Transcriptome analysis reveals dysregulation of immune and inflammatory responses in DRG and spinal cord of a mouse model of acute herpetic neuralgia

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Research Article

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Abstract

Objective.

Herpetic-related neuralgia (HN) caused by varicella-zoster virus (VZV) infection is one of the most typical and common neuropathic pain in the clinic. However, due to the lack of an animal model to simulate HN, the potential mechanisms and therapeutic approaches for the prevention and treatment of HN are still unclear. This study aims to provide a comprehensive understanding of the molecular mechanisms and potential therapeutic targets of HN.

Methods.

We used an HSV-1 infection-induced HN mouse model and screened the differentially expressed genes (DEGs) in the DRG and spinal cord using an RNAseq technique. Moreover, the bioinformatics methods were used to figure out the signaling pathways and expression regulation patterns of the DEGs enriched. In addition, quantitative real-time RT-PCR and western blot was carried out to further confirm the expression of DEGs.

Results.

HSV-1 inoculation in mice resulted in mechanical allodynia, thermal hyperalgesia, and cold allodynia, following the infection of HSV-1 in both DRG and spinal cord. Besides, HSV-1 inoculation induced an up-regulation of ATF3, CGRP, and GAL in DRG and activation of astrocytes and microglia in the spinal cord. Moreover, 639 genes were up-regulated, 249 genes were down-regulated in DRG, whereas 534 genes were up-regulated, and 12 genes were down-regulated in the spinal cord of mice 7 days after HSV-1 inoculation. GO and KEGG enrichment analysis suggested that immune responses and cytokine-cytokine receptor interaction are involved in DRG and spinal cord neurons in mice after HSV-1 infection. In addition, CCL5 and its receptor CCR5 were significantly up-regulated in DRG and spinal cord upon HSV-1 infection in mice. And blockade of CCR5 exhibited a significant analgesic effect and suppressed the up-regulation of inflammatory cytokines in DRG and spinal cord induced by HSV-1 infection in mice.

Conclusions.

We reported a reliable HN model with HSV-1 infection-induced allodynia and hyperalgesia in mice through dysregulation of immune response and cytokine-cytokine receptor interaction mechanism.

Introduction

Herpetic-related neuralgia (HN) caused by varicella-zoster virus (VZV) infection is one of the most typical and common neuropathic pain in clinics [1]. This kind of pain often shows spontaneous pain,
hyperalgesia, and abnormal pain sensitivity [2], mostly moderate or severe pain, which has seriously affected the life quality of patients [3]. To date, the clinical treatment for HN only focuses on symptom control, including the use of topical lidocaine or capsaicin and oral gabapentin, pregabalin, or tricyclic antidepressants [4, 5], as well as the improvement of immunity and antiviral treatments [6, 7]. The present analgesic treatments for HN are still limited due to the poor understanding of the pathogenic mechanism of HN. It is well studied that age, immune deficiencies, malignancies, organ transplantations, autoimmune diseases, emotional stress, and immunosuppressive therapies are the major risks for HN [8, 9].

The initial infection of VZV causes varicella. During initial infection, VZV can invade and transport reversely to dorsal root ganglion (DRG) neurons along peripheral sensory nerve endings, and then establish lifelong latent infection in DRG neurons. During the onset of acute herpes zoster, the latent virus is activated, the virus begins to replicate and spread along the affected nerves, and finally triggers the inflammatory immune response that can damage peripheral and central neurons [10]. Newly synthesized virus particles are transported along the central and distal axons of all types of sensory neurons, which could lead to cell necrosis and apoptosis in the skin and DRG neurons [11]. Meanwhile, patients still suffer pain even after they healed from herpes. In 2019, epidemiological data showed that the incidence of HN was 7.7% in China, and the incidence rate for post-herpetic neuralgia (PHN) was 29.8% [12]. However, it has been reported that the incidence rate of PHN in HN patients is around 5% ~ 30% [13, 14]. The incidence of PHN in elderly patients over 60 years old is 50%-75% [15]. Besides, patients with HN easily get anxiety and depression, lose confidence in life, and even have suicidal tendencies. Therefore, the prevention and treatment of HN are urgently needed.

VZV is a human α herpes virus with strong species specificity that has no animal storage host, and human is the only natural host. VZV reactivates when the individual's immunity is decreased and causes HZ [16, 17]. It is difficult to simulate the typical clinical HN symptoms [18]. Therefore, an animal model of HN is necessary, which could help to elucidate the pathology of HN. A rodent infectable human alpha herpes virus, herpes simplex virus type-1 (HSV-1), which is genetically close to VZV, could be used for developing an animal model that maximumly mimics human herpetic-related pain. HSV-1 has been reported to be a neurotropic virus and becomes latent in the sensory ganglia as well [19]. The virus gains access to the peripheral sensory neurons and spreads to sensory ganglia by axonal transport after infection [20]. HSV infection in humans causes paresthesia symptoms, including dysesthesia, hypoalgesia, and tingling [21], which are very similar to those of VZV infection. Therefore, HSV-1 infection in mice can be used as a valuable model of herpetic pain.

Chemokines are a class of secretory small molecular proteins that regulate inflammatory responses in DRG, spinal cord, and brain [22, 23]. There is increasing evidence that chemokines are associated with chronic pain [24] and chronic pruritus [25] after nerve injury. C-C motif chemokine ligand 5 (CCL5), also known as monocyte chemoattractant protein 1, specifically recruits monocytes to the sites of inflammation, infection, or trauma [26]. It has been reported that CCL5 and its receptor CCR5 are involved
in the development and maintenance of chronic pain [27]. However, the involvement of CCL5 and its receptor CCR5 in human HN is still unknown.

This study aims to explore the transcriptomic changes in mouse DRG and spinal cord following HSV-1 infection and summarized the general transcriptomic changes of DRG and spinal cord in HSV-1 infected mice. The purpose of the present study is to provide a comprehensive understanding of the transcriptome level of the DRG and the spinal cord in mice mimicking human HN. In addition, we tested the involvement of CCL5 and its receptor CCR5 in HSV-1 infected mice.

**Materials And Methods**

**Cells and Viruses**

African green monkey kidney cells (Vero, NO. CRM-CCL-81) and HSV-1 KOS strains (NO. VR-1493) were purchased from ATCC American type culture collection (ATCC). Vero cells were cultured in Dulbecco’s Modified Eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) and penicillin-streptomycin (100 U/ml and 100 ug/ml, Gibco). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. HSV-1 virus particles were amplified on Vero cells, and the virus titer on Vero cells was determined by a plaque test. The steps of viral plaque assays were carried out as follows: Virus stock solutions were diluted in continuous gradient in DMEM. Monolayer Vero cells in six-well plates were exposed to virus dilutions with different dilution gradients at 37 °C for 1 hour. The liquid in the 6-well plate was sucked away, cells were washed with PBS, and DMEM containing 5% FBS and 1% methylcellulose (covering medium) was added to cells. After 72 hours of virus infection, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The virus titer was calculated by scoring the plaque-forming unit (PFU).

**Animals**

Eight weeks, male healthy C57BL/6J mice with an average weight of 25 ± 1g were selected (purchased from Guangdong Medical Experimental Animal Center) for the construction of the pain model. All experimental mice have placed in individually ventilated colony cages and maintained in a normal 12-hour light/dark cycle with free access to food and water. All experimental procedures were carried out following the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Shenzhen University. All efforts were made to minimize the use of animals and alleviate their discomfort.

**HSV-1 infection**

Mice were anesthetized with isoflurane (2%), and then the right mid-flank and right foot fur of the mice were shaved using a scraper. HSV-1 (1×10⁶ PFU in 10 μL) was inoculated subcutaneously in the tibia of the right hind leg of mice with a microsyringe. The contralateral hindlimb was not inoculated with the
virus. The sham group was mock-infected as described for HSV-1 infection, but the viruses used were previously inactivated by heating at 60°C for 1 hour.

**Mechanical nociceptive behavior tests**

As we previously reported, von Frey filaments were used to assess the mechanical injury threshold in mice [28]. Thirty minutes before the test, mice were placed in an acrylic cage (12 × 10 × 17 cm) with a steel mesh floor in advance to acclimate to the test environment. In the mechanical damage threshold test, a series of von Frey filaments with a logarithmic increase in stiffness (0.008² logarithmic force, mg) was applied vertically to the middle of the mouse's right rear paw pad with gradually increasing pressure. These tests elicited responses including flexion, leg raising, or foot licking as indicators of evoked nociceptive pain, and responses disappeared significantly after von Frey filaments were withdrawn. Each von Frey filament was applied to mouse footpads for 3–4 s to induce terminal reflexes, and the smallest filament (force applied) that elicited response was considered the mechanical damage threshold (Log mg).

**Thermal nociceptive behavior tests**

Mice were transferred to the behavioral testing room 30 minutes before testing to acclimate to the experimental environment. Mice were placed in acrylic cages on a temperature-controlled plate and the latency to the first nociceptive response (hind paw licking, hind paw shaking, jumping, and spinning) was measured [29, 30]. The temperature control panel for the hot plate test was set to 53°C, and the temperature of the cold plate test was set to 4°C. To avoid local tissue damage in mice caused by high temperature or low temperature, the test termination time was controlled within 20 seconds.

**Total RNA extraction**

DRG and spinal cord collection were performed as we previously reported [28]. Seven days after HSV-1 inoculation, mice were euthanized. The dorsal skin and muscle of the mouse were opened with dissection shears to expose the spine, and the spinous and transverse processes of the spine were gently dissected with a rongeur. After the spinal cord is exposed, the spinal cord was pulled up with forceps to view the dorsal root of the spinal nerve and its DRG within the spinal canal. The lumbar L4-L5 DRG and spinal cord were collected and placed in a pre-cooled 1.5 mL centrifuge tube. Finally, steel balls were placed in a centrifuge tube, TRIzol reagent (Invitrogen, USA) was added, and then placed in a grinder for cryogenic grinding until the tissue was completely dissolved. Total RNA dissolved in TRIzol reagent was extracted according to the manufacturer’s manual. All RNAs were quantified on a nanodrop spectrophotometer (Thermo Fisher, USA). The total RNA used for subsequent tests should meet the OD - (260) / OD - (280) ratio between 1.8 and 2.0. The RNA samples were stored in a freezer at -80°C until use.

**RT-PCR and RT-qPCR**

The Hifair® III 1st Strand cDNA Synthesis Kit (Yeasen Biotechnology, China) was used for total RNA genomic residue removal and cDNA reverse transcription according to the manufacturer’s instructions. All
primer sequences were designed using primer BLAST from the National Center for Biotechnology Information (NCBI) (Table 1) and synthesized by Tsingke Biotechnology (China, Beijing). PCR was conducted with the 2×Hieff® PCR Master Mix kit (Yeasen Biotechnology, China) according to the manufacturer's instructions. Briefly, 5 μL of each sample template RNA was mixed with 2 μL (10 μM) of each of the primers (Table 1), 16 μL of water, and 2×Hieff® PCR Master Mix. The following thermocycling conditions were used under standard mode as per the manufacturer’s recommendation: 5 min at 94°C, followed by 40 cycles at 94°C for 15 sec and 60°C for 1 min. According to the manufacturer's manual, TB green® Fast qPCR mix (TaKaRa, Japan) was used in the qPCR test to determine gene expression quantification. Data were collected and analyzed using ABI-7500 software (Applied Biosystems, USA). The results were normalized to a housekeeping gene (Gapdh) and relative expression was shown as 2^−△△Ct.

RNA Sequence and Data Analysis

Seven days after HSV-1 inoculation, the dorsal root ganglion and spinal cord tissues of mice were collected, homogenized, and dissolved in TRIzol reagent (Invitrogen, USA). The mRNA library construction and transcriptome sequencing were completed by Beijing Genomics Institute (BGI, Shenzhen, China). The BGSeq-500 high-throughput sequencing platform was used for sequencing to obtain an average of 6.52 G of raw data for each sample, and SOAPnuke (v1.5.2) was used to filter the sequenced raw data [31]. The clean reads were mapped to the GCF_000001635.26_GRCm38.p6 using HISAT2 (v2.0.4) with Q value ≤ 0.05 [32]. Bowtie2 (v2.2.5) was applied to align the clean reads to the reference coding gene set, then the expression level of the gene was calculated by RSEM (v1.2.12) [33, 34]. According to the quantitative results of gene expression, we screened differentially expressed genes among samples based on the DEseq2 algorithm [35]. The results of Q value (Adjusted P-value) ≤ 0.05, and fold change (FC) ≥ 2 were statistically significant and were defined as differentially expressed genes. The gplots R package was used to construct the heatmaps (http://cran.r-project.org/web/packages/gplots/index.html), and Draw Venn Diagram online tool was used to generate the Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) [36]. Functional classification of DEGs between groups was performed using the DAVID 6.8 (https://david.ncifcrf.gov/) [37] and KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/kobas3) [38, 39] online database. The sequencing data set supporting the results of this article has been submitted to the NCBI Gene Expression Omnibus (GEO) database, and the accession number is GSE208282.

Western blot analysis

The tissues of mouse dorsal root ganglion and spinal cord were collected in a 1.5ml centrifuge tube, and an appropriate amount of precooled tissue lysate (Beyotime Biotechnology, China) was added to the tube. The tissue was placed in a precooled tissue grinder for grinding, centrifuged at 4°C and 12000 rpm for 10 minutes, and the supernatant was collected. The protein content in tissue supernatants was quantified with a BCA protein concentration assay kit (Beyotime Biotechnology, China). Tissue lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate total proteins.
and then transferred to the PVDF membrane (Millipore, USA). The PVDF membrane that had been transferred was incubated with TBS-T blocking solution containing 5% nonfat dry milk (Bio-Rad, USA) for 1 h at room temperature. Membranes and antibodies were incubated overnight at 4°C: anti-CCR5 antibody (Abcam, USA; 1:2000) and anti-GAPDH (ProteinTech, USA; 1:2000). The next day, after washing the membrane three times with TBS-T, the HRP-conjugated secondary antibody (ProteinTech, USA, 1:5000) was incubated with the primary antibody for 1 h at room temperature. Finally, the membrane was washed three times with TBS-T to remove residual secondary antibodies, and the protein expression level was observed using an ECL substrate kit (Millipore, USA).

**Immunohistochemistry**

Mice were anesthetized with 1% pentobarbital sodium (50 mg/kg) by intraperitoneal injection. Anesthetized mice were perfused with 0.9% saline and 4% paraformaldehyde sequentially. Spinal cords, L4 and L5 DRGs were removed intact into centrifuge tubes and then fixed in 4% formaldehyde overnight. These tissues were dehydrated with sucrose solutions (10%, 20%, and 30%) and embedded with OCT as they settled to the bottom in a 30% sucrose solution. The embedded tissue was frozen sectioned, and the thickness of DRG and spinal cord sections were 20 μm and 25 μm, respectively. DRG and spinal cord slices were blocked with PBS solution containing 10% goat serum and 0.3% Triton X-100 at room temperature for 1 hour. Next, the sections were incubated overnight with primary antibody ATF3 (Abcam, USA), GFAP (CST, USA), IBA1 (Abcam, USA) at 4°C, and then incubated with Goat anti-rabbit Cy3 or FITC binding secondary antibody (1:500 dilution, Beyotime Biotechnology, China) at 37°C in the dark for 2 hours. Finally, the sections were stained with nuclear dye DAPI (Yesen biotechnology, China) in the dark for 8 minutes. The stained sections were imaged by laser confocal microscope (Olympus, FV3000, Japan).

**Serum cytokine measurements**

When the mice were euthanized, blood was obtained by cardiac puncture, coagulated at room temperature for 30 minutes, and centrifuged at 3000 rpm at 4°C for 10 minutes to collect serum. CCL5, TNF-α, IL-1β, and IL-6 levels were measured using commercially available ELISA kits (Absin Bioscience Inc., China) according to the manufacturer's instructions. Briefly, test samples and serially diluted standards were pipetted into 96-well plates pre-coated with immobilized antibodies and incubated for 2 hours at 37°C. After washing 3 times with washing buffer, the biotynilated antibody was added to the wells and incubated at 37°C for 60 minutes. Then, three final washes were performed. After the addition of streptavidin solution and chromogenic substrate, the intensity detected at 450 nm was measured by a microplate reader. Draw a standard curve based on the concentration and absorbance of the standard. Finally, the cytokine concentration of the sample was calculated according to the standard curve equation.

**Statistical analysis**
All quantitative data were presented as Mean ± Standard Deviations (S.D.) from three or more independent experiments. For differentially expressed gene screening in transcriptome sequencing, genes with q-value below 0.05 and ≥ 2 with fold changes were considered differentially expressed. Quantitative data were analyzed using an independent samples t-test or one-way analysis of variance (ANOVA), and statistical significance was analyzed using Graphpad Prism 8 (GraphPad Software, La Jolla, CA, USA). Results were considered significant when $P$-value was ≤ 0.05.

Results

Behavioral characterization of the mice after HSV-1 inoculation

To mimic human HN, HSV-1 (1×10$^6$ PFU in 10 μL) was inoculated subcutaneously in the tibia of the right hind leg of mice with a microsyringe as shown in Figure 1A. After inoculation of the virus, we examined the mechanical thresholds and paw withdrawal latencies in response to thermal and cold stimulation in mice from HSV-1, HSV-1 inactivated inoculation and naïve groups.

To mimic human HN, HSV-1 (1×10$^6$ PFU in 10 μL) was inoculated subcutaneously in the tibia of the right hind leg of mice with a microsyringe as shown in Figure 1A. After inoculation of the virus, we examined the mechanical thresholds and paw withdrawal latencies in response to thermal and cold stimulation in mice from HSV-1, HSV-1 inactivated inoculation and naïve groups. Behavioral results showed that the mechanical thresholds (ipsilateral) in response to von Frey stimulation in mice 3 days after HSV-1 inoculation were significantly decreased and reached the bottom on the 7th day after HSV-1 inoculation, compared with mice from either inactivated HSV-1 inoculation or naïve groups. Besides, after HSV-1 inoculation for 7 days, the mechanical thresholds (ipsilateral) were gradually increased until 56 days after inoculation, although still significantly lower than the mice from either HSV-1 inactivated or naïve groups (Figure 1B). Moreover, the paw withdrawal latencies in response to either thermal or cold stimulation in mice with HSV-1 inoculation 3 days were significantly decreased and became most sensitive to thermal or cold stimulation in mice on the 7th day after HSV-1 inoculation. Then, the paw withdrawal latencies were gradually recovered to the baseline until the day of the 56th after HSV-1 inoculation (Figure 1C and D). These results suggested that mechanical allodynia, thermal hyperalgesia, and cold allodynia have occurred in mice after HSV-1 inoculation.
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**HSV-1 exists in mouse DRG and spinal cord after inoculation**

We next confirmed the existence of HSV-1 in mouse DRG and spinal cord, since the mechanical allodynia, thermal hyperalgesia, and cold allodynia have occurred in mice after HSV-1 inoculation. qRT-PCR results demonstrated that an HSV-1 gene, *UL30* was detectable in mouse spinal cord and DRG L4, 5, and 6, but not in sham group mice (Figure 2A). Besides, the expression level of *UL30* was significantly increased in the DRG of mice 3 days after inoculation and reached the highest level on the 7th day after HSV-1 inoculation. The mRNA expression of HSV-1 *UL30* became undetectable until 28 days after inoculation (Figure 2B). Moreover, *infected cell protein 0 (ICP0)*, which contributes to efficient virus growth and reactivation from latency, and *thymidine kinase (TK)* from HSV-1 were detected in the spinal cord and DRG from different groups of mice. The results showed that both *ICP0* and *TK* were detectable in the spinal cord and DRG in mice from the HSV-1 inoculation group, but not in the sham group (Figure 2C and D). These results suggested that HSV-1 can be infected and propagated in the sensory neurons in both DRG and spinal cord of mice, although HSV-1 was inoculated subcutaneously in the tibia of the right hind leg of mice.

**ATF3, a neuropathic pain marker, is up-regulated in mouse DRG after HSV-1 inoculation**

It is well-known that human HN is a classic neuropathic pain. To further examine the similarity and characteristics of the HSV-1 inoculation-induced pain model, we examined the expression of neuropathic pain markers in mouse DRG after HSV-1 inoculation. Immunofluorescence staining images showed that the fluorescent signaling of ATF3 is dramatically increased in mouse DRG after HSV-1 inoculation (Figure 3A). Moreover, qRT-PCR results demonstrated that the mRNA expression of *Atf3* (Figure 3B), *Cgrp* (Figure 3C), and *Gal* (Figure 3D) is significantly up-regulated as well in the DRG from mice 7 days after HSV-1 inoculation. These data revealed that peripheral sensory neuron in DRG is injured 7 days after HSV-1 infection.

**The activation of astrocyte and microglia is observed in mouse spinal cord after HSV-1 inoculation**

The activation of spinal astrocytes and microglia in mice after HSV-1 inoculation was also assessed. Immunostaining results showed that the astrocytic marker GFAP is increased in mouse spinal cord 7 days after HSV-1 inoculation (Figure 4A). Moreover, the microglia activation marker IBA1 is dramatically increased as well in mouse spinal cord 7 days after HSV-1 inoculation (Figure 4B). These results further suggested that neuroinflammation in the spinal cord occurred in mice 7 days after HSV-1 infection.

**Transcripts regulated in mouse DRG and spinal cord after HSV-1 inoculation**

The transcriptome data was generated from mouse DRG and spinal cord 7 days after inoculation of VZV using RNAseq technique, averagely generating 44.14 million raw sequencing reads and then 43.45
million clean reads after filtering low quality. Clean reads are mapped to reference. Each data set contained 43 million reads and a mapping rate of 98%. Table 2 briefly summarized the information of sequencing data from each sample. We counted the number of identified genes and calculated its proportion and distribution to the total gene number in the database of each sample as shown in Supplementary Figure S1A. The correlation of gene expression levels among samples is a key criterion to test whether the experiments are reliable and whether the samples chosen are reasonable. We calculated the correlation value between samples based on normalized expression results and draw a correlation heatmap (Supplementary Figure S1B). DEGs screening is aimed at finding DEGs between samples and performing further function analysis on them. The representative distributions of genes up-or down-regulated are shown in the volcano in Figure 5A and B. Our data revealed that 639 genes were up-regulated, 249 genes were down-regulated in mouse DRG (Figure 5A), whereas 534 genes were up-regulated, and 12 genes were down-regulated in mouse spinal cord 7 days after HSV-1 inoculation (Figure 5B). The red dots represent the up-regulated genes, while the green dots represent the down-regulated genes. In addition, the top 50 DEGs, including a series of immune response-associated genes, and chemokines, \( Ccl2 \), \( Ccl5 \), \( Ccl8 \), and \( Ccr5 \), in DRG (Figure 5C) and spinal cord (Figure 5D) were listed in mice for 7 days after HSV-1 inoculation.

**Gene ontology analysis of the differential genes**

To better understand the associated functions of the differentially expressed genes in mouse DRG and spinal cord after HSV-1 inoculation, gene ontology (GO) analysis was used to perform enrichment analysis and classifications (Figure 6). GO analysis identified enriched biological processes associated with “immune system process”, “innate immune response”, “inflammatory response”, “immune response”, and “defense response to virus” in DRG and spinal cord (Figure 6A and B), indicating that strong immune responses were induced in both peripheral and central nervous system in mice after HSV-1 inoculation. Identified enriched cellular component terms associated with “membrane”, “extracellular exosome”, “extracellular region”, “external side of plasma membrane” and “cell surface”, suggesting multifarious extracellular components were involved in immune responses in DRG and spinal cord upon HSV-1 inoculation (Figure 6C and D). Moreover, enriched molecular functions were defined as associated with “protein binding”, “GTP binding”, “cytokine activity”, “GTPase activity”, and “double-stranded DNA binding”, implying that signaling transduction and inflammatory response were induced in both DRG and spinal cord after HSV-1 inoculation (Figure 6E and F). Taken together, the GO analysis results suggested strong immune responses and signaling transduction are mainly response to HSV-1 infection in DRG and spinal cord of mice.

**Analysis of important KEGG pathways**

We next performed KEGG pathway enrichment using KOBAS as previously reported [38, 39]. We listed the top 20 enriched KEGG pathways using DEGs from DRG and spinal cord of mice after HSV-1 inoculation as shown in Figure 7. The differential genes were significantly enriched in the classifications of “Cell adhesion molecules”, “Herpes simplex infection” and “Cytokine-cytokine receptor interaction” in both DRG
and spinal cord after HSV-1 infection (Figure 7A and B). These results suggested that strong immune response and inflammatory response have occurred in DRG and spinal cord after HSV-1 inoculation as well. We further performed a functional analysis of the cytokine-cytokine receptor interaction pathway with data from both DRG and spinal cord as shown in Supplementary Figure S2. The results showed that the DEGs enriched in the cytokine-cytokine receptor interaction pathway were mainly involved in chemokines (e.g. CCL5 and its receptor CCR5, CCL2, and its receptor CCR2), cytokines, and the TNF family in both DRG and spinal cord after HSV-1 inoculation. This result further demonstrated that immune responses and cytokine-cytokine receptor interaction are involved in DRG and spinal cord neurons in mice after HSV-1 infection.

**CCL5 and its receptor CCR5 were significantly up-regulated in DRG and spinal cord upon HSV-1 infection in mice**

We further focused on the chemokine expression of CCL5 and its receptor CCR5 in DRG and spinal cord of mice after HSV-1 inoculation. Our results demonstrated that the mRNA expression level of Ccl5 was significantly up-regulated in either DRG or spinal cord of mice after HSV-1 inoculation. And the mRNA expression level of Ccl5 reached the highest level in the DRG (2.89 ± 0.09 fold) and spinal cord (18.83 ± 1.22 fold) of mice on the 7th day after HSV-1 inoculation (Figure 8A and B). Besides, the mRNA expression level of Ccr5 was up-regulated as well and reached the highest level in the DRG (2.4 ± 0.26 fold) and spinal cord (15.71 ± 2.31 fold) of mice on the 7th day after HSV-1 inoculation (Figure 8C and D). Furthermore, the protein expression level of CCR5 was also up-regulated in both DRG and spinal cord of mice after HSV-1 inoculation (Figure 8E and F). In addition, we determined the serum level of CCL5 in mice after HSV-1 inoculation. The results indicated that the serum level of CCL5 in mice was dramatically increased after HSV-1 inoculation as well. These results further suggested the involvement of CCL5 and its receptor CCR5 in the development of neuropathic pain in mice upon HSV-1 infection.

**Blockade of CCR5 significantly alleviated mechanical allodynia, thermal hyperalgesia, and cold allodynia in mice induced by HSV-1 infection**

Previous results have demonstrated that the mechanical threshold and paw withdrawal latency in response to thermal and cold stimulation reached the lowest on the 7th day after HSV-1 infection. We, therefore, examined the analgesic effects of CCR5 blockade on mice on the 7th day after HSV-1 infection (Figure 9A). A CCR5 blocker, maraviroc, was injected intrathecally with different doses as previously reported [40]. The behavioral results showed that either mechanical allodynia, thermal hyperalgesia, or cold allodynia were significantly alleviated in a dose-dependent manner (Figure 9B, C, and D). Moreover, we noticed that the analgesic effect of maraviroc was the strongest in the time 1 hour after intrathecal injection in either mechanical or thermal and cold stimulation tests (Figure 9B, C, and D). These results suggested that blockage of CCR5 could be a promising approach for alleviation of HSV-1 infection-induced HN.
Blockade of CCR5 suppressed the up-regulation of inflammatory cytokines in DRG and spinal cord induced by HSV-1 infection in mice

It has been reported that CCR5 regulates the expression of cytokines [41, 42]. To further explore the possible analgesic mechanism of CCL5 and its receptor CCR5, we, therefore, examined the expression of cytokines in DRG and spinal cord of mice after HSV-1 inoculation. The results showed that HSV-1 inoculation significantly up-regulated the mRNA expression of Tnf-α, Il-1β, and Il-6 in either DRG or spinal cord of mice (Figure 10A, B, and C). Moreover, blockage of CCR5 with its antagonist, maraviroc (50 mg, i.t.), dramatically prevented the up-regulation of Tnf-α, Il-1β, and Il-6 in either DRG or spinal cord of mice (Figure 10A, B and C). We also examined the serum levels of TNF-α, IL-1β, and IL-6 in mice after HSV-1 inoculation. The results showed that the serum levels of TNF-α, IL-1β, and IL-6 were significantly increased upon HSV-1 infection, whereas prevented by maraviroc (50 mg, i.t.) (Figure 10D, E and F). These results revealed that the inflammatory cytokines might be a possible mechanism mediated by CCL5 and its receptor, CCR5.

Discussion

HN caused by VZV infection is one of the most typical and common neuropathic pain in clinical. This kind of pain often manifests as spontaneous pain, hyperalgesia, and abnormal pain sensitivity, mostly moderate or severe pain, which has seriously affected the life quality of patients. In the present study, we reported an HSV-1 infection-induced HN animal model, which could be used for exploring the potential mechanism of HN. Further, we for the first time use the RNaseq technique to clarify the possible mechanism of HSV-1 infection-induced HN in mice. In the present study, our findings demonstrated that HSV-1 infection in mice induced significant allodynia and hyperalgesia probably through an immune response and cytokine-cytokine receptor interaction mechanism. Moreover, up-regulation of CCL5 and its receptor CCR5 is contributed to HSV-1 infection-induced HN in mice. In addition, the blockade of CCR5 alleviated allodynia and hyperalgesia probably through the suppression of inflammatory cytokines.

VZV is an α herpes virus with strong species specificity, has no animal host, and human is the only natural host. Therefore, it is difficult to simulate the typical HN symptoms in the laboratory similar to clinical symptoms [18]. As a homologous family of VZV, HSV is a neurophilic virus, which can infect animals and replicate in various tissues and organs of mice [43]. After the first infection with HSV-1, HSV-1 can also be latent in the sensory ganglion. The reactivation of HSV-1 latent in the sensory ganglion could lead to herpes simplex, which is very similar to the symptoms observed in HN patients [44]. Therefore, HSV-1 infection-induced herpes simplex can be used as an effective simulation of HN. It has been reported that 5 days after HSV-1 inoculation, the mice developed shingles-like skin lesions accompanied by allodynia and hyperalgesia, and this phenomenon can last for at least 8 days. At the same time, HSV-1 viral DNA can be detected in mouse DRG from the 2nd to the 8th day after HSV-1 infection, and the viral DNA content reaches its peak on the 5th day [45, 46]. Coincidently, our results demonstrated that mechanical allodynia, thermal hyperalgesia, and cold allodynia occurred in mice after HSV-1 infection, which is similar to that of patients with HN. Moreover, immunostaining results
demonstrated that peripheral sensory neuron in DRG is injured 7 days after HSV-1 infection with the evidence of up-regulated neuropathic pain markers, ATF3, CGRP, and GAL in mouse DRG 7 days after HSV-1 infection. In addition, astrocyte and microglia are activated, indicating in HSV-1 infection resulted in neuroinflammation in the spinal cord. These findings further supported that HSV-1 infection-induced pain model has certain characteristics of neuropathic pain, at least 7 days after HSV-1 infection. Taken together, our data suggested that HSV-1 infection-induced HN in mice could be a reliable model to simulate clinical HN.

In addition, HSV-1 viral DNA is detectable in mouse ipsilateral L4-6 DRG 3 days after HSV-1 inoculation and reached the highest level in DRG 7 days after HSV-1 inoculation. Thus, HSV-1 reached DRG in several days and proliferated 7 days after inoculation. Coincidently, it is noteworthy that mechanical allodynia and thermal hyperalgesia also occurred and reached the most sensitive levels in mice 7 days after inoculation. On the other hand, in sham group mice, treatment with inactivated HSV-1, did not induce allodynia and hyperalgesia, excluding the possibility that allodynia and hyperalgesia were due to the inflammatory response to herpetic protein. These data suggested that allodynia and hyperalgesia were most probably due to HSV-1 infection and propagation in sensory neurons.

RNAseq technique was recruited to better understand the pathogenesis of HSV-1 infection-induced HN. The results identified a series of genes that were differentially expressed in mouse DRG and spinal cord. GO analysis revealed that the DEGs enriched biological processes were associated with strong immune responses in both peripheral and central nervous systems in mice after HSV-1 inoculation. Enriched cellular component terms associated with multifarious extracellular components were involved in immune responses in DRG and spinal cord after HSV-1 inoculation. Enriched molecular functions implied that signaling transduction and inflammatory response were induced in both DRG and spinal cord after HSV-1 inoculation. Moreover, KEGG pathways analysis results demonstrated that strong immune and inflammatory responses, and cytokine-cytokine receptor interaction have occurred in DRG and spinal cord after HSV-1 inoculation as well. Taken together, our results demonstrated that immune response and cytokine-cytokine receptor interaction are involved in DRG and spinal cord neurons in mice after HSV-1 infection. It is easy to understand that strong immune and inflammatory responses occurred in DRG and spinal cord in mice after HSV-1 virus infection. In addition, immune response, and cytokine-cytokine receptor interaction, are well-known to be involved in neuropathic pain, such as SNI, and chemotherapy-induced peripheral neuropathy [47, 48]. These data also support our conclusion that HSV-1 infection-induced HN is somehow a kind of neuropathic pain.

There is increasing evidence demonstrating that chemokines are associated with chronic pain after nerve injury [24]. It has been reported that CCL5 and its receptor CCR5 are involved in the development and maintenance of inflammatory and neuropathic pain [49, 50]. Accordingly, our data suggested the expression of CCL5 and its receptor CCR5 were significantly up-regulated as well in mouse DRG and spinal cord after HSV-1 infection. Besides, the serum level of CCL5 is also increased in mice after HSV-1 infection. Moreover, blockade of CCR5 alleviated mechanical allodynia and thermal hyperalgesia in mice induced by HSV-1 infection, suggesting CCR5 could be a therapeutic target for alleviation of HSV-1 infection.
infection-induced HN. This is similar to the role of CCL5 and CCR5 in inflammatory and neuropathic pain [49, 50].

The mechanisms underlying the inoculation of HSV-1-induced allodynia and hyperalgesia are still unknown. Although it is unclear which cell type in DRG and spinal cord was infected by HSV-1. Infection of sensory neurons with HSV-1 results in the infiltration of macrophages and mononuclear cells into DRG and the release of inflammatory mediators such as cytokines [51–53]. Moreover, it is known that CCR5 regulates the expression of cytokines [41, 42]. According to our data, several inflammatory cytokines, accompanied by CCR5, were increased in mice after HSV-1 infection. Moreover, the blockade of CCR5 with its antagonist, maraviroc significantly suppressed the increase of those cytokines, including TNF-α, IL-1β, and IL-6, in mice after HSV-1 infection. These results revealed that the inflammatory cytokines might be a possible mechanism mediated by CCL5 and its receptor, CCR5.

In conclusion, we reported a reliable HN model with HSV-1 infection-induced allodynia and hyperalgesia in mice through dysregulation of immune response and cytokine-cytokine receptor interaction mechanism. Moreover, up-regulation of CCL5 and its receptor CCR5 is contributed to HSV-1 infection-induced HN in mice. In addition, the blockade of CCR5 alleviated allodynia and hyperalgesia probably through the suppression of inflammatory cytokines. Taken together, the HSV-1 infection-induced HN model would be useful for further exploring the pathogenesis of pain syndrome and developing new therapeutic approaches against HN.

**Abbreviations**

CCL5  
C-C motif chemokine ligand 5  
DEGs  
differentially expressed genes  
DMEM  
Dulbecco’s Modified Eagle medium  
DRG  
dorsal root ganglion  
FBS  
fetal bovine serum  
GEO  
Gene Expression Omnibus  
HN  
herpetic-related neuralgia  
HSV-1  
herpes simplex virus type-1  
ICP0  
injected cell protein 0
Declarations

Ethics approval and consent to participate

All experiment procedures in this study were approved by the Ethics Committee of Animal Care and Use of Shenzhen University Health Science Center.

Availability of data and materials

The sequencing data from PIPN rats were available in the GEO database (GSE208282).

Competing interests

The authors declare that they have no conflict of interest.

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Authors’ contributions

The authors’ contributions were as follows: S Wu and W Sun were responsible for the concept and design of the study; S Wu, S Yang, R Li, X Ba, C Jiang, D Xiong, L Xiao, and W Sun were involved with experimental and analytical aspects of the manuscript; S Wu and W Sun performed data interpretation, presentation and writing of the manuscript.
Acknowledgments

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Consent for publication

The paper has been read and approved by all authors. All authors approved the submission of this paper to “Brain, Behavior, and Immunity” for publication. All authors confirmed that neither the manuscript submitted nor any part of it has been published or is being considered for publication elsewhere.

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Tables
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Table 2
The summary of raw RNA sequencing data set.

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<th>Total Clean Reads (M)</th>
<th>Total Clean Bases (Gb)</th>
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It showed the summary of RNA sequencing data of 9 samples, including raw reads number, clean reads number, clean data rate, mapped rate, and percentage of clean reads as well as Q20 (Phred quality scores Q) and Q30.

Figures
Figure 1

The schematic of the experimental design and behavioral tests in mice after HSV-1 inoculation

A Scheme of experimental design and protocol. C57Bl6/j mice were randomly divided into an HSV-1 inactivated group and an HSV-1 activated group. Mice were anesthetized with isoflurane (2%), and then the right mid-flank and right foot fur of the mice were shaved using a scraper. HSV-1 (1×10^6 PFU in 10 μL) was inoculated subcutaneously in the tibia of the right hind leg of mice with a microsyringe. B Mechanical tests in mice after HSV-1 inoculation. C and D Paw withdrawal latencies in response to thermal (C) or cold (D) stimuli in mice after HSV-1 inoculation. Mean ± SD, n = 6; * P < 0.05, ** P < 0.01, *** P < 0.001. Repeated measures ANOVA followed by Dunnett’s post hoc test were performed, and statistical differences by Dunnett’s post hot test were shown.

Figure 2

HSV-1 detection in mouse DRG and spinal cord after inoculation
A and B qRT-PCR results of *UL30* in the spinal cord (SC) and DRG L4, 5, and 6 in mice after HSV-1 inoculation. C and D RT-PCR results of *ICP0* (C) and *TK* (D) in the spinal cord and DRG L4, 5, and 6 in mice after HSV-1 inoculation.

**Figure 3**

The expression of neuropathic pain markers in mouse DRG after HSV-1 inoculation
A The representative immunofluorescence staining images of ATF3 and DAPI in the DRG from mice 7 days after HSV-1 inoculation. The scale bar indicates 100 μm. B, C, and D qRT-PCR results of *Atf3* (B), *Cgrp* (C), and *Gal* (D) in the DRG from mice 7 days after HSV-1 inoculation. Mean ± SD, n = 6; * P < 0.05, ** P < 0.01, *** P < 0.001. Unpaired Student’s *t*-test.

Figure 4
The activation of astrocyte and microglia in mouse spinal cord after HSV-1 inoculation

A and B: The representative immunofluorescence staining images of the expression of astrocyte activation marker GFAP (A) and microglial activation marker IBA1 (B) in the spinal cord from mice 7 days after HSV-1 inoculation. The scale bar indicates 100 μm.

Figure 5
A number of the differentially expressed genes were induced in the DRG and spinal cord in mice after HSV-1 inoculation

**A and B** Volcano plot showing the log2 fold change (x-axis) against adjusted $P$-value (y-axis) for DEGs in the DRG (A) and spinal cord (B) in mice between sham and HSV-1 inoculation group. Significantly upregulated genes are indicated in red and down-regulated genes are indicated in green. DEGs: the differentially expressed genes. **C and D** The top DEGs in DRG (C) and spinal cord (D) were listed in mice 7 days after HSV-1 inoculation.
Figure 6

Functional analyses of DEGs by Gene Ontology classifications

A and B The comparison of Gene Ontology (GO) enrichment ($P<0.05$) in biological process with genes significantly decreased (green) and increased (red) in the DRG (A) and spinal cord (B) in mice between sham and HSV-1 inoculation group. The X-axis represents the enrichment score (-log10 P-value) for each
GO term. **C and D** The comparison of Gene Ontology (GO) enrichment ($P<0.05$) in cellular components with genes significantly decreased (green) and increased (red) in the DRG (C) and spinal cord (D) in mice between sham and HSV-1 inoculation group. **E and F** The comparison of Gene Ontology (GO) enrichment ($P<0.05$) in molecular function with genes significantly decreased (green) and increased (red) in the DRG (E) and spinal cord (F) in mice between sham and HSV-1 inoculation group. The top 10 significantly enriched GO terms including biological process, cellular component, and molecular function were shown. The enriched gene number as the abscissa and GO terms is plotted as the ordinate.

**Figure 7**
Figure 7

KEGG classifications of DEGs in the DRG and spinal cord in mice after HSV-1 inoculation

A and B Histogram shows the distribution of the top 20 enriched KEGG pathways in the DRG (A) and spinal cord (B) in mice between sham and HSV-1 inoculation group. The X-axis represents the number of genes, and the Y-axis represents the name of the enriched pathway. The color indicates the enrichment score (adjust $P$-value).
The expression of CCL5 and its receptor CCR5 in mice after HSV-1 inoculation

**A and B** The mRNA expression of *Ccl5* in the DRG (A) and spinal cord (B) of mice after HSV-1 inoculation.

**C and D** The mRNA expression of *Ccr5* in the DRG (C) and spinal cord (D) of mice after HSV-1 inoculation.

**E and F** The protein expression of CCR5 in the DRG (C) and spinal cord (D) of mice after HSV-1 inoculation.

**G** The serum Ccl5 (pg/mL) expression in mice after HSV-1 inoculation.
The serum level of CCL5 in mice after HSV-1 inoculation. Mean ± SD, n = 6; * P < 0.05, ** P < 0.01, *** P < 0.001. One-way ANOVA followed by a 2-tailed t-test with Bonferroni correction.

Figure 9

The analgesic effect of CCR5 blockade on mechanical allodynia, thermal hyperalgesia, and cold alldodynia in mice induced by HSV-1 inoculation.
A Scheme of experimental design of CCR5 blockade. B Mechanical tests in HSV-1 inoculated mice treated with different doses of a CCR5 inhibitor, maraviroc. C and D Paw withdrawal latencies in response to thermal (C) or cold (D) stimuli in HSV-1 inoculated mice treated with different doses of maraviroc. Mean ± SD, n = 6; * P < 0.05, ** P < 0.01, *** P < 0.001. Repeated measures ANOVA followed by Dunnett’s post hoc test were performed, and statistical differences by Dunnett’s post hoc test were shown.

**Figure 10**

The effect of CCR5 blockade on the expression of inflammatory cytokines in mice induced by HSV-1 inoculation

A, B and C The mRNA expression of *Tnf-α* (A), *Il-1β* (B), and *Il-6* (C) in the DRG and spinal cord in HSV-1 inoculated mice treated with maraviroc (50 mg, i.t.). D, E and F The serum levels of TNF-α (D), IL-1β (E), and IL-6 (F) in HSV-1 inoculated mice treated with maraviroc (50 mg, i.t.) determined by using
commercially available ELISA kits. Mean ± SD, n = 6; * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) vs. sham group; 
\( \# P < 0.05 \), \( \#\# P < 0.01 \), \( \### P < 0.001 \) vs. HN+vehicle group. One-way ANOVA followed by 2-tailed t-test with Bonferroni correction.

**Supplementary Files**

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