Scientific Research Report

New Insights Into the Correlation Between Necroptotic Activation and Neutrophil Infiltration in Pulpitis

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ARTICLE INFO

Article history:
Received 6 December 2024
Received in revised form
19 February 2025
Accepted 20 February 2025
Available online xxx

Keywords:
DPCs
Necroptosis
Neutrophils
Pulpitis

ABSTRACT

Background: Pulpitis is a significant dental concern characterized by inflammation of the dental pulp, often causing severe pain and potential tooth loss. Immune infiltration and necroptosis, both implicated in various inflammatory conditions, have been noted in pulpitis. However, the roles of necroptosis and immune infiltration in pulpitis pathogenesis remain poorly understood.

Methods: Hematoxylin and eosin (HE) staining assessed inflammatory infiltration in dental pulp tissues. Western blotting (WB) and immunohistochemistry (IHC) quantified pMLKL expression. Transcriptome datasets GSE92681 and GSE77459 from GEO identified necroptosis-related differentially expressed genes (NRDEGs) via bioinformatics analysis. Functional enrichment identified 6 hub NRDEGs based on diagnostic capability through machine learning and immune infiltration. Transwell assays evaluated neutrophil infiltration post dental pulp cells (DPCs) necroptosis. The STRING database analyzed CXCL8-activated neutrophil receptors, and Transwell and co-immunoprecipitation (Co-IP) validated ligand-receptor interactions.

Results: Increased pMLKL expression in inflamed dental pulp indicates activation of necroptosis pathways. Functional enrichment analysis revealed that NRDEGs were mainly involved in necroptosis. Both datasets showed significant inflammatory infiltration. Bioinformatics suggested a link between immune infiltration and necroptosis in DPCs, with correlation analyses showing NRDEGs were closely associated with neutrophil infiltration. Necroptotic DPCs produce CXCL8, which recruits neutrophils via the C3AR1 receptor. The CXCL8/C3AR1 axis, validated by Transwell and co-immunoprecipitation assays, is crucial for communication between necroptosis and neutrophil chemotaxis in DPCs.

Conclusion: Necroptosis occurs in pulpitis and correlates with immune cell infiltration. DPCs undergoing necroptosis can recruit neutrophils via the CXCL8/C3AR1 axis, exacerbating inflammation. This study offers new insights into the pathogenesis of pulpitis and potential therapeutic strategies.

Introduction

Pulpitis, an inflammatory condition that specifically affects the pulp tissue, can give rise to detrimental consequences if left untreated. These consequences encompass the potential for damage to the periapical tissue, impairing tooth mobility,

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https://doi.org/10.1016/j.identj.2025.02.016

tooth preservation, and even the maxillofacial structure.[1] Necroptosis plays an important role in inflammatory diseases,[2] but its role in pulpitis remains to be determined. Necroptosis is a typical pattern of programmed cell death.[3] The most typical signal transduction cascade is initiated by tumor necrosis factor receptor 1 (TNFRI).[4-7] Necroptotic cells exhibit a necrotic phenotype, including swelling and membrane rupture, and release damage-associated molecular patterns, inflammatory cytokines and chemokines that mediate extreme inflammatory responses.[8-10] Therefore, it is crucial to investigate the role and underlying mechanisms of necroptosis in pulpitis.

Immune infiltration is 1 of the hot spots in the study of inflammatory diseases in recent years. Pulpitis is mainly caused by the invasion of bacteria and their toxic products.[11] To fight against bacterial infection, the pulp tissue initiates immune responses.[12] An increasing number of studies have shown that immune inflammation is involved in the pathophysiological process of pulpitis,[13] and the presence of immune cell infiltration in dental pulp tissue has been found.[14,15] Previous studies have shown that the degree of neutrophil infiltration correlates with the severity of pulpitis, with only a few neutrophils in healthy pulp tissue and abundant neutrophils in tissue with irreversible pulpitis.[16,17] Hence, it is of great interest to explore the role of necroptosis and neutrophil infiltration in the pathogenesis of inflamed dental pulp.

In this study, we discovered that necroptotic DPCs can exacerbate pulpitis by attracting neutrophils through the CXCL8/C3AR1 axis. Hub necroptosis-related differentially expressed genes (NRDEGs) were screened by a systematic bioinformatics analysis of the GEO dataset. In addition, we found that cells undergoing necroptosis in pulpitis would promote neutrophil infiltration by means of ligand–receptor interactions. We demonstrated that DPCs undergoing necroptosis in inflamed dental pulp may produce CXCL8 and interact with the neutrophils membrane receptor C3AR1 to recruit neutrophils. These results further elucidate the underlying molecular immune processes in the development process of pulpitis.

Materials and methods

Collection of clinical samples

Healthy and inflamed dental pulp tissue was collected from clinical patients who signed an informed consent form. Detailed information on the participants can be found in Additional file 1. The dental pulp samples were obtained from 10 healthy individuals aged 20-50 years, including 6 females and 4 males. A total of 5 healthy third molars and 5 inflamed third molars were collected for subsequent experiments. After the teeth were broken with a hammer, the pulp was removed intact and cleaned 3 times with saline. RNA was extracted from dental pulp using Tissue RNA Purification Kit PLUS (EZBioscience, EZB-RN001-plus). The specific procedures included sonication of tissue, lysis of cells by lysate, RNA precipitation, washing and drying, and dissolution of RNA. Teeth with the following symptoms were diagnosed as pulpitis[18]: (1) Typical pain symptoms of acute pulpitis, including spontaneous and paroxysmal severe throbbing pain, imprecise localization, and radiating pain. (2) The presence of dental lesions or other causes that could be identified as causing pulpal lesions. (3) Thermal stimulation aggravates pulp pain. The pulp tissue from a single tooth was divided into 3 sections. The structurally intact portion was used for paraffin embedding, while the remaining 2 sections were used for RNA and protein extraction, respectively. Thus, each experiment included 5 samples. The study followed the ethical guidelines of the Declaration of Helsinki and was approved by the Research Medical Ethics Committee (NFEC-2023-139). All participants were aware of the study and provided written informed consent.

HE staining

The dental pulp tissue was fixed in 4% paraformaldehyde for 8 hours, dehydrated, and embedded in paraffin. Paraffin blocks were then sectioned using a paraffin microtome to obtain 4μm thick paraffin sections. After dewaxing, the paraffin sections were hydrated and stained according to the instructions provided in the Hematoxylin-Eosin staining kit (Solarbio, G1120). Finally, the stained sections were fixated with neutral balata.

IHC staining

For IHC staining, after dewaxing and rehydration, the paraffin-embedded dental pulp tissue sections were incubated with primary antibodies against Phospho-MLKL (Cell Signaling Technology, 18640, 1:300) at 4 °C overnight and biotin-labeled secondary antibodies (Abmart, A10029S, 1:5000) at 37 °C for 1.5 h. The sections were then stained with DAB (Solarbio, DA1010) and hematoxylin.

Western blot analysis

Tissue or cell samples were washed with cold phosphate buffer solution (PBS), protein solutions were collected after sufficient lysis, and protein concentrations were measured with a BCA assay kit (Beyotime, P0010). All protein samples were separated on SDS–PAGE gels and transferred to PVDF membranes (Merck millipore, SLGV004SL). After blocking with 5% nonfat dry milk solution (Beyotime, P0216) for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies, including pMLKL (Cell Signaling Technology, 18640, 1:1000), CXCL8 (Abcam, ab289967, 1:1000), and GAPDH (Abcam, ab181602, 1:1000). Then, the cells were incubated with secondary antibodies (Abmart, A10029S,1:10000) for 1 h at room temperature. Finally, protein bands on the membrane were detected using chemiluminescence.

qRT–PCR

Collected healthy and inflamed pulp were used to extract RNA by Tissue Total RNA Extraction Kit (Foregene, RE-03011). RNA was reverse transcribed into template DNA using the Color Reverse Transcription Kit (EZBioscience, A0010CGQ). The expression of NRDEGs was verified by qRT–PCR using QuantStudio™ Real-Time PCR software (Thermo Fisher

Scientific, V1.3). Primer sequences were designed using the Primer Blast Tool available on the National Center for Biotechnology Information (NCBI) website and reported in Additional file 2.

Isolation and culture of human primary neutrophils

The procedure utilizes the human peripheral blood neutrophil separation kit (Solarbio, P9040) as follows:

(1) Sample Preparation: Mix 10 ml of fresh anticoagulated blood with an equal volume of diluent, then carefully layer this mixture onto 1 part of Solution A. Centrifuge at 500 g (approximately 1800 rpm) for 25 minutes using a horizontal rotor (15 cm radius). This centrifugation yields 4 layers: plasma, a cloudy white layer of mononuclear cells, a slightly turbid neutrophil-enriched layer, and red blood cells. Discard the plasma and mononuclear layers, collecting the third and fourth layers. (2) Cell Washing: Transfer the collected layers to a tube containing 10 ml of cell wash solution. Mix thoroughly and centrifuge at 500 g (approximately 1800 rpm) for 30 minutes. (3) Red Blood Cell Lysis: After washing, add 6-10 times the volume of red blood cell lysis buffer to the pellet. Gently mix and incubate for 10 minutes at room temperature. Pre-cool the centrifuge to 4 °C, then centrifuge at 400-500 g for 5 minutes and discard the red supernatant. If lysis is incomplete, repeat this step. After 3 washes to remove debris, the pellet contains the desired neutrophils. (4) Final Washes: Wash 1-2 times by adding 20 ml of saline, resuspending the pellet, mixing, and centrifuging at 400-500 g for 2-3 minutes, discarding the supernatant. After resuspending the cells, perform cell counting, seed plates, and proceed with subsequent experiments. The study followed the ethical guidelines of the Declaration of Helsinki and was approved by the Research Medical Ethics Committee (NFEC-2023-139).

Transwell experiments

DPCs were cultured as in the previous study.[19] Dental pulp was obtained from healthy third molars extracted from patients aged 18 to 25 years. The pulp tissue was cleaned with PBS buffer and cut into 1.0*1.0*1.0 mm^3 tissue blocks. Tissue blocks were treated with 1mg/ml collagenase type I for 15 min, and DPCs were cultured in the complete medium (10% fetal bovine serum (FBS) (FBS-UE500, NEWZERUM), 2% penicillin-streptomycin (P/S) (15140122, Gibco)) to obtain DPCs for subsequent experiments. In the Transwell experiment, DPCs were seeded in the lower chamber and pretreated with either DMSO or LAZ (LPS + AZD'5582 + z-VAD-fmk) for 6 hours. The LAZ was administered in the cell culture medium, which consisted of 10% FBS (FBS-UE500, NEWZERUM) and 1% P/S (15140122, Gibco). The LAZ medium contained 20 μg/mL *E. coli* LPS (Sigma-Aldrich, USA), 1 μM AZD05582, and 50 μM z-VAD-fmk (all from MedChemExpress, China). After the pretreatment, discard the previous medium and replace it with cell culture medium (10% FBS, 1% P/S). Neutrophils were then seeded in the upper chamber and incubated at 37 °C with 5% CO2 for 30 minutes. Afterwards, crystal violet staining was performed to observe the migration or invasion of neutrophils on the membrane surface. In the Transwell experiment investigating the interaction between the ligand CXCL8 of DPCs and the membrane receptor C3AR of neutrophils, additional treatment of neutrophils with SB290157 was applied. Trifluoroacetate (SB290157, MedChemExpress) acts as a competitive antagonist against C3aR.[20,21]

Co-IP experiment

DPCs were seeded in T25 cell culture flasks and treated with LAZ. The specific method is the same as mentioned above. After treatments, the cells were washed 3 times with PBS buffer. Fresh culture medium was then added to the flasks, and after 30 minutes, the culture medium was collected. The collected medium was used to culture neutrophils for 30 minutes, and the resulting neutrophils were harvested for subsequent co-immunoprecipitation (CO-IP) experiments. We used BeyoMag™ Anti-GFP Magnetic Beads (Beyotime', P2179M). The neutrophils were collected and lysates were prepared. Lysates were incubated with appropriate control antibodies (IgG) to reduce nonspecific binding. The lysates were incubated with an antibody against CXCL8 (Abcam, ab289967, 1:500) for the target protein. The lysate-antibody mixture was incubated overnight at 4 °C with gentle shaking to form antibody-protein complexes. The beads were washed several times using wash buffer to remove unbound proteins, detergents and other impurities. Proteins were eluted from the beads using elution buffer or by boiling in sample buffer. The eluted proteins were analyzed by Western blotting (C3AR1, Abcam, 1:1000) .

Data acquisition

Microarray data and corresponding clinical data for patients with pulpitis were obtained from the GEO database. Data from the GSE92681 dataset, which contains 7 inflamed pulp tissue and 5 healthy pulp tissue, were collected using GPL16956 (Agilent-045997 Arraystar human lncRNA microarray V3). Data from the GSE77459 dataset containing 6 inflamed pulp tissue and 6 healthy pulp tissue were collected using GPL17692 (Affymetrix Human Gene 2.1 ST Array).

GO and KEGG enrichment analysis

To explore the potential biological functions of the DEGs between inflamed and healthy pulp, GO enrichment analysis and KEGG pathway analysis were performed using the R package clusterProfiler. Only pathways with a false discovery rate < 0.05 were considered statistically significant.

Selection of NRDEGs

We screened DEGs using the R package "limma (v 3.48.3)" and set the thresholds to $P < .05$ and $|\text{Log2FC}| > 0.5$. DEGs volcano plots and heatmaps were created by the ggplot2 R package (https://ggplot2. tidyverse.org/) and the heatmap R package (https://CRAN.R-project.org/package=pheatmap). In addition, we collected 159 necrosis-related genes from the KEGG pathway database (https://www.genome.jp/dbget-bin/www_bget?pathway+hsa04217). Additional file 3 provides relevant

information. We identified NRDEGs by using the VENN package. Functional analysis of NRDEGs was performed using GO analysis and KEGG analysis. To narrow down the number of NRDEGs, a support vector machine (SVM) algorithm was used to identify hub NRDEGs.

Gene correlation analysis

The R language was used to analyze the correlation between the 6 hub genes, and the pearson correlation coefficient was calculated. The results were displayed through the R package "circlize." Red lines represent positive correlations, green lines represent negative correlations, with darker colors showing stronger correlations.

ROC curves

A subject operating characteristic (ROC) curve was used to evaluate the ability of hub NRDEGs to recognize pulpitis. The area under the curve (AUC) was calculated, and an AUC>0.7 indicated suitable predictive ability.

Immune infiltration analysis

Comprehensive predictive analysis of immune cell abundance was conducted using the ssGSEA feature of the R language GSVA package. ssGSEA was used to estimate the abundance of 23 immune cell types by scoring gene expression data based on gene signatures.

Relationship between immune infiltration and necroptosis

The data in GSE77459 were screened for the top 50 genes highly associated with 6 NRDEGs, and Reactome-based ssGSEA analysis of the enriched immune-related pathways was performed to estimate the relative abundance of different immune cell types in each sample.

STRING analysis

The STRING database (https://string-db.org/) was used with C3AR1 as the query and target protein ("https://cn.string-db.org/cgi/network?taskId=boctkCG1dCbp&sessionId=bdGYo3h3 × 2vl").

Statistical analysis

GraphPad Prism 8 (San Diego, USA) was used for statistical analysis and graphing. All experiments were repeated 3 or more times. we employed t-tests for comparisons between 2 groups, depending on the data characteristics and research questions. To assess the normality of our data, we utilized Kolmogorov-Smirnov test, and visual assessments such as Q-Q plots. All results with a $P < .05$ were considered statistically significant. *, **, ***, and **** represent $P < .05$, $P < .01$, $P < .001$, and $P < .0001$, respectively.

Results

Increased necroptosis in human inflamed dental pulp

We collected samples of healthy and inflamed pulp, and HE staining showed that there was a large amount of immune cell infiltration in the inflamed pulp tissue, and sections of the tissue were disorganized and disordered (Figure 1A). The role of phosphorylated MLKL in the process of necroptosis was crucial, and it was considered to be a key node in the necroptotic signaling pathway. Therefore, we performed IHC and WB experiments on healthy pulp tissue and inflamed pulp tissue, which showed that pMLKL protein expression was significantly upregulated in inflamed pulp tissue compared to healthy pulp tissue gt(Figure 1A-C). We also established the LPS-induced inflammation DPCs model and observed a significant upregulation in pMLKL protein expression (Figure 1D, E). The above experimental results demonstrated that inflammatory cell infiltration and necroptotic signals were activated in inflamed dental pulp.

Enrichment analysis of DEGs

We collected 2 datasets, GSE77459 and GSE92681, related to pulpitis from the GEO database. To characterize the transcriptome of patients with pulpitis, we screened for differentially expressed genes ($|log2FC|>0.5$, $P < .05$). A total of 3861 differentially expressed genes (DEGs) were found in the GSE77459 dataset between pulpitis and healthy samples, of which 1984 genes were upregulated and 1877 genes were downregulated (Figure 1F). In another dataset, GSE92681, there were 3789 DEGs between pulpitis samples and healthy samples, of which 1852 genes were upregulated and 1937 genes were downregulated (Figure 1F). We screened the overlapping upregulated DEGs and downregulated DEGs of the 2 gene sets for further analysis, of which 481 were upregulated and 292 were downregulated (Additional file 4). Venn diagram analysis revealed the shared genes (Figure 1G).

Subsequently, we performed GO and KEGG analysis to determine the functions and associated pathways of the DEGs, with a total of 761 GO terms and 266 KEGG pathways (Additional file 5). GO enrichment analysis showed that these genes were enriched in several entries related to immune infiltration, including leukocyte cell–cell adhesion, immunological synapses, and MHC class II protein complexes. Terms related to necroptosis were also identified, including secretory granule membrane, endocytic vesicle, and specific granule (Figure 1H). Notably, it was also observed that inflammatory cells may act through ligand–receptor interactions (Figure 1H). Moreover, the KEGG analysis showed similar results, including enrichment of pathways associated with cytokine–cytokine receptor interactions, chemokine signaling, cell adhesion and phagosomes (Figure 1I). The results provided preliminary evidence suggesting the presence of immune infiltration and necroptosis in inflamed dental pulp. Additionally, our findings suggested that cellular communication mechanisms may significantly contribute to these processes.

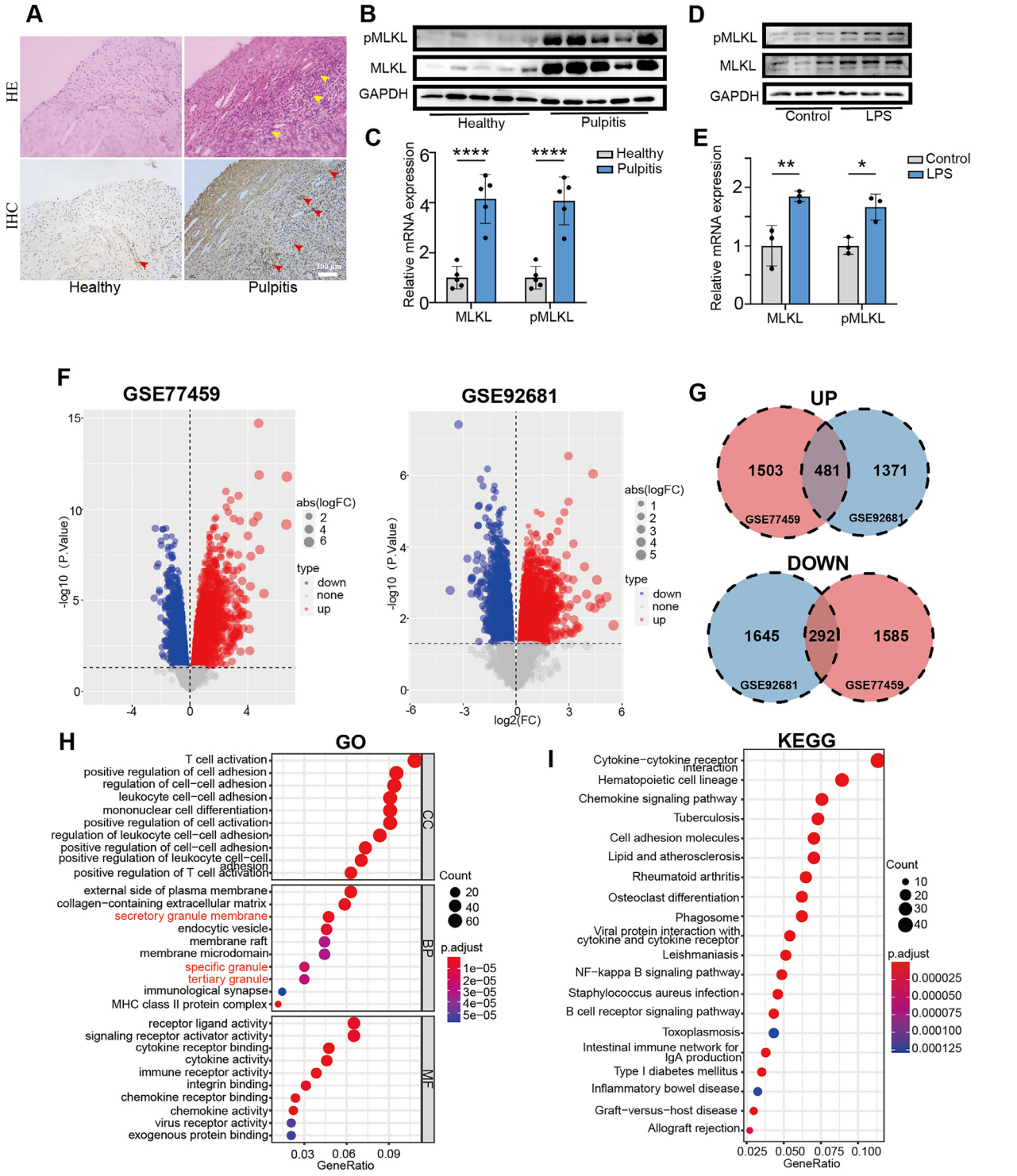

Fig. 1 – Increased necroptosis in human inflamed dental pulp. **(A) Inflammatory cell infiltration and pMLKL expression levels in healthy and inflamed pulp tissue. Yellow arrows indicate immune cell infiltration. Red arrows indicate regions that are positive for pMLKL. (B, C) Quantification of MLKL and pMLKL levels in healthy and inflamed pulp tissue by WB. (D, E) Quantification of MLKL and pMLKL levels in Control and LPS-stimulated DPCs by WB. GAPDH was used as a loading control, and relative protein expression was normalized to the loading control. Data are presented as the mean ± SEM. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$. (F) Volcano plot showing the GSE77459 and GSE92681 DEGs. (G) Venn analysis screening for shared up- or downregulated genes between the 2 gene sets. (H, I) GO and KEGG functional enrichment analysis of DEGs.**

Screening of Hub NRDEGs

We compared the screened necroptosis-related genes with the DEGs using Wayne analysis to obtain 10 NRDEGs, shown in the Venn diagrams (Figure 2A). These NRDEGs were all upregulated genes, including *MLKL*. By GO and KEGG enrichment analysis of the NRDEGs, we identified a total of 554 GO terms and 86 KEGG pathways (Additional file 6).

GO enrichment analysis showed that the NRDEGS were enriched in 4 entries, including regulation of inflammatory response, tumor necroptosis factor production, and cytokine receptor binding (Figure 2B). In addition, KEGG pathway enrichment indicated that the NRDEGs were enriched in necroptosis, the TNF signaling pathway, cytokine activity, and cytokine receptor binding (Figure 2C). The functional analysis results of the NRDEGs further suggested that the activation of necroptotic cell death and immune cell infiltration in the inflamed pulp are closely related and that cytokine and cytokine receptor interactions may be a potential mechanism of action for both.

To further refine our analysis, we implemented the support vector machines (SVMs) algorithm to narrow down the 10 NRDEGs. We initially screened 10 genes from the GSE92681 dataset and an additional 6 genes from the GSE77459 dataset. Subsequently, we performed a Venn diagram analysis to identify overlapping genes, resulting in the identification of 6 hub NRDEGs (Figure 2D, E, F).

Identification and validation of Hub NRDEGs

Heatmaps were used to show the differentially expression of these hub NRDEGs in the 2 datasets (Figure 3A). Additionally, we assessed the relationship between these hub NRDEGs and pulpitis by plotting receiver operating characteristic (ROC) curves. The AUC values of the hub NRDEGs in the GSE77459 dataset were all 1. The results of the analysis in the GSE92681 dataset also suggested that NRDEGs also showed high accuracy in identifying pulpitis (Figure 3B). Gene expression correlation analysis was conducted to explore the interactions among the hub NRDEGs, and robust positive correlations were found (Figure 3C, D). Moreover, we obtained both healthy and inflamed pulp tissue and isolated tissue RNA for qRT–PCR experiments. The experimental outcomes revealed that the mRNA levels of all 6 hub NRDEGs were significantly increased in the inflamed dental pulp tissue (Figure 3E).

Necroptotic DPCs promote neutrophil migration

We conducted an immune infiltration analysis on the dataset to evaluate the degree of immune cell infiltration in pulpitis (Figure 4A, B). It was observed that a variety of immune cells infiltrated the tissue, with neutrophil infiltration being significantly increased. In the enrichment analysis, we found that NRDEGs were significantly enriched in immune-related entries and pathways. Therefore, we conducted an investigation to examine the association between necroptosis and immune infiltration in pulpitis, aiming to uncover the potential interactions between these 2 processes.

We discovered a significant correlation between the hub NRDEGs and immune cell infiltration in the context of pulpitis. Specifically, these hub NRDEGs displayed a noteworthy association with the infiltration of neutrophils (Figure 4C). The results of the analysis suggest a potential interaction between necroptosis and neutrophil immune infiltration in pulpitis. The primary cell type within dental pulp is DPCs, and our earlier experimental findings further support that necroptosis is triggered at the specific site where these cells are positioned. The results of the transwell experiment demonstrated that DPCs promote the migration of neutrophils after undergoing programmed necroptosis (Figure 5A, B).

Necroptotic DPCs release CXCL8 to engage with the neutrophil transmembrane receptor C3AR1

To further elucidate the relationship between necroptotic DPCs and neutrophil migration, we performed an in-depth analysis of the dataset. First, the top 50 genes highly associated with each hub NRDEG were filtered in the GSE77459 dataset (Figure 5C). Next, we downloaded the all 30 genes included in the "positive regulation of neutrophil chemotaxis" entry from the GO website (https://amigo.geneontology.org/amigo/term/GO:0090023). C3AR1 was identified after overlaying the 2 gene sets. We further predicted the proteins that interact with C3AR1 using the STRING website and obtained a total of 10 proteins with the highest potential (Figure 6A, B).

Based on the results of our previous analysis, we were concerned that the mechanism underlying the promotion of neutrophil migration following the necroptosis of DPCs may involve cellular communication, particularly through the cytokine pathway. Among these proteins, CXCL8 is an extracellularly secreted cytokine that can mediate the inflammatory response by chemotaxis of neutrophils, basophils and T cells. Therefore, we hypothesized that necroptotic DPCs could produce CXCL8 and recruit neutrophils through interactions with C3AR1. We observed significant upregulation of CXCL8 in necroptotic DPCs and inflamed pulp (Figure 6C). The Co-IP experiment confirmed the potential interaction between CXCL8 and C3AR1 (Figure 6D). We also observed that the SB290157 could block neutrophil chemotaxis by transwell assay (Figure 6E).

Discussion

Progressive destruction of DPCs can lead to pulpitis and a lack of restorative capacity, ultimately resulting in pulp necrosis. In recent years, there has been increasing evidence that programmed cell death, including apoptosis,[22,23] pyroptosis[24,25] and NETosis,[26] plays an important role in pulpitis. For example, lipophosphatidic acid from *Streptococcus mutans* causes DPCs apoptosis and is an important cause of pulpitis.[23] The upregulated expression of IL-1β, IL-18 and caspase-1 in LPS-treated DPCs also confirms the presence of pyroptosis in hDPSCs.[24] Furthermore, NETs can induce apoptosis in DPCs and attract macrophages in infected dental pulp.[26] However, the role of necroptosis, a form of programmed death, in pulpitis is unclear.

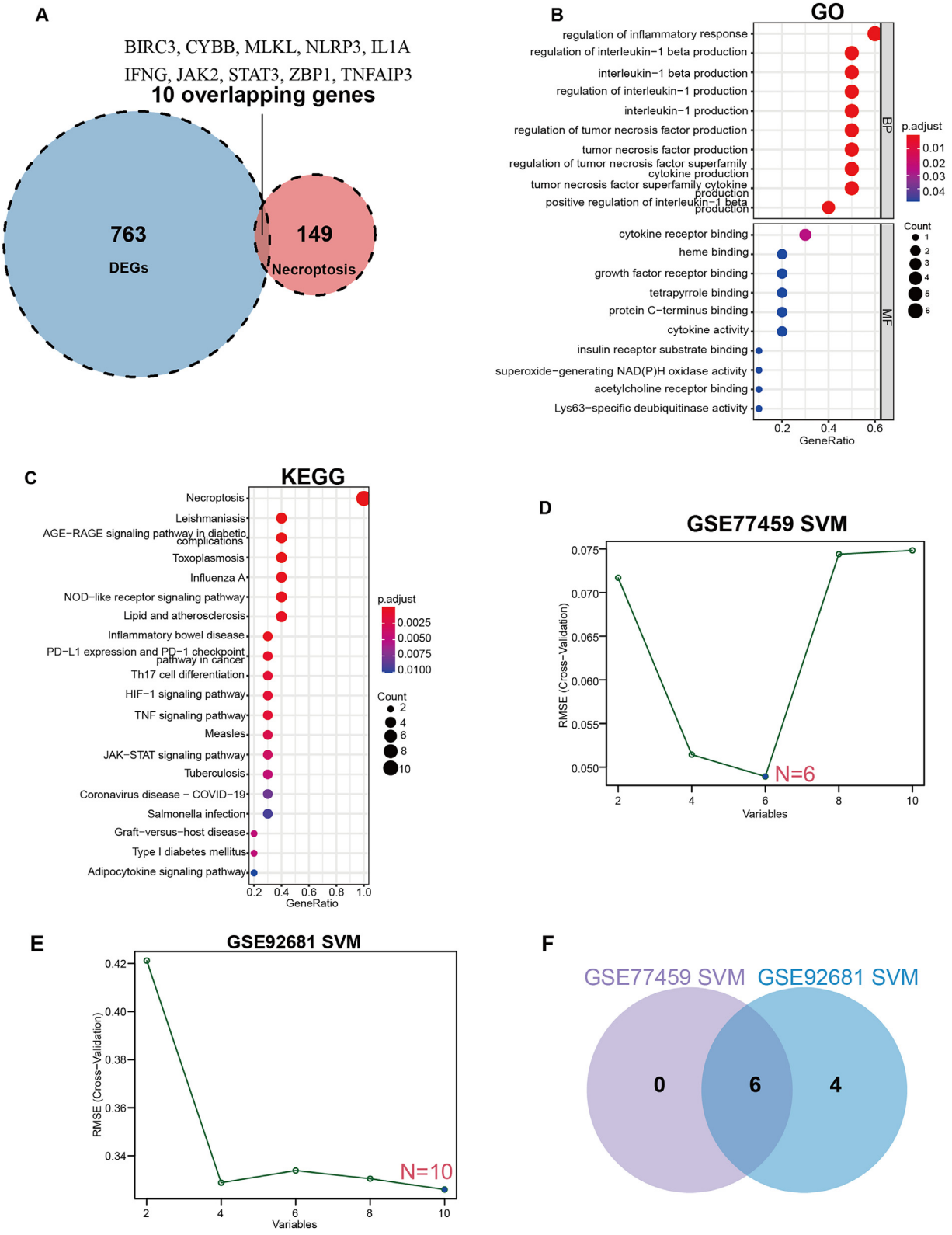

Fig. 2 – Enrichment analysis of hub NRDEGs. **(A) Venn diagram showing the shared necroptosis-related genes and DEGs. (B) GO and (C) KEGG functional enrichment analysis of NRDEGs. (D, E) SVM algorithm to narrow down NRDEGs. (F) Wayne analysis screening yielded 6 hub NRDEGs.**

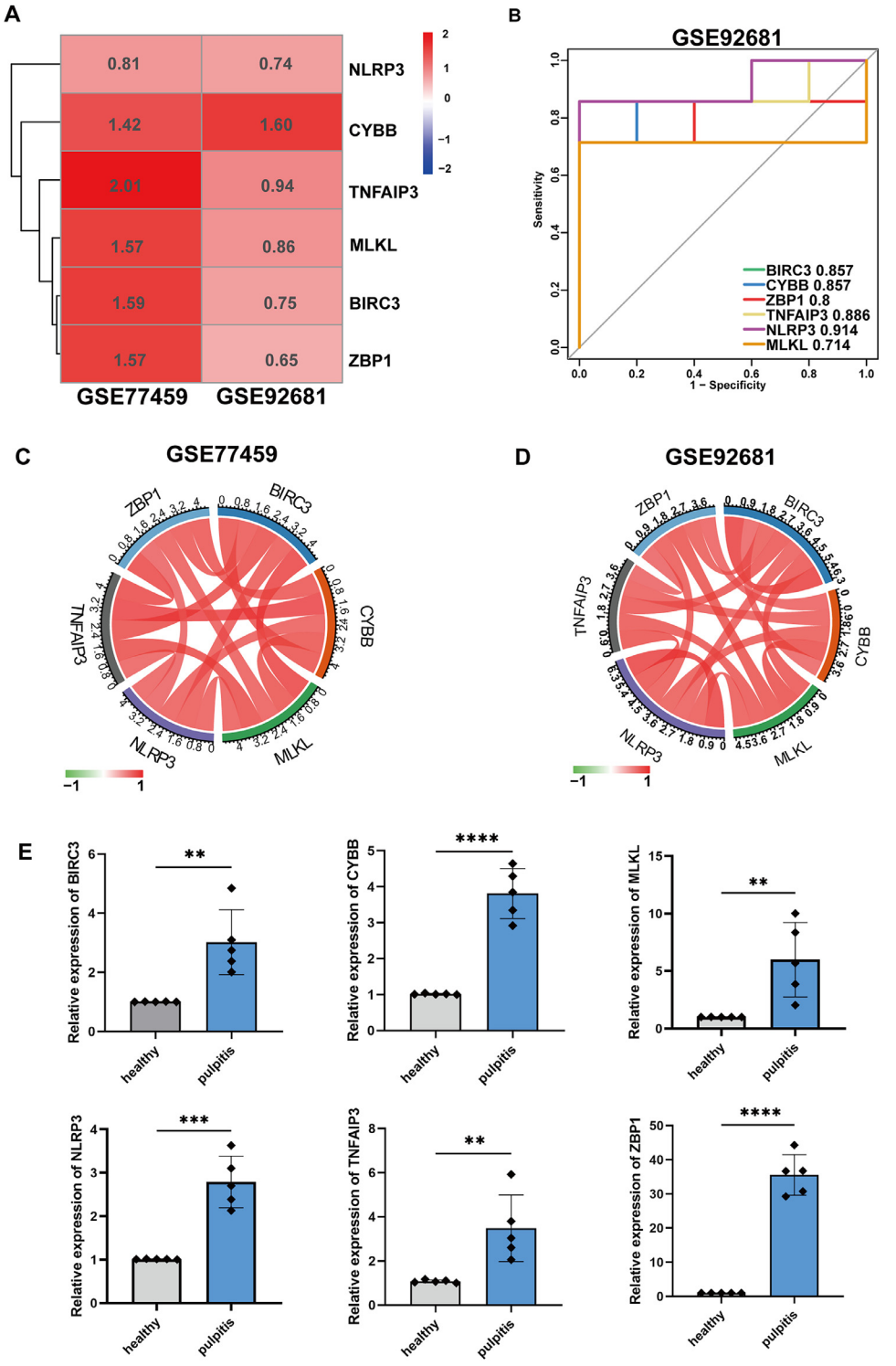

Fig. 3 – Screening of hub NRDEGs. **(A) Heatmap showing differential expression of 6 hub NRDEGs in the 2 datasets. (B) ROC curves for assessing the diagnostic potential of hub NRDEGs for pulpitis. (C, D) Gene expression correlation analysis identified interactions between hub NRDEGs. (E) qRT–PCR validation of hub NEDEG expression in dental pulp tissue.**

Necroptosis plays an important role in various inflammatory diseases.[27] For example, RIPK3/MLKL-mediated necroptosis in gingival fibroblasts leads to the migration and polarization of THP-1 macrophages, which exacerbates periodontitis.[28] However, no study has demonstrated the presence of necroptosis in pulpitis. Therefore, our research aimed to explore the occurance of necroptosis in pulpitis. First, we observed increased protein expression pMLKL levels

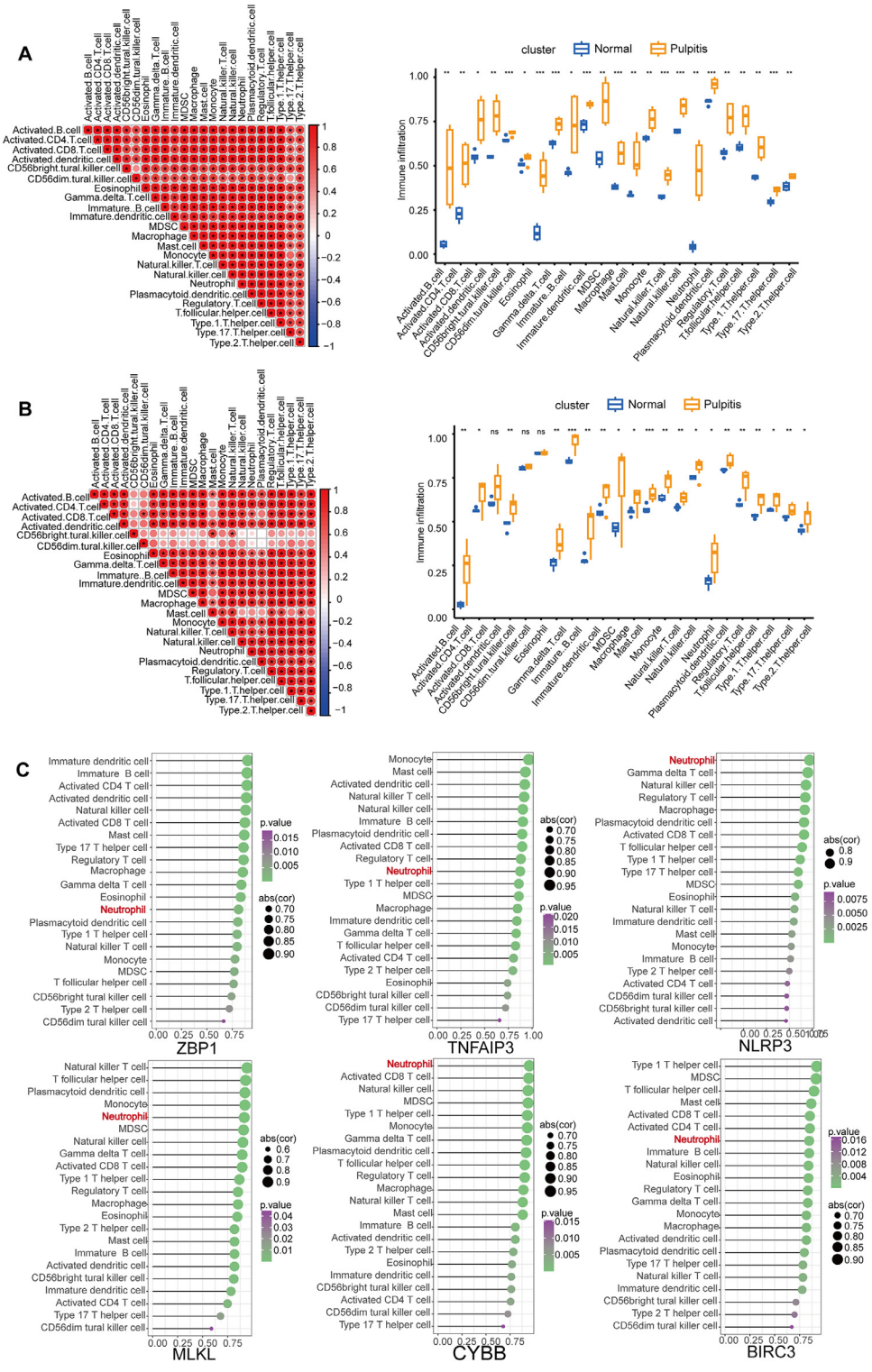

Fig. 4 – Immune infiltration analysis. **(A, B) Immune infiltration analysis of the pulpitis gene set. (C) Correlation analysis between hub NRDEGs and immune cell infiltration.**

in inflamed dental pulp, confirming that necroptosis may be activated in inflamed dental pulp. Six hub NRDEGs, including MLKL, were screened by bioinformatic analysis of a GEO dataset. Functional enrichment also showed that the regulatory pathway of necroptosis may be involved in the pathogenesis of pulpitis.

Pulp tissue transcriptome data were obtained from the GEO database and further analyzed. The results of the

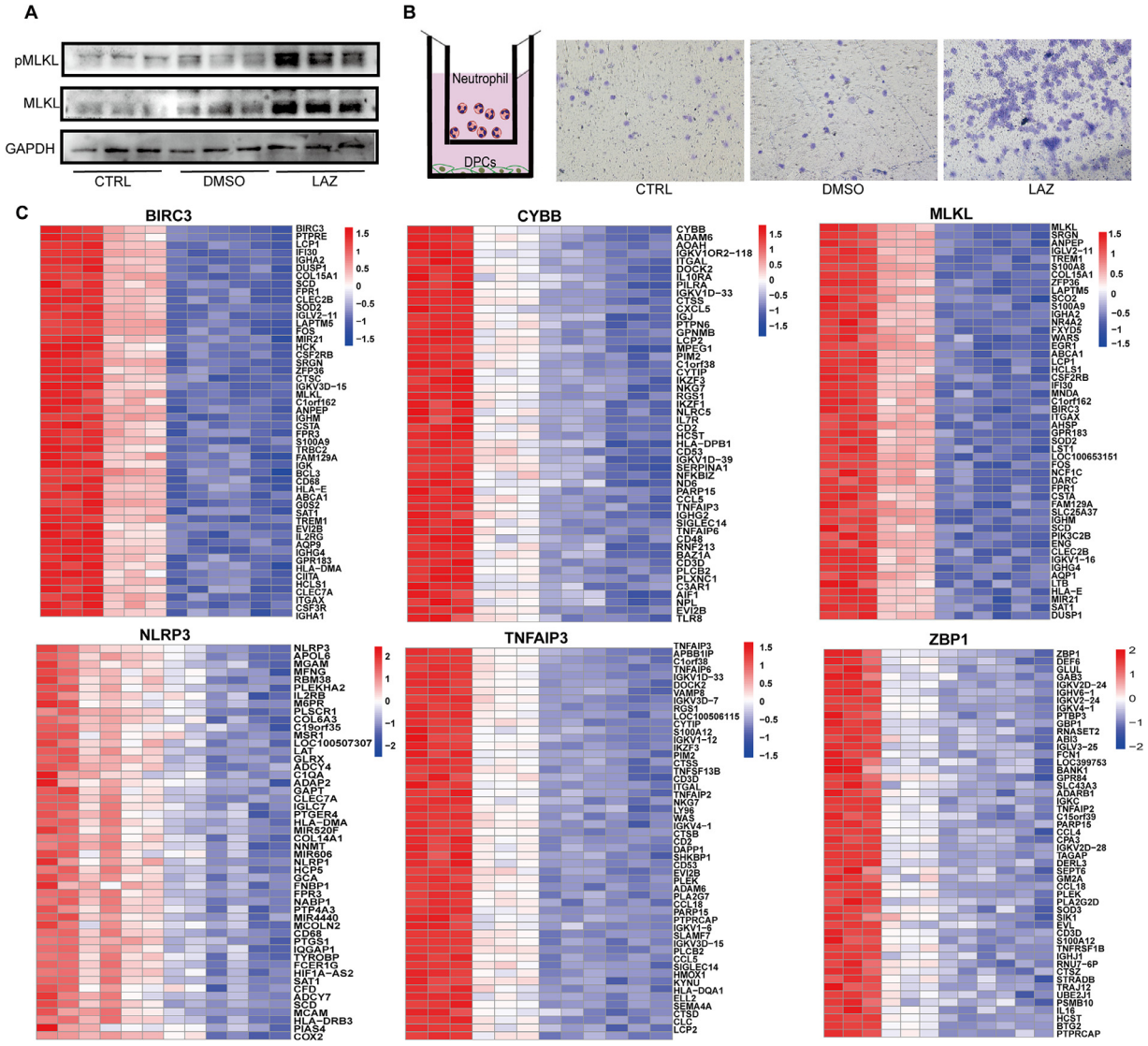

Fig. 5 – Necroptotic DPCs promote neutrophil migration. **(A) MLKL and pMLKL protein expression levels in LAZ-stimulated DPCs were determined. (B) Transwell assays were used to verify the effect of necroptotic DPCs on neutrophil migration. (C) The 50 genes with the highest correlation with the hub NRDEGs in GSE77459.**

enrichment analysis of DEGs indicated that immune infiltration and cellular communication could be important mechanisms in pulpitis. It is worth noting that no previous study has reported on the relationship between the necroptosis of DPCs and immune infiltration in pulpitis. Immune cells play a crucial role in the progression of pulpitis.[29] On the 1 hand, the recruitment and activation of immune cells such as macrophages and neutrophils can mediate pathogen clearance and limit tissue damage.[30,31] On the other hand, the overactivation of these immune cells can lead to pulpal damage and impede the process of tissue regeneration. Recent studies have shown that the proportions of M0 macrophages, neutrophils and follicular helper T cells are significantly higher in inflamed dental pulp than in normal pulp tissue.[32]

Therefore, to further explore the relationship between necroptosis of DPCs and immune infiltration in the inflamed dental pulp, we performed an immune infiltration analysis on the dataset. Significant inflammatory infiltration, including the presence of neutrophils, was observed in the inflamed pulp. Notably, the relationship between necroptosis and immune infiltration is complex.[33] Necroptosis can accelerate the death of immune cells. For example, necroptosis of some infiltrating immune cells has been found in polymyositis, leading to the lysis of muscle tissue.[34] On the other hand, necroptotic cells can secrete relevant cytokines, undergo chemotaxis and activate immune cells.[35,36] Tumor cell necroptosis is a major cause of tumor cell death. It has now been found that tumor cell necroptosis induces the immune system to attack tumors and has become a novel antitumor therapeutic strategy.[37] Based on our data analysis, we formulated a hypothesis that DPCs necroptosis could enhance neutrophil chemotaxis, thereby exacerbating dental pulp inflammation.

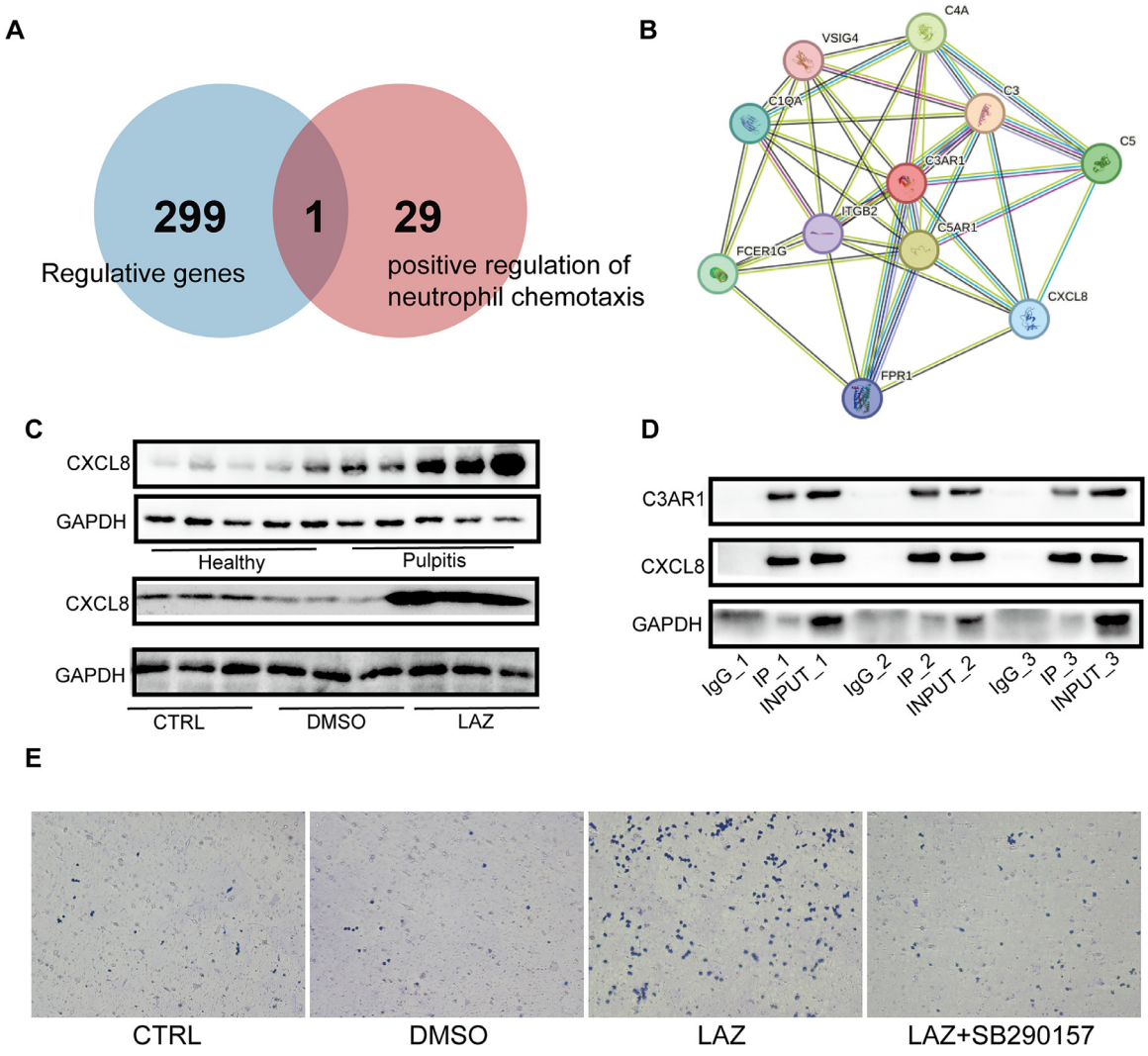

Fig. 6 – Necroptotic DPCs release CXCL8 to engage with the neutrophil transmembrane receptor C3AR1. (A) Three hundred hub genes related to NRDEGs overlapped with genes related to the "positive regulation of neutrophil chemotaxis" functional entry screening for key molecules in cell communication. (B) STRING site prediction of proteins that interact with C3AR1. (C) WB detection of CXCL8 expression levels in dental pulp (above) and necroptotic DPCs (below). (D) The supernatant of necroptotic DPCs was incubated with neutrophils, and an anti-CXCL8 antibody was used to pull down CXCL8 and C3AR1. Co-IP assay validating the interaction of CXCL8 with C3AR1. (E) C3AR1 inhibitors block DPCs necroptosis and neutrophil chemotaxis.

Subsequently, we investigated the correlation between DPCs necroptosis and neutrophil infiltration.

However, the specific mechanism by which necroptotic DPCs promote neutrophil migration has not been elucidated. The enrichment analysis of the 2 datasets showed that cellular communication may be an important mechanism in pulpitis. Therefore, through a rigorous screening sequence of bioinformatics analysis, we screened out C3AR1 as our target. C3AR1 is a membrane receptor protein that plays a vital role in promoting neutrophil chemotaxis, which is important in numerous inflammatory diseases. We predicted proteins that interact with C3AR1 using the STRING website and constructed a protein interaction network. CXCL8 is a cytokine that can be secreted and exert extracellular effects.[38] Previous studies have shown that CXCL8 functions as a chemotactic factor for neutrophils, attracting them to sites of inflammation.[39] In addition, MLKL-active cells in renal ischemia-reinjury and advanced cancer express high levels of CXCL8.[40] Therefore, we demonstrated by Transwell and co-IP experiments that necroptotic DPCs may recruit neutrophils through the ligand–receptor interaction of CXCL8/C3AR1.

The present study does have certain limitations that should be acknowledged. First, while we did observe an elevation in pMLKL protein levels in inflamed dental pulp, further experiments are required to provide comprehensive evidence confirming the occurrence of necroptosis in pulpitis from multiple perspectives. In addition, while we demonstrated that the release of CXCL8 from necroptotic DPCs may

interact with C3AR1 to recruit neutrophils, the specific molecular mechanism underlying this interaction requires further investigation. In the future, research should focus on identifying and characterizing the molecular mechanisms through which CXCL8 and C3AR1 interact to recruit neutrophils to the site of inflammation. Detailed proteomics and transcriptomics approaches could provide valuable insights into the signaling pathways and regulatory networks involved in this process. Furthermore, exploring the potential therapeutic implications of targeting these pathways could open up new avenues for the treatment of pulpitis and other inflammatory diseases.

Conclusion

Activation of pMLKL in the necroptotic pathway was observed in inflamed dental pulp. Bioinformatics analysis revealed significant differences in the expression levels of NRDEGs in inflamed and healthy pulp samples and screened out 6 hub NRDEGs, including MLKL. In addition, we found that the lopsided immune cells and NRDEG expression in pulpitis were associated with the infiltration of multiple immune cell types. Additionally, we provided evidence demonstrating that the release of CXCL8 following necroptosis in DPCs may interact with the neutrophil membrane receptor C3AR1, resulting in the promotion of neutrophil chemotaxis.

Conflict of interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Xiaolan Guo: Writing – original draft, Validation, Software, Methodology, Investigation, Data curation. **Xinyan Ma:** Writing – original draft, Software, Investigation, Methodology, Data curation. **Peng Liu:** Writing – original draft, Software. **Xiaoxin Chen:** Writing – review & editing, Validation. **Sitong Liu:** Writing – review & editing, Validation. **Longrui Dang:** Writing – review & editing, Data curation. **Buling Wu:** Writing – review & editing, Supervision, Conceptualization. **Zhao Chen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Data availability

Microarray data and corresponding clinical data for patients with pulpitis were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) .The data that support the findings of this study are openly available in Gene Expression Omnibus (GEO), reference number GSE77459 and GSE92681.

Funding

This project was supported by the National Natural Science Foundation of China (82100996); the Fundamental Research Project of Shenzhen Science and Technology Innovation Committee (JCYJ20210324121007020); the General Project of President Scientific Research Fund of Shenzhen Stomatological Hospital of Southern Medical University (Pingshan) (2021B001); and the Medical Scientific Research Foundation of Guangdong Province (B2021074). Funding was received from Science and Technology Projects in Guangzhou (2024A04J5188).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.identj.2025.02.016.

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