglia–$GRPR^+$ neuron interactions.

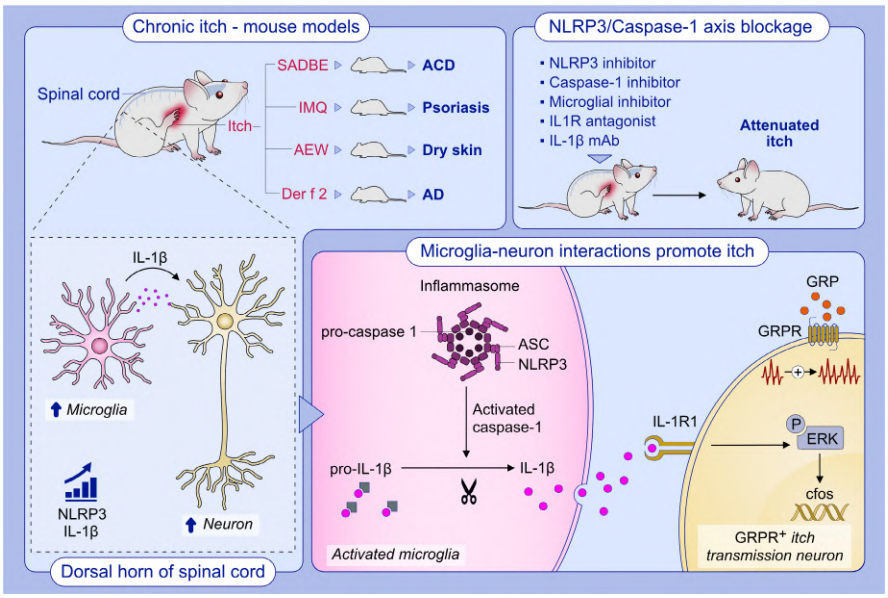
Results: We observed NLRP3 inflammasome activation and IL-1β production in spinal microglia under chronic itch conditions. Blockade of microglial activation and the NLRP3/caspase-1/IL-1β axis attenuated chronic itch and neuronal activation. Type 1 IL-1 receptor (IL-1R1) was expressed in $GRPR^+$ neurons, which are essential for the development of chronic itch. Our studies also find that $IL\text{-}1\beta^+$ microglia are localized in close proximity to $GRPR^+$ neurons. Consistently, intrathecal injection of IL1R1 antagonist or exogenous IL-1β indicate that the IL-1β-IL-1R1 signaling pathway enhanced the activation of $GRPR^+$ neurons. Furthermore, our results demonstrate that the microglial NLRP3/caspase-1/IL-1β axis contributes to several different chronic itches triggered by small molecules and protein allergens from the environment and drugs.

Conclusion: Our findings reveal a previously unknown mechanism in which microglia enhances the activation of $GRPR^+$ neurons through the NLRP3/caspase-1/IL-1β/IL1R1 axis. These results will provide new insights into the pathophysiology of pruritus and novel therapeutic strategies for patients with chronic itch.

KEYWORDS
chronic itch, gastrin-releasing peptide receptor, IL-1β, microglia, NLRP3 inflammasome

Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; AEW, acetone–ether–water; ASC, the adapter protein apoptosis-associated speck-like protein containing CARD; Der f 2, group 2 major mite allergen from *Dermatophagoides farina*; ERK, extracellular regulated protein kinases; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; IL, interleukin; IMQ, imiquimod; mAb, monoclonal antibody; NLRP3, NOD-like receptor thermal protein domain-associated protein 3; SADBE, squaric acid dibutylester.

Xueting Liu, Yanmei Wang and Yueling Zeng contributed equally.



GRAPHICAL ABSTRACT

NLRP3 inflammasome and IL-1β are selectively expressed in spinal microglia of four types of clinically related allergic itch models. Blockade of the NLRP3/Caspase-1 axis attenuated chronic itch and neuronal activation. Microglia enhances the activation of GRPR$^+$ neurons through the IL-1β-IL1R signaling pathway.

1 | INTRODUCTION

Itch is a sensation that brings an urge to scratch.[1] Chronic itch is a principal manifestation of skin diseases, including psoriasis, allergic contact dermatitis (ACD), and atopic dermatitis (AD), dramatically reducing the quality of life.[2,3]

Small molecules and protein allergens are the main causes of skin inflammation and chronic itch from the environment and drugs. Imiquimod (IMQ) was first identified as a compound with anti-herpes virus activity during screening.[4] It was reported that the most common adverse side effect is itching and mild burning.[4] A small molecule, the hapten SADBE (squaric acid dibutylester), is used in the therapy of patients with alopecia areata or alopecia totalis but often induces skin inflammation and persistent itch.[5] In previous studies, imiquimod was used to establish a chronic pruritus model of psoriasis,[6] and SADBE was used to establish a chronic itch model of allergic contact dermatitis.[7,8] The house dust mite (HDM) in the environment is one of the main causes of atopic dermatitis.[9] Der f 2 is a major dust mite allergen among HDM-sensitized AD patients.[10] In this study, Der f 2 was used to develop allergic skin inflammation associated with chronic itch and the histopathological features of an exacerbated type 2 immune response similar to that seen in human AD. Acetone–ether–water (AEW) was used to establish a dry skin model that was similar to chronic pruritus of unknown origin without robust inflammation.[11,12] The pathogenesis of chronic pruritus remains unclear and hinders the development of effective treatments.[2,3] In this study, we used IMQ, SADBE, AEW, and Der f 2-induced itch models to investigate the common mechanism of itch.

Gastrin-releasing peptide receptor (GRPR) plays an essential role in itch transmission of the spinal cord.[1,7,13] Meanwhile, GRPR-expressing neurons were confirmed as a key component of the spinal itch circuit.[7,14,15] Previous studies demonstrated that the excitability of spinal GRPR$^+$ neurons is enhanced by astrocyte-derived lipocalin-2.[16] In addition, spinal IL-33 promotes chronic itch via STAT3-dependent reactive astrocytes and TNF-α secretion, which regulates the activation of GRPR$^+$ neurons.[17]

Increasing evidence suggests that spinal microglia contribute to chronic itch. Microglia contributed to chronic itch through the CX3CR1/p38 MAPK pathway in a DNFB-induced ACD model.[18] Furthermore, minocycline, the microglial inhibitor, suppressed itch-related behavior in the IMQ model and the atopic dermatitis model.[19,20] However, the underlying molecular mechanisms remain unexplored. Microglia are the major source of several inflammatory mediators that drive central sensitization in the central nervous system.[21–24] Recent studies have indicated that microglia release platelet-derived growth factor B, which promotes neuronal potassium currents via PDGFRα.[25] Microglia-derived IL-1β regulates neuron activity and facilitates insulin release.[26] However, whether microglia–neuron interactions contribute to itch remains unclear.

Inflammasome is a complex comprising a variety of proteins, including NLRs (nod-like receptors), ASC (the adapter protein apoptosis-associated speck-like protein containing CARD), and the cysteine protease caspase-1.[27] It has been demonstrated that morphine sustained neuropathic pain in rats by enhancing NLRP3 inflammasome activation in the superficial microglia of the spinal dorsal horn.[28] However, the role of NLRP3 inflammasome and IL-1β in chronic itch remains unclear.

In this study, we aimed to explore how microglia contribute to the activation of GRPR$^+$ neurons and promote chronic itch. We report that microglia enhanced GRPR$^+$ neurons' activation through the NLRP3-caspase-1-IL-1β-IL1R Axis. NLRP3 inflammasome activation and IL-1β production were observed in the spinal microglia of several

types of chronic itch models. Blocking NLRP3 inflammasome activation, IL-1β, or receptor IL1R1 signaling can effectively attenuate chronic itch and the activation of $GRPR^+$ neurons. Thus, these findings facilitate our understanding of microglia–neuron interactions that promote chronic itch, providing advantages that inform therapeutic interventions for chronic itch.

2 | RESULTS

2.1 | NLRP3 inflammasome is activated in the spinal cord after IMQ treatment

To investigate the crucial central mechanisms of chronic itch, RNA sequencing analysis was performed in the spinal cord of mice using an IMQ-induced psoriasis model[6] (Figure 1A–D). The IMQ-treated mice exhibited spontaneous scratching behavior and thickening of the epidermis (Figure 1B,C), consistent with previous reports.[6,15,29] The results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and the gene set enrichment analyses (GSEAs) indicated that the NOD-like receptor signaling pathway (MMU04621) was highly enriched in the IMQ groups (Figure S1A,B). Further analysis found that 58 differentially expressed genes (DEGs) in the NOD-like receptor signaling pathway (MMU04621) were enriched in the IMQ groups (Figure S1C). Consistently, we confirmed the RNA-seq results by qPCR and found that *Gsdmd*, *Tlr4*, *Myd88*, *Tnf*, *Irf9*, and *Nod1* were upregulated under chronic itch conditions (Figure S1D,E). *Tlr4* and *Myd88* are essential for the transcriptional expression of *Nlrp3* and *Il1b*.[28] GSDMD is a pore-forming protein that controls IL-1β release.[30]

Gene ontology (GO) enrichment analysis results indicated that the NLRP3 inflammasome complex assembly (GO:0044546) was highly enriched in the IMQ groups (Figure 1E). Accordingly, the results of the GSEAs revealed that inflammasome complex (GO:0061702) gene sets were enriched in the IMQ groups (Figure 1F). The heatmap results revealed that *Nlrp3* expression was increased after IMQ treatment (Figure 1G). As shown in the volcano plot, IMQ increased the expression of several genes of the NLRP3 inflammasome components, including *Pycard*, *Casp1*, and *Nlrp3* (Figure 1H). The qPCR and immunoblot results confirmed that the expression of *Pycard* (ASC), *Casp1* (caspase-1), and *Nlrp3* (NLRP3) were increased in the spinal cord after IMQ treatment (Figure 1I,J,K). The immunostaining results revealed that IMQ treatment increased the number of $NLRP3^+$ cells or cells with ASC specks (Figure 1L,M). Consistently, the enzymatic activity of caspase-1 was increased after IMQ treatment (Figure 1N).

2.2 | IL-1β expression is increased in the spinal cord after IMQ treatment and contributes to chronic itch

The GO analysis results showed that interleukin-1 beta secretion (GO:0050702) was highly enriched (Figure 1E). Using GSEA analysis, we found significant enrichment of the interleukin-1 beta production (GO_0032611) and interleukin-1 family signaling (R-MMU-446652) gene sets after IMQ treatment (Figure 2A,B). The immunoblot (Figure 2C,D), ELISA (Figure 2E), qPCR (Figure 2F), and immunofluorescence (Figure 2G,H) results demonstrate that the protein and mRNA expression levels of IL-1β were increased after IMQ treatment. To investigate the critical role of IL-1β in IMQ-induced chronic itch, we used pharmacological (IL-1β mAb[31]) or genetic ($Il1b^{-/-}$ mice) disruption of IL-1β signaling (Figure S2A). The IMQ-induced chronic itch was significantly alleviated after IL-1β mAb treatment (1 μg per mouse, intrathecal injection) (Figure 2I). In addition, the itch was significantly reduced in $Il1b^{-/-}$ mice (Figure 2J). Decreased IL-1β levels in the spinal cords of $Il1b^{-/-}$ and WT mice were confirmed by qPCR (Figure 2K).

2.3 | NLRP3 inflammasome and IL-1β are selectively expressed in spinal microglia contribute to chronic itch

Strikingly, the GO analysis and GSEA results showed that microglial cell activation (GO_0001774) was highly enriched in the IMQ groups (Figure 1E and Figure 3A). The RNAscope ISH results showed that the mRNA expression of *Cd11b* (a microglia marker) was increased after IMQ treatment (Figure 3B,C). The results of the qPCR (Figure 3D) and immunofluorescence (Figure 3E) showed that the mRNA and protein expressions of Iba1 (ionized calcium-binding adaptor molecule 1, a microglial marker, and *Aif1* is the gene name of Iba1) were increased after IMQ treatment. In addition, we found that the number of proliferating microglia ($Ki67^+$ $Iba1^+$ cells) increased significantly (Figure 3E–G).

To identify the cell types of NLPR3 inflammasome activation, we performed immunofluorescence double staining for NLRP3, ASC, or IL-1β with Iba1 (a microglia marker), NeuN (Neuronal nuclei, a neuronal marker), and GFAP (Glial fibrillary acidic protein, an astrocyte marker)[17] (Figure 3H–J). The results of the immunofluorescence showed that NLRP3, ASC, and IL-1β were coexpressed in the microglia but not in astrocytes and neurons after IMQ treatment (Figure 3H–J).

Next, we used the RNAscope double in situ hybridization (ISH) technique to confirm the cellular distribution of *Nlrp3* or *Casp1* expression (Figure 3K,L). Our results indicate that *Nlrp3* mRNA was coexpressed with *Cd11b* mRNA (a marker of microglia) but not coexpressed with *Gfap* (a marker of astrocytes) or *Rbfox3* (a marker of neurons) (Figure 3K). The RNAscope ISH double staining showed that 63.7% of $Casp1^+$ cells were colocalized with the microglia marker (*CD11b*), 33.2% of $Casp1^+$ cells were colocalized with the marker of neurons (*Rbfox3*), and 11.8% of $Casp1^+$ cells were colocalized with the marker of astrocytes (*Gfap*) (Figure 3L). These results suggest that NLRP3 inflammasome activation is predominantly distributed in microglia but not in astrocytes and neurons.

CD11b and Iba1 are expressed in microglia and infiltrating monocytes, and CD45 is used as a distinction marker between

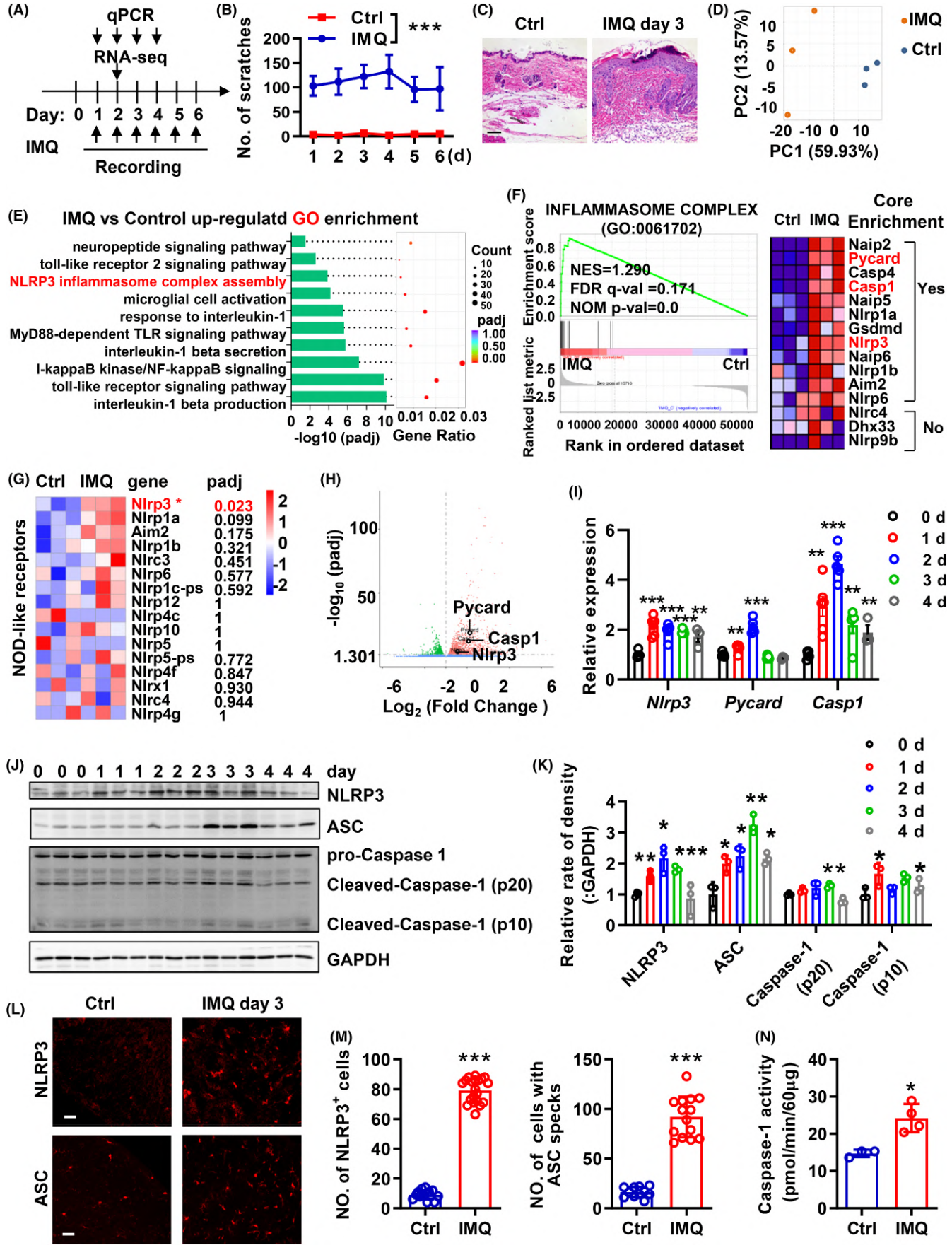


FIGURE 1 NLRP3 inflammasome activation in the spinal cords of IMQ-treated mice. (A) A schematic of the scheduled behavioral recording, RNA-seq, and qPCR analysis of the spinal cords of IMQ treated-mice; (B) Scratching behavior of IMQ-treated mice; (C) Representative H&E staining of the neck skin sections after 3 days IMQ treatment (Scale bar = 100 μm); (D) PCA of cervical spinal cord tissues from the IMQ and control groups; (E) the GO analysis of upregulated genes; (F) Gene set enrichment analysis (GSEA) and a heat map of the inflammasome complex (GO:0061702) gene sets. NES, normalized enrichment score; (G) Heatmap of representative genes related to NOD-like receptors; (H) *Nlrp3*, *Pycard*, and *Casp1* in volcano plots; (I) mRNA levels of *Nlrp3*, *Pycard*, and *Casp1* in the spinal cord were confirmed by qPCR; (J, K) IMQ treatment increased the protein expression of NLRP3, ASC, and caspase-1 in the spinal cord, $n = 3$; (L, M) Three days IMQ treatment increased the number of $NLRP3^+$ and cells with ASC^+ specks in the spinal cord (Scale bar = 25 μm); (N) Increased activity of caspase-1 was observed in the spinal cord after IMQ treatment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA in B, one-way ANOVA followed by Dunnett's test in I and K, unpaired t test in M and N. Values are presented as mean ± SEM. n = 12–15 sections from 3 mice.

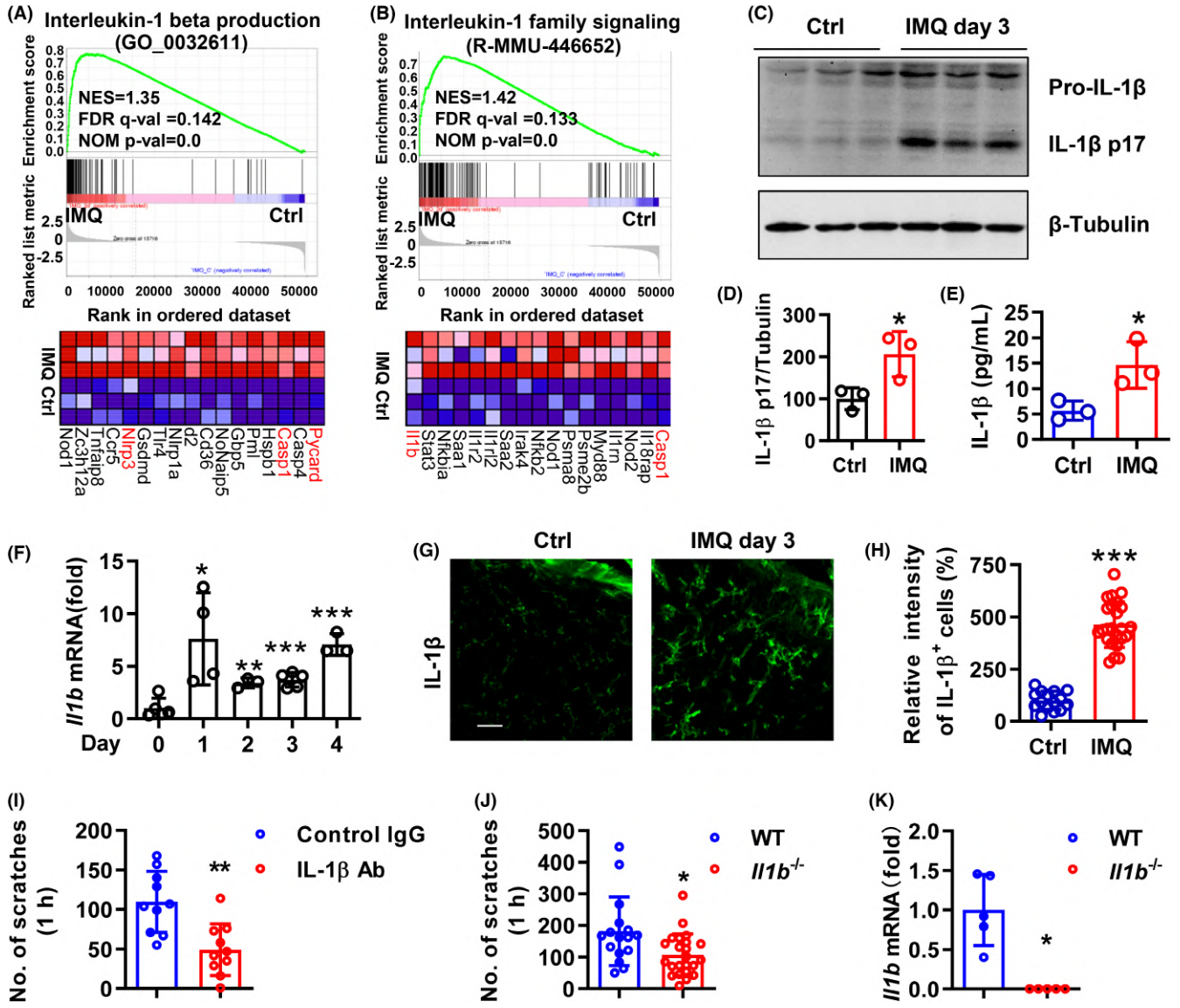


FIGURE 2 Spinal IL-1β contributes to chronic itch. (A–B) Gene set enrichment analysis (GSEA) and a heat map of the (A) interleukin-1 beta production (GO_0032611) and (B) interleukin-1 family signaling (R-MMU-446652) gene sets; (C-D) Three days IMQ treatment increased the protein expression of IL-1β in the spinal cord, n = 3; (E-F) The IL-1β mRNA level and concentration in the spinal cord were detected by (E) ELISA and (F) qPCR assay; (G–H) IMQ treatment increased the protein expression of IL-1β in the spinal cord on day 3; n = 15–20 sections from 3 mice; (I) IMQ-induced chronic itch was significantly attenuated by intrathecal injection of IL-1β mAb (1 μg per mouse) at 15 minutes before recording; (J) IMQ-induced chronic itch was impaired in *Il1b*$^{-/-}$ mice; (K) Decreased IL-1β levels in the spinal cord of Il1b$^{-/-}$ mice were detected by qPCR. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA followed by Dunnett's test in F, unpaired *t*-test in D, E, H, I, J and K. Values are presented as mean ± SEM. Scale bar, 25 μm in G.

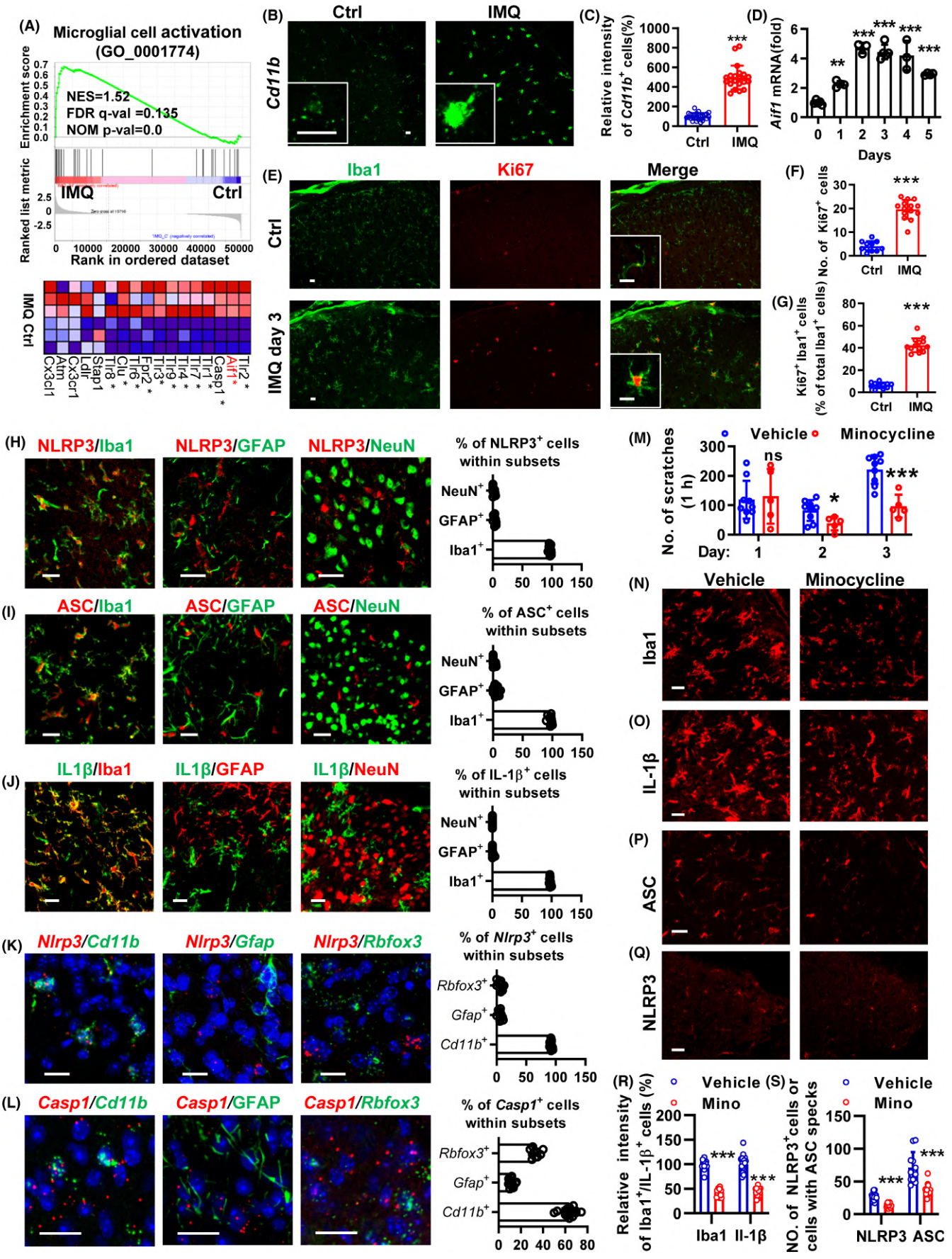


FIGURE 3 NLRP3 inflammasome activation and IL-1β production in the spinal microglia of mice with chronic itch. (A) Gene set enrichment analysis (GSEA) and a heat map of the microglial cell activation (GO_0001774) gene sets; (B-C) Representative (B) RNAscope ISH images and (C) the quantification graphs showing that IMQ treatment increased *Cd11b* mRNA levels in spinal cords; (D) Increased *Aif1* mRNA levels in spinal cord of IMQ-treated mice were detected by qPCR; (E) Co-localization of Iba1 (green) and Ki67 (red) after 3 days IMQ treatment compared to the control group; (F) Quantification graphs showing that IMQ treatment increased $Ki67^+$ cells in the spinal cords; (G) The percentage of co-labeled $Ki67^+$ and $Iba1^+$ cells of the total $Iba1^+$ cells were significantly increased after IMQ treatment; (H-J) Double immunostaining of (H) NLRP3, (I) ASC, or (J) IL-1β with markers of microglia (Iba1), astrocytes (GFAP), and neurons (NeuN) in spinal dorsal horn of mice after IMQ treatment; (K-L) RNAscope ISH staining of (K) *Nlrp3* or (L) *Casp1* with markers of microglia (*CD11b*), astrocytes (*Gfap*), or neurons (*Rbfox3*) in spinal dorsal horn of IMQ-induced mice; (M) IMQ-induced chronic itch was significantly attenuated after treatment with minocycline (i.p. 33 mg/kg). (N-O) Representative immunostaining images and (R) quantification graphs showing the protein expression levels of (N) Iba1 and (O) IL-1β were significantly decreased in the spinal cords after minocycline treatment; (P-Q) Representative immunostaining images and (S) quantification graphs showing the numbers of (P) cells with ASC specks or (Q) $NLRP3^+$ cells were significantly decreased in the spinal cords after treatment with minocycline; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA followed by Dunnett's test in D, unpaired *t*-test in C, F, G, M, R and S. Values are presented as mean ± SEM. $n = 10$–15 sections from 3 mice. Scale bar, 25 μm.

resident microglia and recruited blood monocytes.[32] We next investigated whether IL-1β or ASC expressed in microglia ($CD11b^+CD45^{dim}$) and infiltrating monocytes ($CD11b^+CD45^{high}$) using flow cytometry. Our results showed that 83.0% $IL\text{-}1\beta^+$ or 88.3% ASC^+ cells are $CD11b^+CD45^{dim}$ microglia and 17.0% $IL\text{-}1\beta^+$ or 11.7% ASC^+ cells are $CD11b^+CD45^{high}$ monocytes (Figure S3A–D). In addition, TMEM119 (transmembrane protein 119) is specifically expressed in microglia but not in other immune/neural cells.[32] Our immunofluorescence double staining results showed that 95.5% $IL\text{-}1\beta^+$ cells were observed in $TMEM119^+$ microglia (Figure S3E–F). Therefore, microglia are the main cellular source of IL-1β.

Minocycline inhibits microglia activation in vivo.[15] Consistent with previous studies,[20] we found that IMQ-induced scratching behavior was significantly suppressed after minocycline treatment (Figure 3M). Furthermore, the protein expression levels of Iba1 and IL-1β were significantly decreased in the spinal cords after minocycline treatment (Figure 3N,O,R). In addition, the numbers of cells with ASC specks or $NLRP3^+$ cells were significantly decreased in the spinal cords after treatment with minocycline (Figure 3P,Q,S). Consistently, the mRNA expressions of transcription factor interferon regulatory factor 8 (*Irf8*, a critical transcription factor for transforming microglia into a reactive phenotype[23,33]) and *Il1b* were decreased after minocycline treatment (Figure S3G–I). These data suggested that blockade of microglia activation attenuated NLRP3 inflammasome activation and IL-1β production.

2.4 | Blockade of the NLRP3/Caspase-1 axis attenuated chronic itch and neuronal activation

To elucidate the role of the NLRP3/caspase-1 axis in chronic itch, caspase-1 inhibitor, VX-765,[34] or NLRP3 inhibitor, MCC950,[27] were used. IMQ-induced chronic itch was significantly attenuated after treatment with VX-765 (Figure 4A,B, intrathecal and intraperitoneal injection) and MCC950 (Figure 4A,C, intrathecal and intraperitoneal injection). The immunoblot results showed that cleaved IL-1β p17 expression in the spinal cord was significantly decreased after VX-765 (Figure 4D,E) or MCC950 (Figure 4F,G) treatment.

To further explore the role of caspase-1 in chronic itch, $Casp1^{-/-}$ mice were used (Figure S2C). IMQ-induced chronic itch (Figure 4H) and cleaved IL-1β p17 production were significantly attenuated in $Casp1^{-/-}$ mice (Figure 4I,J). The mRNA expression of *Casp1* in the spinal cords of $Casp1^{-/-}$ and WT mice were detected by qPCR (Figure 4K). These results indicate that NLRP3 inflammasome activation contributes to IMQ-induced chronic itch.

To elucidate how NLRP3 inflammasome activation contributes to chronic itch, we tested neuronal activation after the blockade of the NLRP3/caspase-1 axis. Previous studies revealed both Fos[35] and pERK[29] expression in the spinal cord associated with spontaneous scratching. In this study, we found that the numbers of both Fos^+ and $pERK^+$ cells were significantly increased in the spinal cords after IMQ treatment (Figure S4A,B). Consistently, we detected $c\text{-}Fos^+$ and $pERK^+$ cells after pharmacological and genetic blockade of the NLRP3/caspase-1/IL-1β axis (Figure 4L–Q). We found that the numbers of $c\text{-}Fos^+$ (Figure 4R and Figure S4C,D) and $pERK^+$ (Figure 4S and Figure S4E,F) cells were significantly decreased in the spinal cords after IL-1β mAb, VX-765, or MCC950 treatments (pharmacological blockade) or in the spinal cords of $Il1b^{-/-}$ or $Casp1^{-/-}$ mice (genetic blockade). Furthermore, we found that the numbers of $c\text{-}Fos^+$ and $pERK^+$ (Figure 4N,R,S) cells and the mRNA expression of *Fos* were significantly decreased after minocycline treatment (Figure S3H). These data suggest that microglia, NLRP3 inflammasome, and IL-1β contribute to chronic itch via neuronal activation in the spinal cord.

To determine whether microglia-derived IL-1β activates neurons, we tested the location of the microglia, IL-1β, and activated neurons ($c\text{-}Fos^+$ and $pERK^+$ cells). The results of immunostaining showed that both microglia and $IL\text{-}1\beta^+$ cells were localized in close proximity to the $c\text{-}Fos^+$ and $pERK^+$ cells in the dorsal horn of the spinal cord (Figure S5A–D).

2.5 | IL-1R1 is expressed in $GRPR^+$ neurons and contributes to chronic itch

To explore how IL-1β contributes to chronic itch and neuronal activation, we focused on IL-1R1. The RNAscope ISH (Figure 5A,B) and qPCR (Figure 5C) results showed that the mRNA expression of *Il1r1*

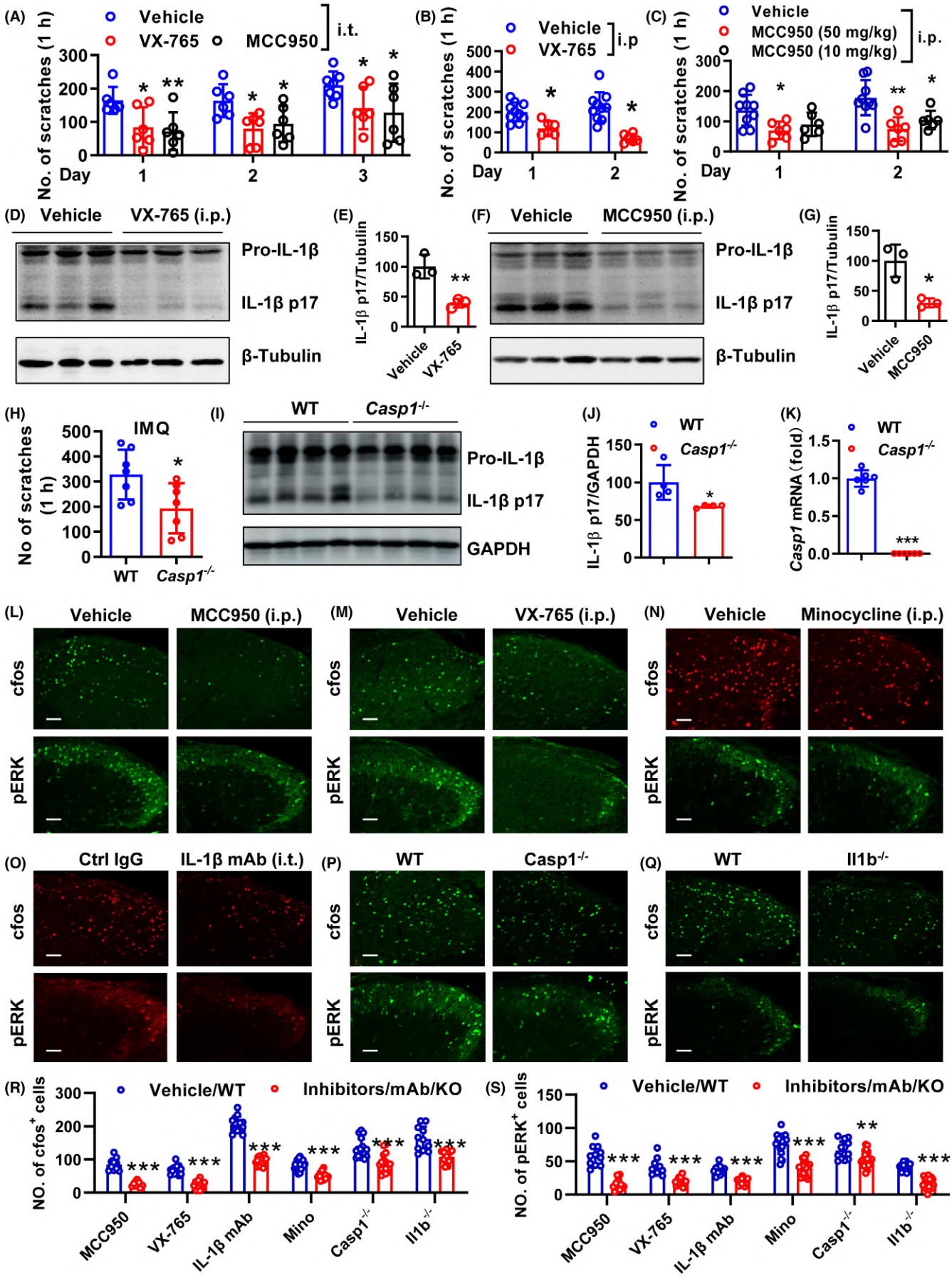


FIGURE 4 Blockade of the NLRP3/Caspase-1 axis attenuated IMQ-induced chronic itch and neuronal activation. (A) IMQ-induced chronic itch was significantly attenuated after intrathecal (i.t.) injection of VX-765 (25 n mol) and MCC950 (10 n mol); (B) IMQ-induced chronic itch was significantly attenuated after intraperitoneal (i.p.) injection of VX-765 (100 mg/kg); (C) IMQ-induced chronic itch was significantly attenuated after treatment with MCC950 (i.p. 10 and 50 mg/kg); (D–E) The protein expression of cleaved IL-1β p17 was decreased after VX-765 treatment (i.p. injection) in the spinal cord, $n = 3$; (F–G) The protein expression of IL-1β was decreased after MCC950 treatment (i.p. injection, 50 mg/kg) in the spinal cord, n = 3; (H) IMQ-induced chronic itch was attenuated in *Casp1*$^{-/-}$ mice; (I-J) Decreased cleaved IL-1β p17 levels in the spinal cord of *Casp1*$^{-/-}$ mice were detected by western blot analysis; (K) Decreased *Casp1* mRNA levels in the spinal cord of *Casp1*$^{-/-}$ mice were detected by qPCR; (L–Q) Representative immunostaining images and (R-S) quantification graphs showing the numbers of (R) c-Fos$^+$ or (S) pERK$^+$ cells that were decreased in the spinal cords after (L) MCC950 (i.p. injection, 50 mg/kg), (M) VX-765 (i.p. injection, 100 mg/kg), (N) minocycline (i.p. injection, 33 mg/kg) or (O) IL-1β mAb (i.t. injection, 1 μg) treatment or in (P) Casp1$^{-/-}$ and (Q) IL1b$^{-/-}$ mice; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA followed by Dunnett's test in A and C, unpaired t test in B, E, G, H, J, K, R and S. Values are presented as mean ± SEM. $n = 9$–15 sections from 3 mice. Scale bar, 50 μm.

was increased in the spinal cord after IMQ treatment. To determine the critical role of IL-1R1 in chronic itch, interleukin 1 receptor antagonist (IL1Ra) was used.[28] We found that IMQ-induced chronic itch was significantly attenuated after treatment with IL1Ra (Figure 5D).

We next explored the cellular distribution of *Il1r1* expressed in the spinal dorsal horn. The RNAscope ISH double staining showed that 81.88% of Il1r1$^+$ cells were colocalized with the neuronal marker (*Rbfox3*), 12.31% of *Il1r1*$^+$ cells were colocalized with the marker of astrocytes (*Gfap*), and 4.34% of *Il1r1*$^+$ cells were colocalized with the marker of microglia (*CD11b*) (Figure 5E–J,Q,R). Remarkably, 54.48% of *Il1r1*$^+$ cells were excitatory neurons (vesicular glutamate transporter 2, *Vglut2*$^+$), and 31.28% of *Il1r1*$^+$ cells were inhibitory neurons (vesicular GABA transporter, *Vgat*$^+$) (Figure 5K–N,Q,S).

To identify which itch-specific neuronal pathway was relevant to IL-1β-IL1R signaling, we examined whether IL1R1 was expressed in GRPR$^+$ neurons using the RNAscope double ISH. Our RNAscope ISH data revealed that 26.6% (46 of 173 cells) of *Grpr*$^+$ cells were co-expressed with *Il1r1* (Figure 5O,P). This result confirms that the two receptors were coexpressed in the same neurons in the spinal cord.

2.6 | GRPR is essential for IMQ-induced chronic itch

Next, we tested the critical role of GRPR in IMQ-induced chronic itch. The results of the RNAscope in situ hybridization showed that the mRNA level of *Grpr* was increased significantly after IMQ treatment (Figure 6A,B). Consistent with previous studies,[15] IMQ-induced chronic itch was significantly attenuated in *Grpr* KO mice (Figure 6C and Figure S2B) or was abolished after neurotoxin bombesin-saporin (BB-sap) treatment (Figure 6D).

2.7 | Microglia enhances the activation of GRPR$^+$ neurons through the IL-1β-IL1R signaling pathway

To explore whether microglia activates GRPR$^+$ neurons, we tested the location of the microglia, IL-1β, and GRPR$^+$ neurons. We took advantage of GRPR-eGFP mice that allow for the detection of GRPR$^+$ neurons by anti-GFP staining[7,29] (Figure S2D) and found that IL-1β or microglia were localized in close proximity to GRPR$^+$ neurons (Figure S5E,F) and activated GRPR$^+$ neurons (c-Fos$^+$ GRPR GFP$^+$ and pERK$^+$ GRPR GFP$^+$ cells) after IMQ treatment (Figure 6E–J).

To demonstrate that microglia-derived IL-1β can enhance the activation of GRPR$^+$ neurons directly, we detected the number of c-Fos$^+$ GFP$^{+35,36}$ and pERK$^+$ GFP$^{+29,35}$ cells (activated GRPR$^+$ neurons) after intrathecal injection of exogenous IL-1β. We found that the number of activated GRPR$^+$ neurons (c-Fos$^+$ GFP$^+$ and pERK$^+$ GFP$^+$ cells) was increased after intrathecal injection of IL-1β under the conditions of co-injection with GRP (Figure 6K,M) or IMQ treatment (Figure 6L,M). In addition, we found that the number of activated GRPR$^+$ neurons was decreased after intrathecal injection of IL1Ra (Figure 6N–Q).

To confirm that IL-1β-mediated chronic itch and neuronal activation depend on the GRPR pathway, we tested the effect of IL1Ra in *Grpr* KO mice. We found that IL1Ra had no effect on the number of c-Fos$^+$ and pERK$^+$ neurons (Figure 6O,R,S) or IMQ-induced scratching behavior in *Grpr* KO mice (Figure 6T).

Taken together, these results reveal that microglia enhanced the activation of GRPR$^+$ neurons through IL-1β-IL1R1 signaling.

2.8 | The microglial NLRP3 inflammasome-IL-1β axis contributes to neuronal activation and chronic itch in different itch models

At last, we expanded our studies to examine whether microglia contribute to chronic itch via the NLRP3/ASC/IL-1β axis in several murine models of chronic itch, including the HDM allergen Der f 2-induced allergic dermatitis (Figure S6A), the SADBE-induced ACD model (Figure S6C), and the AEW-induced dry skin model (Figure S6E). *Nlrp3*, *Pycard*, and *Caspase-1* were involved in the NLRP3 inflammasome components, and *Tlr4* and *Myd88* were involved in the transcriptional expression of *Nlrp3* and *Il1b*. Using qPCR, we found that *Nlrp3*, *Pycard*, *Tlr4*, *Myd88*, *Caspase-1*, and *Il1b* were upregulated in the spinal cords of the Der f 2-, SADBE-, and AEW induced-itch models (Figure S6B,D,F).

The immunofluorescence results showed that the numbers of cells with ASC specks (Figure S6G–I), the expression of IL-1β and Iba1 (Figure S6G–I and Figure S7), the numbers of c-Fos$^+$ and pERK$^+$

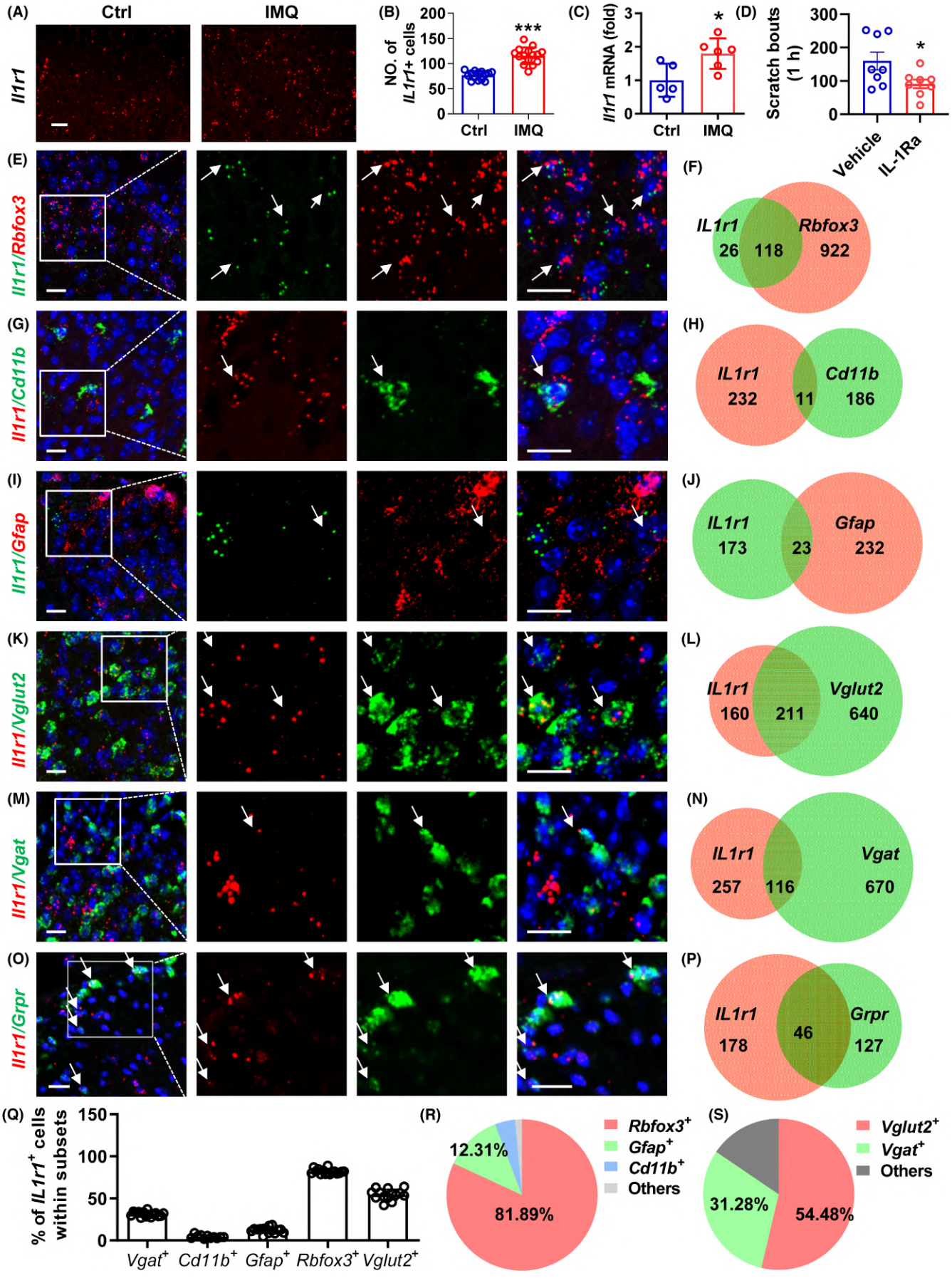


FIGURE 5 IL-1R1 in GRPR$^+$ neurons contributes to chronic itch. (A–B) Representative (A) RNAscope ISH images and (B) quantification graphs showing that the mRNA expression of *Il1r1* increased in the spinal cords of IMQ mice; (C) *Il1r1* mRNA levels in spinal cords of IMQ-treated mice and control mice were detected by qPCR; (D) IMQ-induced chronic itch was significantly attenuated by intrathecal injection of IL1Ra (100 ng per mouse) at 15 minutes before recording; (E, G, I, K, M, O) RNAscope ISH double staining of *Il1r1* with (E) Rbfox3 (neurons), (G) *CD11b* (microglia), (I) *Gfap* (astrocytes), (K) *Vglut2*, (M) *Vgat*, or (O) *Grpr* in spinal dorsal; (F, H, J, L, N, P) Venn diagrams for the quantification of *Il1r1* overlaps with the indicated markers; (Q–R) Quantification of the percentage of *Il1r1*$^+$ cells coexpressing commonly used markers of microglia, astrocytes, and neurons subtypes, and the data are presented as a percentage of all *Il1r1*$^+$ cells; (S) Quantification of *Il1r1* overlaps with *Vglut2* or *Vgat*. Data are presented as a percentage of all *Il1r1*$^+$ cells. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired *t*-test in B, C and D. Values are presented as mean ± SEM. $n = 10–15$ sections from 3 mice. Scale bar, 25 μm.

cells (Figure S8) were increased in the spinal cords of the Der f 2-, SADBE-, and AEW-induced itch models.

Furthermore, in the SADBE, Der f 2, and AEW models, the chronic itch was significantly attenuated in the Casp1$^{-/-}$ (Figure S9A–C) or Il1b$^{-/-}$ mice (Figure S9D–E) or after IL-1β mAb treatment (Figure S9F–H).

Taken together, these results demonstrate that microglial NLRP3 inflammasome activation is critical for neuronal activation and multiple types of chronic itch.

3 | DISCUSSION

Itching is the most disturbing symptom of allergies. Children and adults with atopic dermatitis either suffer from an intractable chronic itch or experience acute itching flares, which further significantly increase the intensity of itching. More complicated, chronic itching can be one of the most troublesome symptoms of other non-allergic conditions.[37] However, fewer studies have paid effort to figure out the central neuron-related mechanisms underlying the chronic itch. Sensory neurons, which trigger the migration of allergy-skewing DCs, act as primary sensors of allergens and initiate an allergic immune response,[38] have been the focus of a large number of studies. At the cellular level, allergens directly activate TRPV1$^+$ sensory neurons, leading to itch and pain behaviors.[38] A subset of basophils activate sensory neurons that drive allergen-induced itch flare-ups in atopic dermatitis.[39] Macrophage-expressed TRPV4 selectively participates in the inflammation and reflex pain-related responses through activation of the NLRP3 inflammasome.[40] The upregulated expression of the P2Y12 receptor after the activation of satellite glial cells induces an increase in ROS content, activation of NLRP3 inflammasome, elevated inflammatory cytokine release, and peripheral nerve damage in the dorsal root ganglion.[37] In the present study, we first demonstrated on spinal microglia level that microglia contribute to chronic itch through GRPR$^+$ neuron sensitization via the IL-1β/IL1R1 signaling pathway. A previous study reported that IL-1β modulated excitatory neurotransmission by enhancing the frequency and amplitude of sEPSCs.[41] Using GRPR-eGFP mice,[7,29] we found that activated GRPR$^+$ neurons (cfos$^+$ or pERK$^+$ GRPR$^+$ neurons) were localized in close proximity to microglia or the IL-1β$^+$ cells. Furthermore, we observed that the number of activated GRPR$^+$ neurons was increased after intrathecal injection of exogenous IL-1β, suggesting that IL-1β can enhance GRPR$^+$ neuron activation.

Turning to the stimuli, it has been shown that the proinflammatory mediators contribute to chronic itch.[11,17,42] Central IL-33 contributes to DNFB-induced chronic itch, and increased expression of IL-33 was found in the spinal dorsal horn in DNFB-induced ACD mice.[17] A previous study demonstrated TNF-α was upregulated in the spinal cord after AEW treatment, and intrathecal injection of TNF-α antagonist etanercept reduced AEW-induced itch.[42] Besides protein allergens, endogenous molecules such as keratinocyte-derived alarmin thymic stromal lymphopoietin (TSLP), endogenous phospholipid lysophosphatidylcholine in blood, etc. can induce epithelia-sensory neuron cross-talk to promote itch.[43,44] Cytokine IL-22, significantly increased in the skin lesions of HDM allergen-induced AD mice model, differentially upregulates the expression of GRP and epithelial-derived type-2 cytokines (TSLP and IL-33) in primary keratinocytes. GRP strongly induces TSLP, IL-33 and GRPR synergistically with IL-22.[45] Consistently, TSLP promotes elevated expression of IL-22 in patients with AD.[46] Therefore, TSLP-GRP-IL-22 would create a vicious circle of positive feedback on the inflammation response, similar to the state of substance P and sensory neuron-related pathway.[38]

However, few studies addressed the role of spinal NLRP3/IL-1β/IL-1R signaling in the context of skin inflammation-induced itch. NLRP3 and IL-1β play essential roles in psoriatic skin inflammation and the innate immunity-related pathways, including NOD-like receptor and inflammasome-related pathways, were enriched in psoriatic skin.[47,48] The IL-1β/IL-1R signaling pathway has been shown to regulate psoriatic inflammation.[49] A recent finding demonstrates the activation of another inflammasome, NLRP1, in the spinal cord and its contribution to the AEW-induced chronic itch.[50] Spinal microglia are known to contribute to chronic itch.[18–20] Previous studies have shown that astrocytes may contribute to the excitation of GRPR$^+$ neurons via LCN2 in the spinal cord under chronic itch conditions.[16] However, how microglia contribute to neuronal activation and chronic itch, as well as itch-related factors derived from microglia, are poorly identified.[51] We showed that NLRP3, ASC, and IL-1β were expressed in spinal microglia but not in astrocytes or neurons and identified a mechanism by which microglia-derived IL-1β promotes chronic itch via the IL1R1-GRPR axis. Importantly, we have used distinct types of chronic itch models, including the IMQ-induced psoriasis model, the SADBE-induced ACD model, and the HDM allergen Der f 2-induced allergy model, all of which are all induced by protein allergens and small molecules from the environment or drugs. Therefore, it is demonstrated that spinal-activated NLRP3 inflammasome and its downstream factor, IL-1β, play an essential role in chronic itch. This mechanism would be common in the

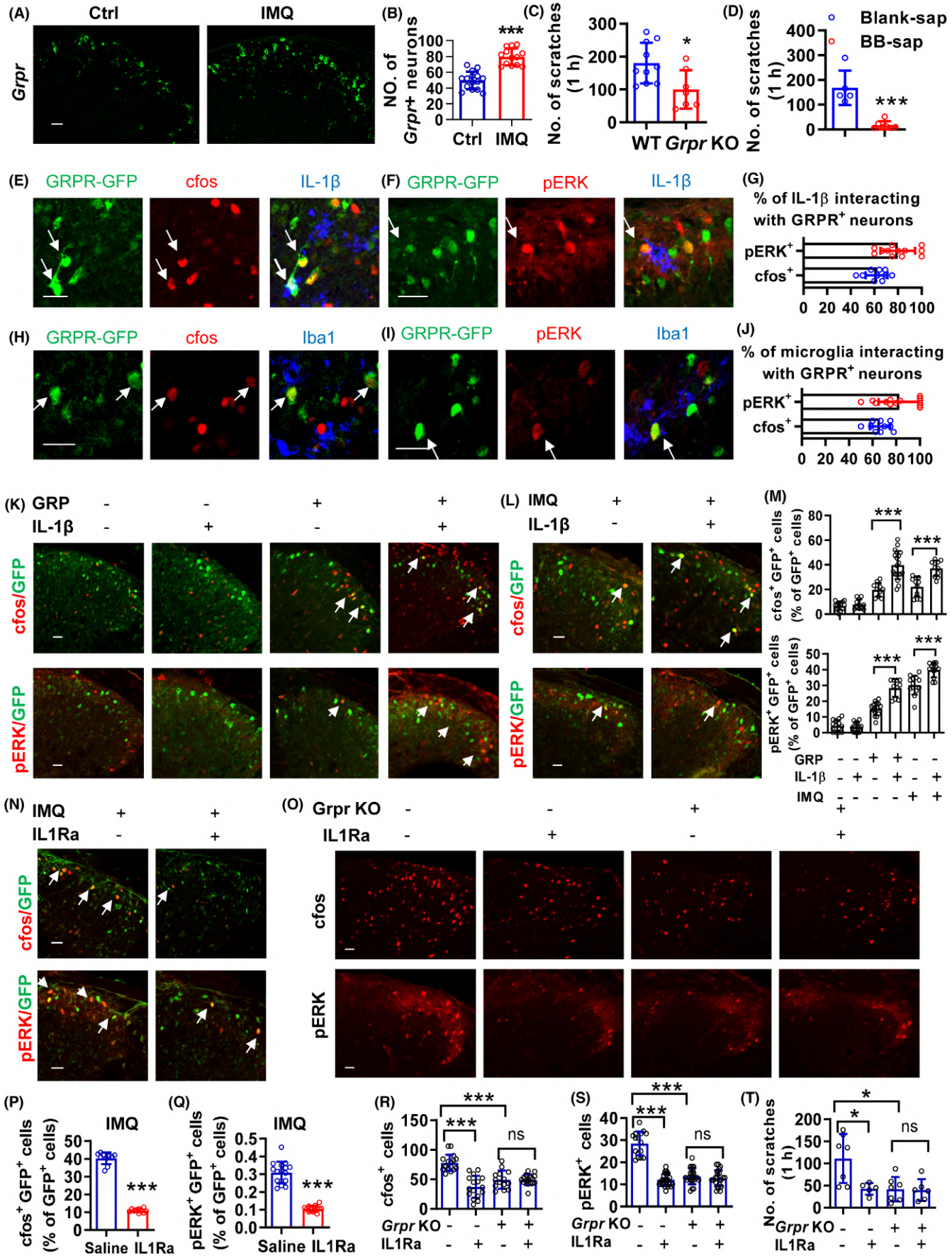


FIGURE 6 Microglia crosstalk with GRPR$^+$ neurons via the IL-1β-IL-1R1 pathway. (A-B) Representative (A) RNAscope ISH images and (B) quantification graphs showing that IMQ treatment increased the mRNA expression of *Grpr* in the spinal cords; (C) the number of scratches decreased in *Grpr* KO mice after IMQ treatment; (D) IMQ-induced chronic itch was significantly reduced after treatment with BB-sap (400 ng, intrathecal injection); (E–F) Immunostaining of GFP/IL-1β with (E) c-Fos or (F) pERK in the spinal cords of GRPR-eGFP mice; (G) Quantification of the percentage of IL-1β$^+$ cells interacting with activated GRPR$^+$ neurons (/activated GRPR$^+$ neurons); (H–I) Immunostaining of GFP/Iba1 with (H) c-Fos or (I) pERK in the spinal cords of GRPR-eGFP mice; (J) Quantification of the percentage of microglia interacting with activated GRPR+ neurons (/activated GRPR$^+$ neurons); (K, M) Representative (K) immunostaining images and (M) quantification graphs showing GRPR-GFP$^+$/c-Fos$^+$ or GRPR-GFP$^+$/pERK$^+$ cells in the spinal cords after intrathecal injection of IL-1β and GRP; (L, M) Representative (L) immunostaining images and (M) quantification graphs showing GRPR-GFP$^+$/c-Fos$^+$ or GRPR-GFP$^+$/pERK$^+$ cells in the spinal cords after intrathecal injection of IL-1β in IMQ-treated mice; (N, P, Q) Representative (N) immunostaining images and (P, Q) quantification graphs showing (N, P) GRPR-GFP$^+$/c-Fos$^+$ or (N, Q) GRPR-GFP$^+$/pERK$^+$ cells in the spinal cords after intrathecal injection of IL1Ra in IMQ-treated mice; (O, R, S) Representative (O) immunostaining images and (R, S) quantification graphs showing (O, R) c-Fos$^+$ or (O, S) pERK$^+$ cells in the spinal cords after intrathecal injection of IL1Ra in IMQ-treated WT and *Grpr* KO mice; (T) IMQ-induced scratching behavior in WT and *Grpr* KO mice after intrathecal injection of IL1Ra. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, unpaired *t*-test in B, C, D, M, P, Q, R, S and T. All quantitative data are presented as the mean ± SEM. $n = 10$–15 sections from three mice. Scale bar, 25 μm.

spinal microglia of several types of itch models induced by allergens and small molecules widely related to allergies. The common mechanisms may have broader implications for the development of effective therapies for multiple types of chronic itch.

Therefore, NLRP3 inflammasome and its downstream mediators may be therapeutic targets for the treatment of chronic itch. According to the contribution of NLRP3, Caspase-1 and IL-1β/IL1R1, we applied pharmacological (MCC950, VX-765, IL-1β mAb, and IL1Ra) and genetic approaches (Casp1$^{-/-}$ and Il1b$^{-/-}$ mice) to investigate the treatment efficacy. We did demonstrate that chronic itches and neuronal activation were significantly reduced after pharmacological or genetic blockade of the NLRP3/caspase-1/IL-1β axis. This result is corroborated by previous studies. Anti-IL-1β canakinumab can be used to treat severe pustular psoriasis with no recurrent systemic manifestations.[52] Bavachin exerts anti-neuroinflammatory effects through inhibiting NF-κB signaling mediated by regulation of ubiquitin-editing enzyme A20 complex,[53] which downregulates NLRP3 inflammasome activation in macrophages.[54] However, we found that the number of scratches in Il1b$^{-/-}$ or Casp1$^{-/-}$ mice was significantly reduced but not completely abolished. Our RNAseq results suggest that a wide range of proinflammatory and chemotactic mediators such as Tnf, Mif, Ccl2, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl9, and Ccl12 are upregulated in the spinal cord after IMQ treatment.[29] Central TNF-α has been demonstrated to contribute to acute itch and AEW-induced chronic itch.[42] Actually, by analyzing several samples of skin patients, Moy AP et al. showed that CD3$^+$ T cells in biopsy specimens from chronic AD lesions were blended with T_H2 (64.6%), T_H17 (30.4%), T_H22 (3.3%) and T_H1 cells (4.8%),[55] implying that all kinds of inflammatory mediators would contribute to the AD symptoms and itch. Therefore, it is tempting to speculate that in addition to inflammasome-mediated IL-1β, other inflammatory factors might also contribute to IMQ-induced chronic itch.

However, the receptors responsible for neuronal activity have not been elucidated. Previous studies showed that IL-1β promotes neuronal excitability through IL1R1$^+$ neurons.[41,56] Our RNAscope ISH data revealed that IL1R1 was mainly expressed in neurons, especially in GRPR$^+$ neurons, inferring that IL-1β promoted chronic itch through the sensitization of IL1R1$^+$ GRPR$^+$ neurons.

Intrathecal injection of the IL1R1 antagonist IL1Ra inhibited GRPR$^+$ neuron activation. Moreover, the inhibitory effect of IL1Ra on chronic itch and neuronal activation was abolished in Grpr ko mice. Thus, a key finding of our work is that IL1R1 facilitates itch modulation through its crosstalk with GRPR. Our studies raise several critical questions concerning the underlying mechanism: How does IL1R1 modulate the activity of GRPR neurons? Do IL1R1 and GRPR form a heteromeric complex that initiates signaling crosstalk? Further studies are needed to address these issues.

Our results highlighted the specificity of NLRP3 inflammasome activation and IL-1β production in spinal microglia to induce itch. Importantly, the mechanisms of itch and pain in the spinal cord share some commonalities, but nonetheless present notable differences. Previous studies have shown that NLRP3 inflammasome activation is observed in the superficial microglia of the spinal dorsal horn in a morphine-sustained neuropathic pain model.[28] IL-1β was released by activated microglia under chronic pain conditions,[22–24] while our studies proved that NLRP3 and IL-1β were expressed in spinal microglia. Thus, microglial activation, NLRP3 activation, and IL-1β production are shared under itch and pain conditions. However, GRP-GRPR signaling is involved only in itch transmission, but not in pain. It is important to note that the cell types involved in inflammasome activation are diverse across various pain models mediated by distinct mechanisms.[57,58] A recent study demonstrated that NLRP2 and IL-1β were overexpressed in the spinal astrocytes at the peak of complete Freund adjuvant-induced inflammatory pain.[57] In addition, a previous study showed that IL-1β was upregulated in astrocytes after spinal cord injury.[58] As a result, inflammasome activation is cell-type specific and could vary under chronic itch and chronic pain conditions.

In summary, we demonstrated that central IL-1β/IL1R signaling is essential for facilitating itch transmission in different allergic itch models in mice. Our results reveal a previously unknown mechanism in which microglia enhance the activation of GRPR$^+$ neurons through NLRP3 inflammasome activation and IL-1β/IL1R signaling. Blockade of microglia/NLRP3 inflammasome/IL-1β axis may provide new options for the management of chronic disease patients.

4 | MATERIALS AND METHODS

4.1 | Mice

Breeding breeders of *Grpr* KO mice and GRPR-eGFP mice were kindly provided by Zhoufeng Chen at the Washington University School of Medicine.[7,13,29] IL-1b$^{-/-}$ and Casp1$^{-/-}$ mice were purchased from Cyagen Biosciences (CA, USA). The primers used for genotyping are listed in Table S2. All animal experiments were carried out in accordance with the guidelines approved by the Animal Research Committee of the Second Affiliated Hospital of Guangzhou Medical University.

4.2 | Statistical analyses

In this study, we used GraphPad Prism 8.0 for statistical analysis of the data. The *t*-test was used to compare the differences between the two groups. The data are presented as the mean ± SEM, and $p < 0.05$ was considered statistically significant.

AUTHOR CONTRIBUTIONS

Xueting Liu and Ainlin Tao conceived the study and designed the experiments. Xueting Liu, Yanmei Wang, Yueling Zeng, De Wang, Yuhuan Wen, Limin Fan, Ying He, Junyan Zhang, Weimin Sun, and Yongping Liu performed experiments and analyzed data. Xueting Liu performed RNAseq analysis. Xueting Liu and Yanmei Wang drafted the manuscript. Ainlin Tao reviewed and critiqued the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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