



Proteome Profile of Trigeminal Ganglion in Murine Model of Allergic Contact Dermatitis: Complement 3 Pathway Contributes to Itch and Pain Sensation

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Abstract

Allergic contact dermatitis (ACD) is a common inflammatory dermatosis characterized by persistent itch and pain after topical contact with reactive chemicals. Although it has been long recognized as a type-IV hypersensitivity, its complexity of pathophysiology mechanism makes it still a clinical aporia in treatment. In this study, we aimed to identify crucial proteins involved in the nociceptive sensation of ACD. Based on a chemical-induced ACD murine model, we collected trigeminal ganglions of ACD and control mice for quantitative tandem mass tag (TMT)-labeling proteomic analysis. Immunohistochemistry was further practiced to validate the bioinformatic analysis. A total of 7685 proteins were identified and analyzed. Sixty-four proteins were significantly upregulated, and 75 proteins were downregulated in ACD mice. GO analysis demonstrated that the changed proteins were significantly enriched in terms of immune and peptidase activity in ACD mice. Proteins involved in the complement and coagulation cascades were notably changed in the KEGG enrichment analysis. The upregulation of complement component 3 (C3) in trigeminal satellite cells of ACD mice was further confirmed by immunohistochemistry. ACD upregulated C3 in trigeminal satellite cells. The complement system in sensory ganglion might play an essential role in forming pruritic and nociceptive sensations in ACD.

Keywords Complement component 3 · Itch · Pain · Allergic contact dermatitis · Proteome

Introduction

Allergic contact dermatitis (ACD) is a clinically irritating disease characterized by its persistent itch and pain after topical contact with an allergen among sensitized people. The most canonical mechanism of ACD is considered a type-IV delayed hypersensitivity reaction, involving both innate and adaptive immune responses in a way that has not been fully elucidated despite extensive research (Ahlström et al. 2019; Alinaghi et al. 2019; Kaplan et al. 2012). Treatments of ACD often contain systemic and topical immunosuppressive medications, including corticosteroids, tacrolimus, and cyclosporine. However, these medications

bring considerable adverse effects that prevent long-term use in clinical settings. Therefore, more detailed pathophysiological mechanisms of ACD, especially the itch and pain symptoms, should be understood to improve the treatment regimen.

Primary sensory ganglions, including dorsal root ganglion and trigeminal ganglion, are exquisitely formed by sensory neurons that are circled by satellite glial cells. The interaction between sensory neurons and satellite glial cells has been found responsible for the formation and persistence of pain and itch, involving multiple immune factors (Ji et al. 2016; Zhang et al. 2017). In addition, the complement system, especially complement component 5 and complement component 3 in the skin and sensory ganglion, was associated with neuropathic pain and inflammatory pain (Jang et al. 2010; Levin 2008; Nie et al. 2013). Also, the activation of the complement system is involved in the pathophysiology of ACD in humans and the murine model of ACD (Niebuhr et al. 2012; Tončić et al. 2011). However, the role of complement in ACD-associated itch and pain symptoms is obscure.

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Proteome study provides researchers a more potent tool to understand the thorough picture of protein expression in specific tissues or cells, presenting noticeable molecules in comprehensive pathways (Cho 2014; Dove 1999). Researchers have been trying to find biomarkers in plasma or dermal tissue of ACD patients through proteomic strategy. Serum proteomic study showed an increased immune cell activation, signaling, and trafficking in contact dermatitis (Wang et al. 2017). However, no proteome analysis of the ACD model was done in the primary nociceptive ganglion.

In this study, we adopted a tandem mass tags (TMTs) labeling and an LC–MS/MS platform for quantitative proteomics analysis to elucidate the protein profile in the trigeminal ganglion of ACD mouse and control. Bioinformatics analyses were performed regarding proteins that differed significantly between the two groups. Immunohistochemistry (IHC) was further used to validate results from proteomics analysis. We aim to identify changes in protein expression and associated signaling pathways in ACD mice to better understand the pathophysiological mechanism in the nociceptive sensation of ACD.

Materials and Methods

Animals

All experiment procedures were approved by the Animals Ethics Committee of our institution. Six adult male C57BL/6 mice (2–3 months old, from HFK Bioscience Co., Ltd, Beijing, China) were housed in a controlled environment with food and water ad libitum. Housing conditions were maintained at 21 ± 4 °C and 12 h light/dark cycles.

Model of ACD

The ACD model induced by squaric acid dibutyl ester (SADBE) was conducted according to previously established protocols and experience of our team (Meixiong et al. 2019; Su et al. 2020). In order for sensitization, 25 µl of 1% SADBE in acetone was superficially applied on shaved abdomen skin for 3 days. After another 5 days, 25 µl of 1% SADBE was superficially applied on the shaved cheek for two consecutive days as elicitation. For control mice, acetone of equal volume was applied both in the sensitization phase and elicitation phase.

Behavior Test Assay

For behavioral testing, according to previous studies, mice were placed in a test chamber with all sides mirrors and were recorded for 30 min as an observation period (Jiang et al. 2019). A laboratory administrator blinded to the grouping

method counted the scratching and wiping behavior of mice. All records were performed after 24 h of the second SABDE challenge. One scratching bout was defined as a mouse using its hind paw to scratch the treated cheek multiple times within seconds and then putting its hind paw back to the ground. One wiping bout was defined as a mouse using its forepaw to wipe the treated cheek area and then returning to its original position.

Sample Preparation and TMT Labeling

Ipsilateral trigeminal ganglions (TGs) were collected from mice perfused transcardially with 0.1 M PBS under deep anesthesia with isoflurane. Specimens were homogenized with ice-cold homogenization buffer (8 M urea with 1% SDS and protease inhibitor). The protein solution was collected after centrifuging at 25,000 g at 4 °C temperature. The concentration of protein from each sample was determined by a BCA kit, followed by extracting 100 µg protein from each sample. Triethylammonium bicarbonate buffer (TEAB, 100 mM) and iodoacetamide (IAM, 40 mM) were applied to reduce alkylation. The peptides were harvested after enzymolysis by trypsin (PROTEOMEGA Corporation, Beijing). 6-plex TMT (Thermo Fisher, 90,064) was used to label the peptides from each sample.

High-Performance Liquid Chromatography and LC–MS/MS

Thermo Scientific Vanquish Flex UHPLC was used to separate peptides for the following LC–MS/MS. The column was ACQUITY UPLC BEH C18 Column 1.7 µm, 2.1 mm × 150 mm (Waters, USA) with phase A (2% acetonitrile) and phase B (80% acetonitrile). Another column C18 column (75 µm × 25 cm, Thermo, USA) was applied for LC–MS/MS with phase A (2% acetonitrile, 0.1% formic acid) and phase B (80% acetonitrile, 0.1% formic acid). EASY-nLC 1200 (Thermo, USA) together with Q_Exactive Plus (Thermo, USA) was used for LC–MS/MS, and the mass spectrum information was collected by Thermo Xcalibur 4.0 (Thermo, USA).

Bioinformatic Analysis

For bioinformatics analysis, Thermo Fisher Scientific Proteome Discover software suite 2.4 was used for LC–MS/MS data analysis under the control of the SEQUEST search engine. For each protein, the average abundance of the three ACD samples and three control samples were calculated separately and used for further analysis. Gene ontology (GO) annotation was performed by the DAVID tool (<https://david.ncifcrf.gov/>); furtherly, GO enrichment analysis was performed by Cytoscape and attached plug-in. The pathway

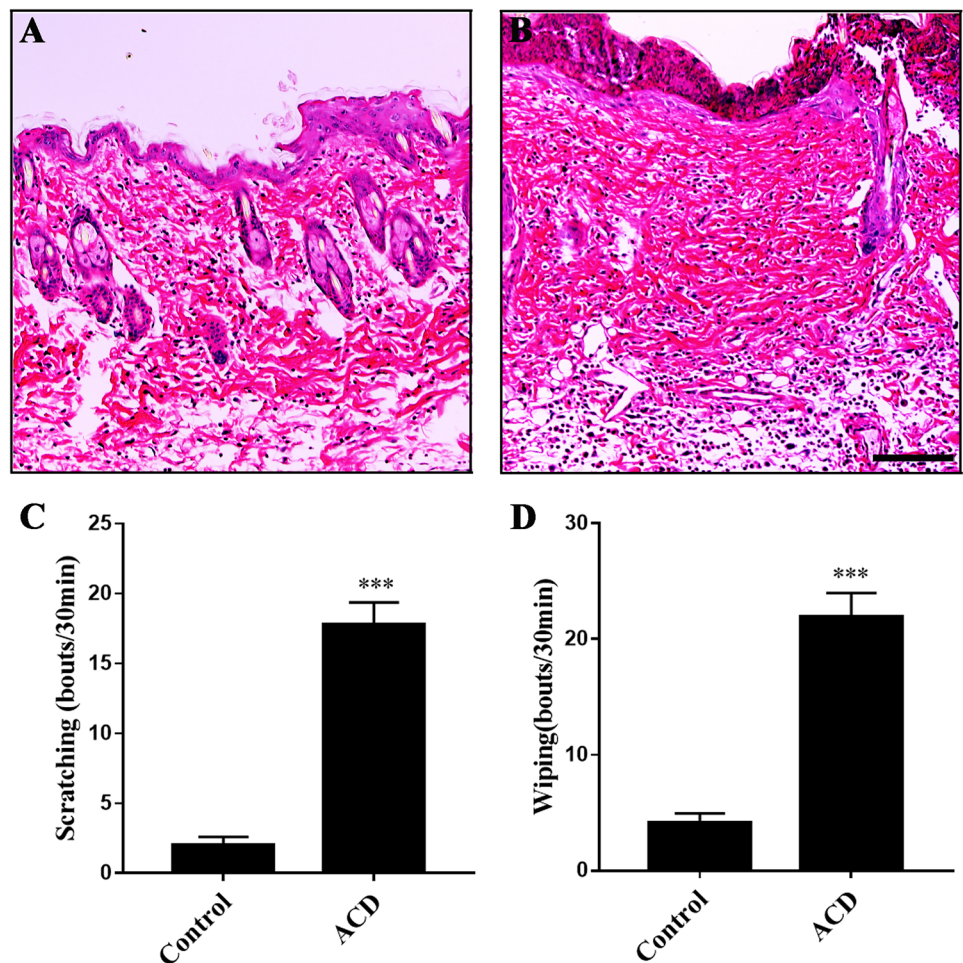
analysis was conducted according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (<http://www.genome.jp/kegg>). The STRING database (<https://string-db.org>) was applied to analyze the network among differently expressed proteins.

Histology and Immunohistochemistry

Experimental mice were perfused with 0.1 M phosphate-buffered saline (PBS) via left ventricle under deep anesthesia with isoflurane. The outflow of blood depended on an incision by iris scissors on the right atrium. After blood evacuation, 4% paraformaldehyde was perfused for fixation. Treated cheek skin was then resected, and skin specimens were further fixed in 10% formalin at room temperature, transferred sequentially to 100% methanol, and eventually embedded with paraffin and sectioned into 3- μ m-thick slices. Skin sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. For immunohistochemistry staining, sections were treated with microwave heat-induced antigen retrieval (121 °C, 15 min) to reveal endogenous biotin activity and were transferred

into 5% hydrogen peroxide to block endogenous peroxidase. After blocking the non-specific binding of the antibody with 5% normal horse serum, sections were incubated with primary antibody (rabbit anti-complement 3, 1:1000, Abcam, USA) at 4 °C overnight and then rinsed with PBS. Then, sections were incubated with proper secondary antibodies (Goat Anti-Rabbit IgG H&L, 1:1000, Abcam). Immunofluorescence was performed following previous studies (Jiang et al. 2019). Briefly, following blocking the non-specific binding of the antibody with 5% normal horse serum, sections were incubated with primary antibody (Rabbit anti-Complement 3, 1:1000, Abcam, USA) at 4 °C overnight and then rinsed with PBS. Then, sections were incubated with proper secondary antibodies (Goat Anti-Rabbit IgG H&L, 1:1000, Abcam). For sections applied to immunofluorescence, primary antibodies (Rabbit Anti-Complement 3, 1:1000, Abcam, USA; Chicken Anti-GFAP, 1:1000, Abcam, USA) was incubated overnight at 4 °C, followed by the incubation of corresponding secondary antibody (Goat Anti-Rabbit IgG Alexa Fluor® 488, 1:400, Abcam, USA; Goat Anti-Chicken IgY Alexa Fluor® 594, 1:400, Abcam, USA). All of the sections were cover-slipped with Mounting Medium with

Fig. 1 Pathology and behavioral changes in ACD mice. **A, B** Representative skin biopsies for control and ACD mice, respectively. ACD skin was prominently swollen and infiltrated with inflammatory cells, and proliferation of stratum spinosum cells was also observed. Scale bar 200 μ m. **C, D** ACD mice manifested with significantly more spontaneous scratching and wiping behavior. $n=8$ for each group, ***Student's t-test. $P<0.001$, ACD vs. control group



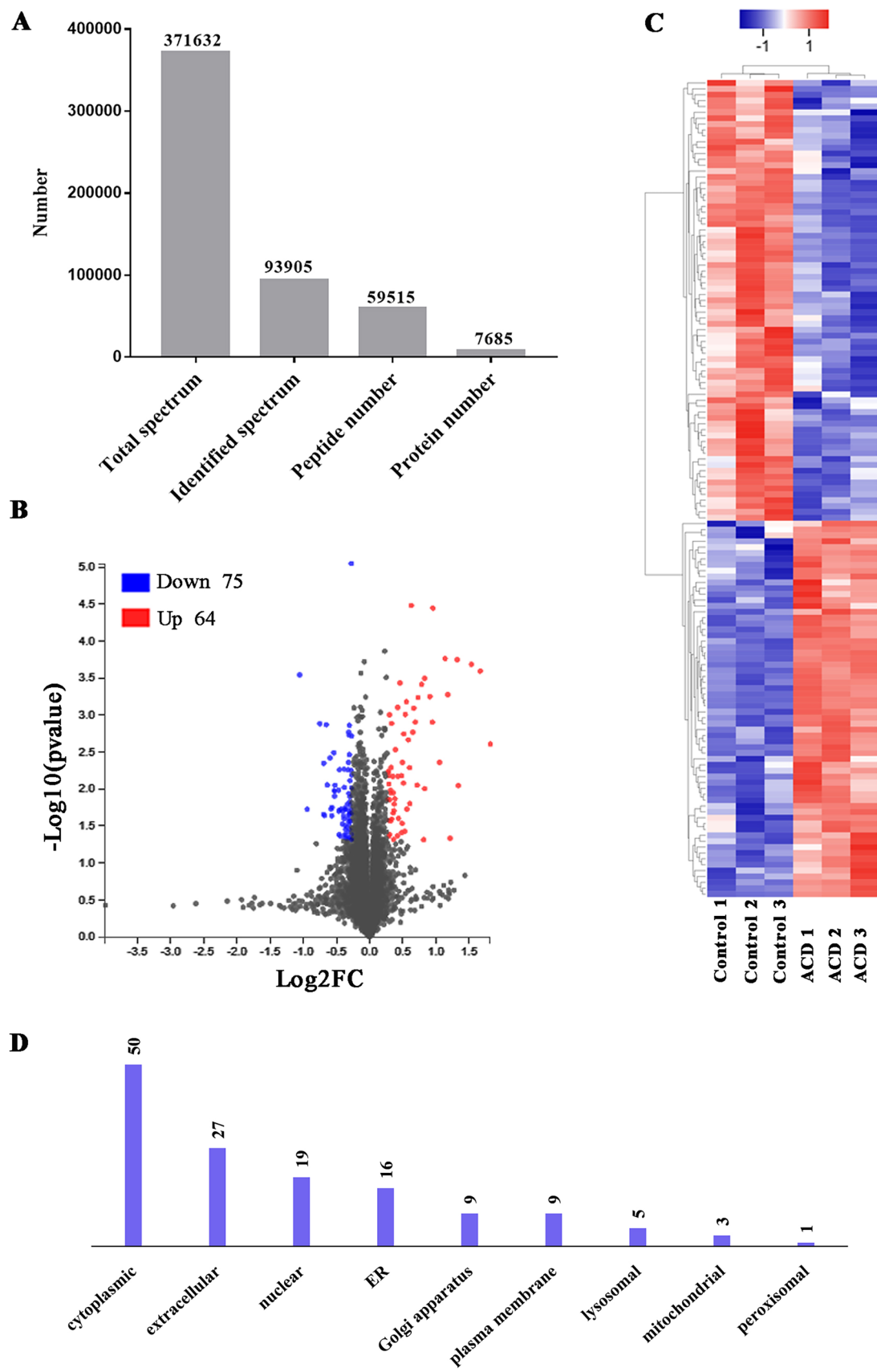


Fig. 2 Distinct protein profile of ACD and control mice. **A** The basic information of detected spectrums, peptides, and proteins. **B** The volcano plot showed the differently expressed proteins in ACD samples. A total of 64 proteins were upregulated in ACD mice trigeminal ganglion (red dots, fold change > 1.2, $p < 0.05$), 75 proteins were downregulated in ACD mice TG (blue dots, fold change < 0.83, $p < 0.05$). **C** Heatmap showed different expression of proteins between ACD and control. **D** The subcellular distribution of 139 differently expressed proteins in TG from ACD mice

DAPI (ZSJB-Bio, China). Images were acquired with a laser confocal microscopic imaging system (FV1000 and Olympus FluoView software, Olympus, Japan) in a dark room.

Trigeminal Injection

We applied trigeminal injection to deliver C3 antibody in order to neutralizing C3 in TG. We performed trigeminal injection follow previous study (Zhang et al. 2016). The drugs were injected from the infraorbital foramen to the foramen rotundum with a 30-G needle. The tip of the needle terminated at the medial part of the trigeminal ganglion, and the reagent (10 μ g in 5 μ l, isotype rabbit IgG or rabbit anti-C3 antibody) was slowly delivered to the trigeminal

ganglion. Drug delivery was performed 6 h before behavioral test.

Statistical Analysis

IBM SPSS Statistics 21.0 for Windows (Armonk, NY, USA) was used for statistical analysis. All data are presented as the mean and its standard error (mean $\pm$ SEM). Shapiro–Wilk test was applied to determine the normality for parametric test. Behavioral results and immunohistochemistry results were analyzed using Student's t-test. A p value < 0.05 was considered statistically significant. Statistical figures were drawn with GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA).

Results

Spontaneous Itch and Pain Behavior in ACD Mice

Cutaneous histology showed typical changes of allergic contact dermatitis in the ACD group of our study. ACD mice presented with prominent epidermal thickening and edema,

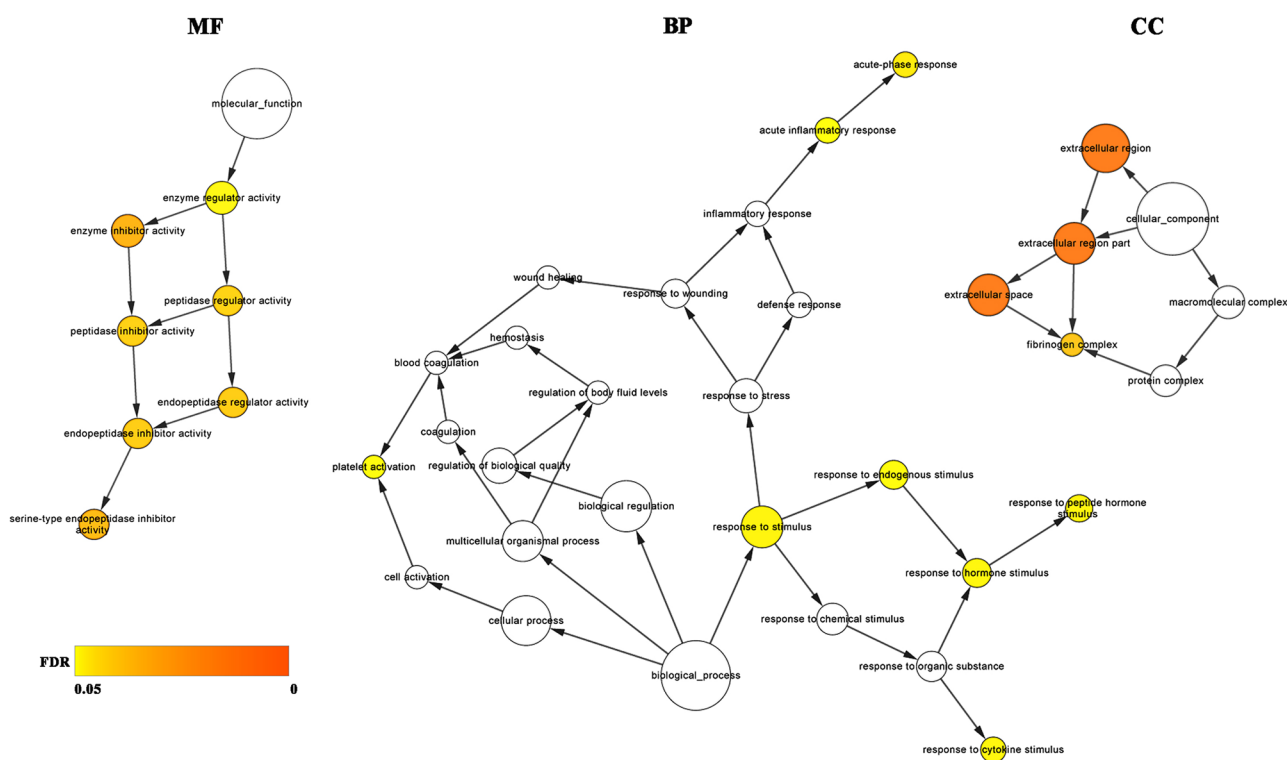


Fig. 3 The gene ontology analysis of DEPs. The color depth of nodes refers to the corrected P-value (FDR) of ontologies as shown in the scale, and FDR < 0.05 was considered statistically significant. The

size of nodes refers to the number of genes that are involved in the terms. BP biological process. CC cellular component; MF molecular function

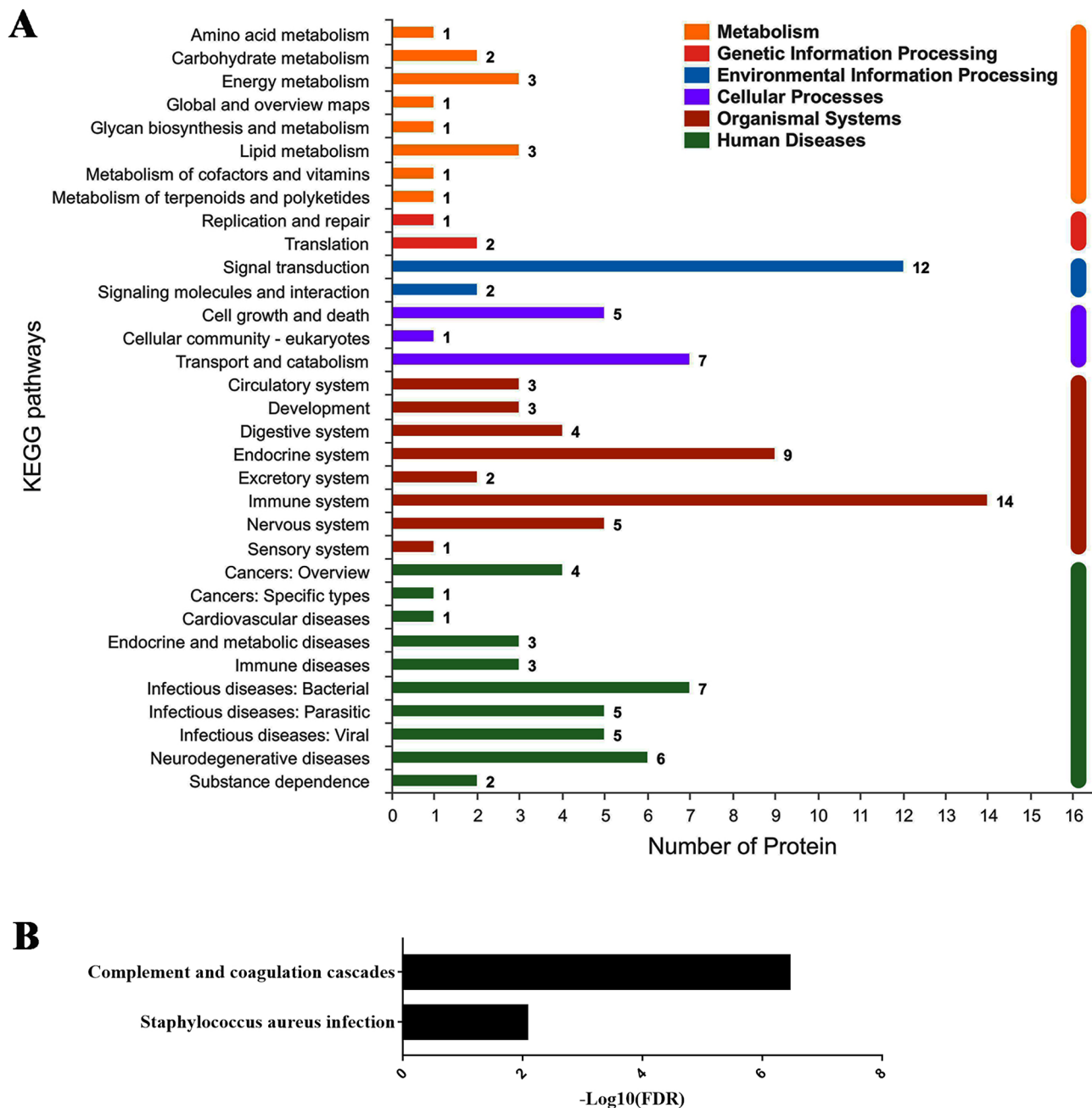


Fig. 4 The KEGG analysis of DEPs. **A** The KEGG pathway annotation of DEPs was listed and classed by the broad headings of the KEGG database, and the numbers of proteins in each KEGG term

were labeled. **B** Enrichment analysis of the KEGG pathway showed the pathways that DEPs enriched to (FDR < 0.05)

immune cell aggregation, and spinous cell proliferation in treated cheek skin after elicitation compared to control mice (Fig. 1A, B). We used the cheek model to determine the behavioral effect of ACD. The counts of wiping and scratching towards the treated area, 24 h after the second challenge, were regarded as the presentation of pain and itch, respectively. Our results showed that ACD mice showed significantly increased pain- and itch-like behavior, indicating the

construction of the ACD model ($n = 8$, $P < 0.001$, Fig. 1C, D).

Distinct Protein Profiles in ACD Murine Model

According to the results of Protein Discovery, 93,905 spectrums were determined, which identified 59,515 peptides (Fig. 2A). A total of 7685 proteins were detected in

samples with the 1% protein FDR levels, among which 139 proteins were dysregulated in the TG from the ACD model. For ACD group, 64 (0.83%) proteins showed significant upregulation (fold change > 1.2, $p < 0.05$) and 75 (0.98%) proteins showed significant downregulation (fold change < 0.83, $p < 0.05$) (Fig. 2B). Cluster analysis was performed to present the changes in protein expression between control and ACD mice. As shown by the heatmap for the differently expressed proteins, the samples in the control and ACD group aggregated into two subclusters, respectively (Fig. 2C). In order to fully understand the function of the differently expressed proteins (DEPs), we analyzed the subcellular location for these 139 DEPs by WoLF PSOR tool. A majority of these proteins were located in cytoplasmic ($n = 50$, 35.97%), extracellular ($n = 27$, 19.42%), and nuclear ($n = 19$, 13.67%), indicating the acute alteration of corresponding subcellular structure (Fig. 2D).

Gene Oncology Analysis of DEPs

In order to functionally identify the DEPs, firstly, we performed gene ontology (GO) analysis for these proteins. The results of GO annotation were shown in Fig. S1 by listing the GO terms in molecular function, cellular component, and biological process. For molecular function, DEPs are mainly annotated to binding, catalytic activity, and molecular function regulator, while DEPs annotated to the cellular anatomical entity, protein-containing complex, and intracellular for the cellular component. Response to stimulus, metabolic process, and immune system process were the main terms in biological process, indicating ACD-induced molecular changes in metabolism and immunity in TG. Further, we performed the GO enrichment analysis by the BINGO plug-in in Cytoscape for the DEPs. In all, the DEPs were enriched into 19 GO terms under the control of FDR < 0.05. As shown in Fig. 3, the colorful circle presented the GO

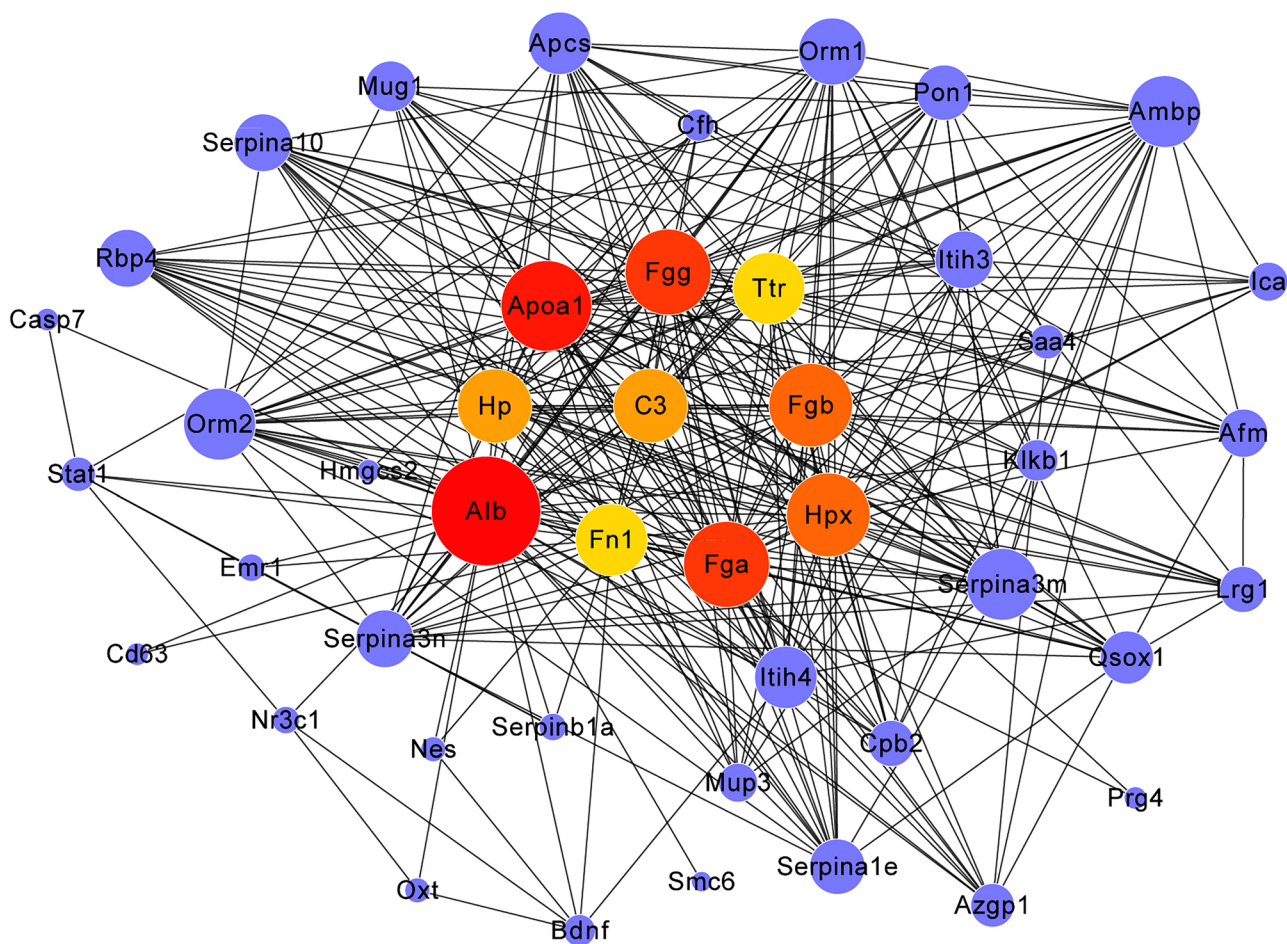


Fig. 5 The construction of protein–protein interaction network for DEPs. Each node was scored based on the linked intensity with other nodes, presented by the size of the circle. The top-10 highest-scoring

proteins were colored, and the color depth of nodes refers to the scores of the node, reflecting the connectivity with other nodes

terms, in which the DEPs significantly enriched. GO terms in molecular function, including enzyme regulator activity, enzyme inhibitor activity, peptidase regulator activity, peptidase inhibitor activity, endopeptidase regulator activity, endopeptidase inhibitor activity, and serine-type endopeptidase inhibitor activity, indicated the changes of enzyme

activity induced by ACD. GO terms in the biological process like acute inflammation response and acute-phase response indicated the inflammatory process in TG induced by ACD. For the cellular component terms, such as extracellular region and extracellular region part, reflected the changes of extracellular structure.

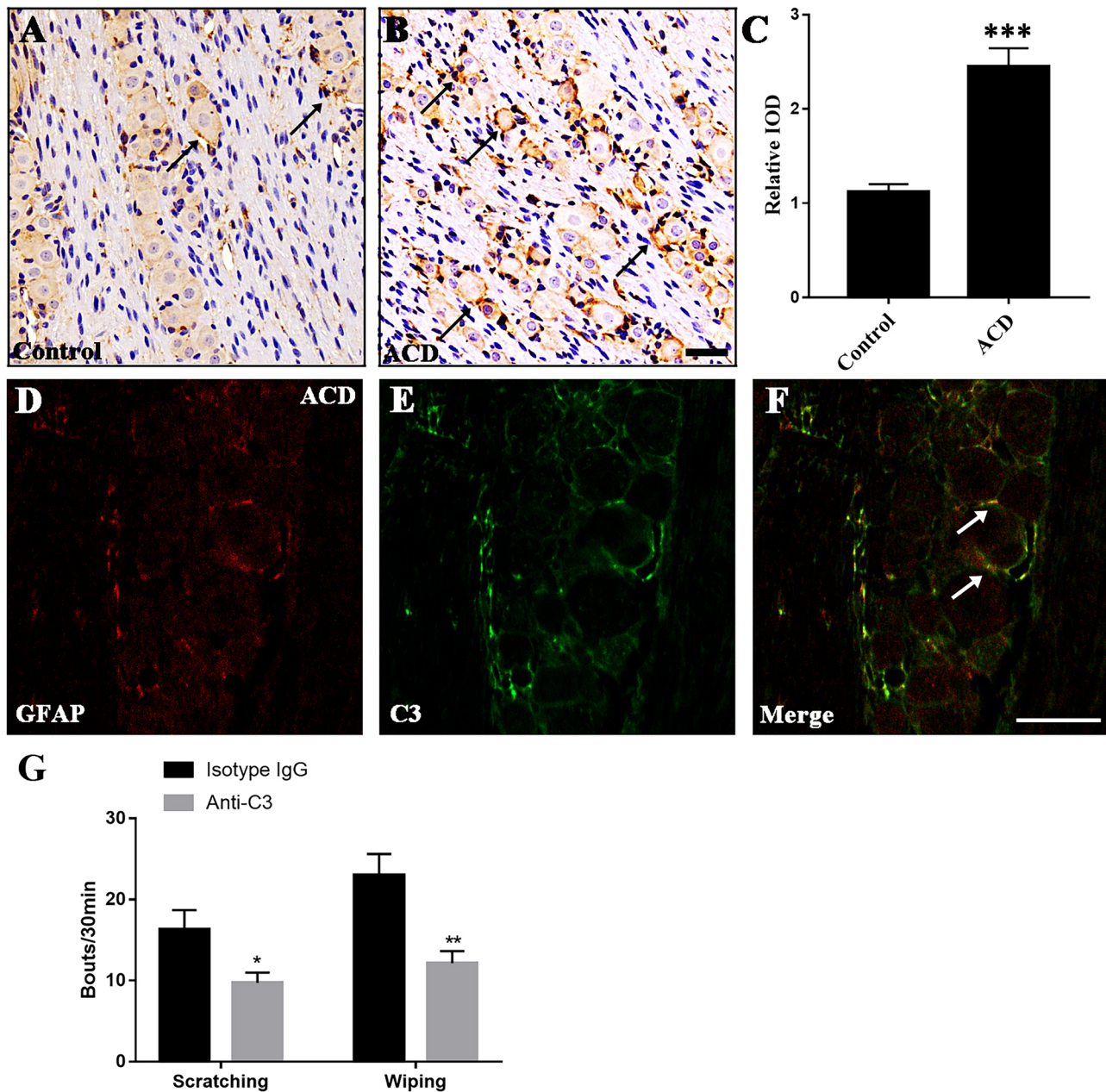


Fig. 6 ACD upregulated the expression of C3 in satellite glial cells. **A** Immunohistochemical staining showed the existence of C3 in TG of control mice. **B** More abundant C3 was observed in TG of ACD mice. **C** Quantitative analysis showed that integrated optical density (IOD) of C3 was significantly increased in ACD mice. $N=3$, ***Student's t-test. $P<0.001$. Scale bar 50 μm . **D–F** Immunofluorescence

staining showed the co-localization of complement 3 and GFAP. Scale bar 50 μm . **G** Trigeminal injection of C3 antibody alleviated scratching and wiping behavior compared with isotype IgG. $n=7$ for each group, * $P<0.05$, ** $P<0.01$, Student's t-test, C3 antibody vs. isotype IgG

Pathway Analysis of DEPs

The pathway analysis of DEPs was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The annotation of DEPs to KEGG pathways was summed and listed in Fig. 4A. Signal transduction and the immune system are the main categories to which the DEPs are annotated. Under the control of $FDR < 0.05$, we performed the enrichment analysis of the KEGG pathway for the DEPs, discovering the complement and coagulation cascades pathway. Detailed information of this pathway was shown in Fig. 4B. For the detailed information in this signaling, we performed enrichment analysis based on the wiki pathway by WebGestalt. All related proteins in the complement and coagulation cascades pathway were mapped and represented with the same color series but different saturations according to their expression level (e.g., strongly upregulated proteins are colored dark red, whereas those slightly upregulated are colored light red) (Fig. S2).

Protein–Protein Interaction Network

The PPI network was constructed according to the STRING database and reanalyzed by cytoHubba plug-in in Cytoscape. Forty-five DEPs were tightly connected and showed interaction with other proteins. The top-10 proteins by the evaluated score were colored, including complement 3 and fibronectin-1. In addition, Stat1, Bdnf, and Casp7 were also showed tight connections with other proteins, which has been demonstrated to be involved in itch and pain (Fig. 5).

Complement Component 3 Was Upregulated in Satellite Glial Cells Induced by ACD

Previous studies indicated that the complement system was involved in inflammatory and neuropathic pain. As C3 was one of the hub genes in the DEPs network, to validate the proteomic data and determine the expression pattern of C3 in TG, we performed immunohistochemistry to compare the expression between control and ACD mice. To our best knowledge, we first time determined that C3 was expressed in satellite glial cells in the TG (Fig. 6A, B). The relative integrated optical density (IOD) was applied to compare the expression between control and ACD mice, showing that ACD significantly upregulated the expression of C3 in TG (Fig. 6C). Considering that the immunohistochemistry results identified the satellite glial cells just based on the relative location, the immunofluorescence was applied to accurate the cellular subtype by the molecular marker. Our immunofluorescence results for TG from ACD mice showed that C3 was co-expressed with the glial fibrillary acidic protein (GFAP), one molecular marker

for satellite glial cells (Fig. 6D–F). In addition, neutralizing C3 in TG by trigeminal injection suppressed itch- and pain-like behavior induced by ACD (Fig. 6G).

Discussion

In the proteome analysis of trigeminal ganglion tissue in allergic contact dermatitis (ACD) murine model, 139 proteins were identified with significantly different expressions between ACD and control mice. Bioinformatics analysis of the DEPs was performed to investigate associated biological changes in a functional perspective. GO analysis demonstrated that immune-associated processes were significantly changed in TG from the ACD model. Complement and coagulation cascades were revealed as the main changes signal pathway that might be involved in the pain and itch symptoms in ACD. In addition, the construction of the PPI network shown that complement 3 served as one of the hub genes in this network. Finally, immunohistochemistry revealed that complement 3 was expressed in the satellite glial cells and was upregulated by ACD.

ACD, orchestrated by multiple proinflammatory mediators, is an inflammatory skin disease with characteristic pain and itch symptoms in the affected skin. Recent studies indicated that ACD not only evoked type-IV delayed hypersensitivity reaction in the affected skin but induced molecular changes in the primary sensory ganglion, which might be related to sensory symptoms (Jiang et al. 2019; Qu et al. 2014, 2015). The primary sensory ganglion was constructed by the sensory neuron that served to relay the somatosensory information and the surrounding satellite glial cells. Under the ACD pathological condition, the inflammatory processes in sensory neurons had been widely investigated, including chemokines (CCL2, CXCL9, CXCL10, and CXCL12), chemokine receptors (CCR2, CXCR3, and CXCR4), which might be responsible for the enhanced activity of sensory neuron (Su et al. 2020). Also, satellite glial cells always interact with sensory neurons through mediators and corresponding receptors, which might maintain the nociceptive sensations (Ma and LaMotte 2005; Song et al. 2018). In this study, through the proteomic strategy, we detected many DEPs related to an acute inflammatory response like complement factor H, serum amyloid A-4, and alpha-1-acid glycoprotein 2. However, the relative cellular subtype of these DEPs should be identified furtherly. Our KEGG pathway revealed that DEPs significantly enriched in the complement and coagulation cascade pathway. C3, the main effector of the complement system, was upregulated in the trigeminal ganglion of ACD mice, according to the proteomic data. Interestingly, we detected C3 in satellite glial cells, and the upregulation of C3 was also determined by immunohistochemistry. To our best knowledge, this was the

first study that discovered the changes of C3 in satellite glial cells under ACD pathology.

The complement system works as a bridge between innate and adaptive immune responses. As the complement system and inflammatory mediators connect closely, the activation of the complement system might lead to the accumulation of inflammatory factors, while inflammation, in turn, upregulates complements synthesis. Among all the components in the complement system, C3 plays a central role, staying at the crossroads of classical, alternative, and lectin pathways. In the classic complement pathway, activation of C3 results in a cascade reaction leading to the formation of membrane attacking complex (MAC) and dissolve of cells and upregulating inflammation. C3 also plays an important role in chemical-induced inflammatory diseases. C3a receptor signaling promotes inflammatory response and accumulation of Th17 cells in trichloroethylene hypersensitivity syndrome (Wang et al. 2019), while antagonists of C3a reduced the secretion of IL-1 β , IL-6, and IL-23. It has been previously discovered that C3 knock-out mice presented with increased inflammation in response to both dermal Th1 sensitizer and Th2 sensitizer, corresponding to type-IV delayed hypersensitivity and type I immediate hypersensitivity. More prominent soft tissue swelling and IFN- γ were observed in C3 knock-out mice than wild-type (Purwar et al. 2011), indicating the protective effect of C3 for skin inflammation. Further experiments adopting C3a receptor-deficient mice showed that C3aR functioned in allergic sensitization but not the elicitation phase (Niebuhr et al. 2012). This result seemed to show that C3 functioned as an anti-inflammatory factor. However, as C3 knock-out influences a great variety of downstream factors, its mechanism should be further illustrated. As a downstream factor of C3, C5aR was found to play a proinflammatory role in ACD (Wang et al. 2013).

There has been quite a little evidence showing that the complement system is involved in nociceptive sensations, especially in the spinal dorsal horn. Significantly, C3 functions as a biomarker of active astrocytes that might be associate with pain (Ji et al. 2019; Li et al. 2019). The nociceptive effect of complement component 5 and C5aR had been identified in neuropathic pain and inflammatory pain associated with microglia activation. In addition, C3 was considered an indicator for complement system activation in both induction and development of hyperalgesia in neuropathic pain. In the chronic constriction injury (CCI) model, increased expression of C3 mRNA was observed in the spinal dorsal horn in association with hyperalgesia (Nie et al. 2013). It was also considered that the neuronal complement pathway played an important role in promoting microglia activation by C3R and contributed to bone cancer pain (Huang et al. 2018). In addition, activation of C3aR was found to be

involved in nociceptive sensitization by facilitating capsaicin-induced calcium influx in neurons of dorsal root ganglion (Jang et al. 2010). Our findings suggested that the complement system might play an important role in the formation of pain and itch in the allergic contact dermatitis model at the primary sensory ganglion, specifically the satellite glial cell. This study highlighted the proteomic changes in sensory ganglion under ACD condition, and the infiltrated DEPs provided an insightful idea of clinical intervention for the nociceptive symptoms of ACD patients.

Limitations

This proteomic study revealed that activation of fibrinolysis and complement system might play an important role in pruritus and nociceptive sensation in ACD. However, specific mechanisms still need to be explored. More experiments, especially in vivo and pharmacologically, would reveal the molecular mechanism of ACD-associated itch and pain furtherly.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12640-021-00384-4>.

Author Contribution Wenliang Su performed the behavioral test, immunohistochemistry, and proteomic analysis. Wenliang Su and Jiawen Yu drafted the manuscript. Lulu Ma designed this project and drafted this manuscript. Xiuhua Zhang and Yuguang Huang reviewed this manuscript and offered kind suggestions. All these authors have approved this manuscript and agreed to its publication.

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Declarations

Ethics Approval All experimental procedures were approved by the Institutional Animal Care and Use Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College and were in accordance with the guidelines provided by the National Institute of Health and the International Association for the Study of Pain.

Consent for Publication All authors have approved the manuscript and agreed for its publication.

Conflict of Interest The authors declare no competing interests.

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