Investigation of pyroptosis-related hub genes and the immune microenvironment in primary Sjögren's syndrome

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Abstract

Objective

Primary Sjögren's syndrome (pSS) is an inflammatory systemic autoimmune disease, while the role and mechanisms of pyroptosis in pSS remain largely undefined.

Methods

Pyroptosis-related genes and gene expression data were obtained from the Molecular Signatures Database and NCBI GEO databases. Differentially expressed genes (DEGs) and pyroptosis-related hub genes were identified by R software. Functional enrichment analyses were conducted using the "ClusterProfiler" R package and WebGestalt7. CIBERSORTx was used to calculate the correlations between immune cells and pyroptosis. Subsequently, histological staining was performed on salivary gland samples from non-pSS and pSS patients to identify the expression of pyroptosis-related genes. Immunofluorescence double staining was conducted to validate the correlation between immune cells and pyroptosis.

Results

A total of 1494 DEGs were identified between eight pSS samples and 10 healthy volunteer samples. Five pyroptosis-related hub genes (AIM2, CASP1, CASP3, IL6, TNF) were recognised. DEGs were mostly enriched in immunity-related terms and several immune cells were associated with the hub genes in pSS. Among them, delta gamma T cell was significantly positively correlated with CASP3. Finally, the protein levels of these hub genes were validated to be elevated in the labial minor salivary gland biopsies of pSS patients compared to those of healthy volunteers using immunohistochemical staining. Immunofluorescence double staining further showed that IL-6, AIM2, CASP1and CASP3 were related to delta gamma T cells, and TNF was related to dendritic cells.

Conclusion

This study uncovered a significant interaction between pyroptosis and the immune microenvironment in pSS patients. Besides, we identified five pyroptosis-related hub genes that might play a role in the pathogenesis of pSS. These findings could offer valuable insights for the development of novel treatment strategies for pSS.

Key words

pyroptosis, immune microenvironment, primary Sjögren's syndrome, CASP3, delta gamma T cell

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Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease that mainly involves secretory gland, leading to dryness of the main mucosal surfaces (1). The prevalence of pSS is estimated to be 0.3/1000-1/1000, and 30–40% of pSS patients present with systemic complications such as palpable purpura, cryoglobulinaemia-associated glomerulonephritis and peripheral neuropathy (2), leading to a heavy burden on healthcare and society.

The pathogenesis of pSS stemmed from the interaction between the activated immune system and epithelial cells (3). Patients with extra-glandular manifestations exhibited increased expression of genes associated with both innate (including apoptosis, TLR, and interferon signalling) and adaptive (T and B cell activation) immune reactions, which are crucial in the progression of pSS (4). Recent research underscored that Sjögren's syndrome (SS) patients showed a higher ratio of Tfr to Tfh cells compared to healthy individuals, indicating an imbalance between pro-inflammatory and immunoregulatory pathways (3). Although the abnormal activation of both innate and adaptive immune pathways is well-established in pSS pathogenesis, the function of abnormally activated immunity in pSS is still unclear (5).

Pyroptosis is a newly recognised type of programmed cell death, and is characterised by membrane pore formation, cell swelling and rupture, and the outflow of IL-1b, IL-18, and cytoplasmic contents (6). Recent studies on pyroptosis and autoimmune diseases have garnered increasing attention (7). Researches indicated that SS patients had an increased expression of NLRP3 inflammasome-related elements in peripheral blood mononuclear cells (PBMC), or in macrophages infiltrating in salivary glands (8, 9). Furthermore, in the ductal epithelial cells of SS patients, excessive damaged cytoplasmic DNA due to DNase1 deficiency could activate AIM2 inflammasome, leading to increased pyroptosis (10). Moreover, caspase-1 is observed to be elevated due to type I IFN in pSS, implying its potential role in SS pathogenesis (11). However, there are few studies on pyroptosis-related genes in pSS, and the role and mechanisms of pyroptosis in pSS has yet to be further explored.

To investigate the role and specific mechanism of pyroptosis in the pathogenesis of pSS, we would comprehensively analyse the gene expression profiles of pSS patients and healthy volunteers using bioinformatic methods. Specifically, we first identified pyroptosis-related differentially expressed genes (DEGs) using R software. Then, enrichment analysis was conducted through Gene Ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to uncover underlying mechanisms. Furthermore, we explored the immune infiltration and discovered the relationship between pyroptosis and the immune microenvironment in pSS using CIBERSORTx and ssGSEA. Finally, we compared the immunohistochemical staining for the proteins of the five hub genes of salivary gland specimens between pSS patients and healthy volunteers to further validate our findings. Immunofluorescence double staining was performed to confirm the association between immune cells and pyroptosis. The exploration of the pyroptosis in the salivary glands of pSS patients could offer fresh perspectives for establishing novel treatment strategies.

Materials and methods

Data acquirement

We retrieved the gene expression data of GSE127952 from GEO databases on September 10, 2023. GSE127952 (Microarray, platform GPL20995) encompassed gene expression profiles of minor salivary glands from eight female patients with pSS and six healthy volunteers. The dataset provided a standardised matrix file, facilitating the extraction and analysis of the microarray data. For our investigation into pyroptosis, we sourced a list of pyroptosis-related genes from the Molecular Signatures Database (MSigDB, version 7.5.1).

Identification of pyroptosis-related hub genes

In the GSE127952 dataset, we quantified the pyroptosis signature score through single-sample Gene Set Enrichment

Analysis (ssGSEA). The comparison of pyroptosis signature scores between healthy volunteers and pSS patients was conducted using the Wilcoxon test via the "ggpubr" R package.

For the identification of differentially expressed genes (DEGs), we used the "limma" R package with inclusion criteria of $p<0.05$ and $|logFC|>1$ (12). Subsequently, we determined pyroptosis-related hub genes by overlapping DEGs and pyroptosis-related genes. This overlap was visually represented using a Venn diagram through the Jvenn website.

Visualisation of expression discrepancies was achieved with a volcano plot and heatmap, using the "ggplot2" and "pheatmap" R packages, respectively. To better understand data variance and distinct sample groupings, a principal component analysis (PCA) was plotted using the "FactoMineR" and "plyr" R packages.

Functional enrichment analysis of DEGs

To elucidate the biological functions and pathways of DEGs, we performed Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using the "ClusterProfiler" R package (13-15). Then we conducted a more in-depth pathway analysis using Gene Set Enrichment Analysis (GSEA). GSEA is a computational method to detect statistical significance, and the reference gene sets selected the KEGG gene set (c2.cp.kegg.v7.4.symbols.gmt) from the Molecular Signatures Database (MSigDB) (16). Our approach entailed 1,000 permutations for the random sampling algorithm. Pathways were deemed statistically significant with $p<0.05$.

Functional annotation of pyroptosis-related hub genes

To further explore the function of the pyroptosis-related hub genes, we performed GO and KEGG pathway enrichment analyses using WebGestalt7. We set a threshold of FDR <0.05.

Investigation of immune cell infiltration

To further investigate the immune cell infiltration and associations in pSS, we processed and uploaded our dataset to CIBERSORTx (http://cibersortx.stanford.edu). CIBERSORTx was a computational tool that estimate the relative proportions of specific immune cell types within a mixed cell population using gene expression data. We used CIBERSORTx to estimate the fraction of 22 immune cell types in the salivary glands from GSE127952.

For visualisation, heatmaps were generated using the "ComplexHeatmap" 2.17.0 package in R to show the proportions of the 22 immune cells in pSS patients and healthy volunteers, as well as the correlation of these immune cells with each other in pSS (17). We utilised the Spearman correlation to discern relationships between different immune cells, using the "psych" R package for this analysis. Lastly, to assess differences in immune cell representation between healthy volunteers and pSS patients, we employed the Wilcoxon test using the "ggpubr" package in R.

Relationship between pyroptosis and immune microenvironment in pSS

We analysed the expression patterns of pyroptosis-associated hub genes in both healthy volunteers and pSS patients, employing the "ggpubr" package for the Wilcoxon test. In the GSE127952 dataset, we derived pyroptosis pathway signature scores through single-sample Gene Set Enrichment Analysis (ssGSEA). With the signature score of pyroptosis, the correlations between the immune cells and pyroptosis were calculated using Spearman's rank correlation and visualised using the "ggplot2" R package (18).

Patients and labial minor salivary gland biopsy

The patients were diagnosed with pSS according to the 2016 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for pSS (19) and without any treatment. The healthy volunteers in this study were individuals who reported subjective symptoms of oral dryness but did not meet any of the objective criteria necessary for a diagnosis of pSS.

Labial minor salivary gland biopsies for immunohistochemistry were obtained from six untreated pSS patients and six healthy controls. Biopsies for Immunofluorescence were obtained from 12 untreated pSS patients. All participants provided written informed consent before clinical information gathering and biopsy procedures. The study received approval from the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University and performed according to the Declaration of Helsinki.

Immunohistochemistry

Fresh biopsy samples were fixed overnight in 4% neutral formaldehyde and subsequently embedded in paraffin. The slides were dewaxed and cooled to room temperature. Then slides were washed in PBS to repair the antigen. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Serum closure was applied using 3% BSA for 30 minutes before incubation with primary antibodies at 4°C overnight. After a PBS wash, slides were incubated with secondary antibodies for 50 minutes at room temperature. Coloration was achieved using DAB, and the sections were counterstained with haematoxylin.

Finally, slides were dehydrated and mounted for observation under a light microscope. Slides were digitised and analysed using Aipathwell (Servicebio), an AI-based digital pathology image analysis software. It automatically locates and outlines areas of interest in tissue samples, determines positivity, computes cell quantities and areas, and generates reports based on algorithmic analysis. The positive cells density, a measure of the number of positive cells divided by the total area of the tissue being examined, was computed and utilised for comparison (20).

Immunofluorescence

Paraffin sections were prepared as described above and the tyramide signal amplification (TSA) was used for the immunofluorescence. The primary antibodies were mixed and incubated under the same conditions. The secondary antibodies were conjugated with different fluorochromes and both

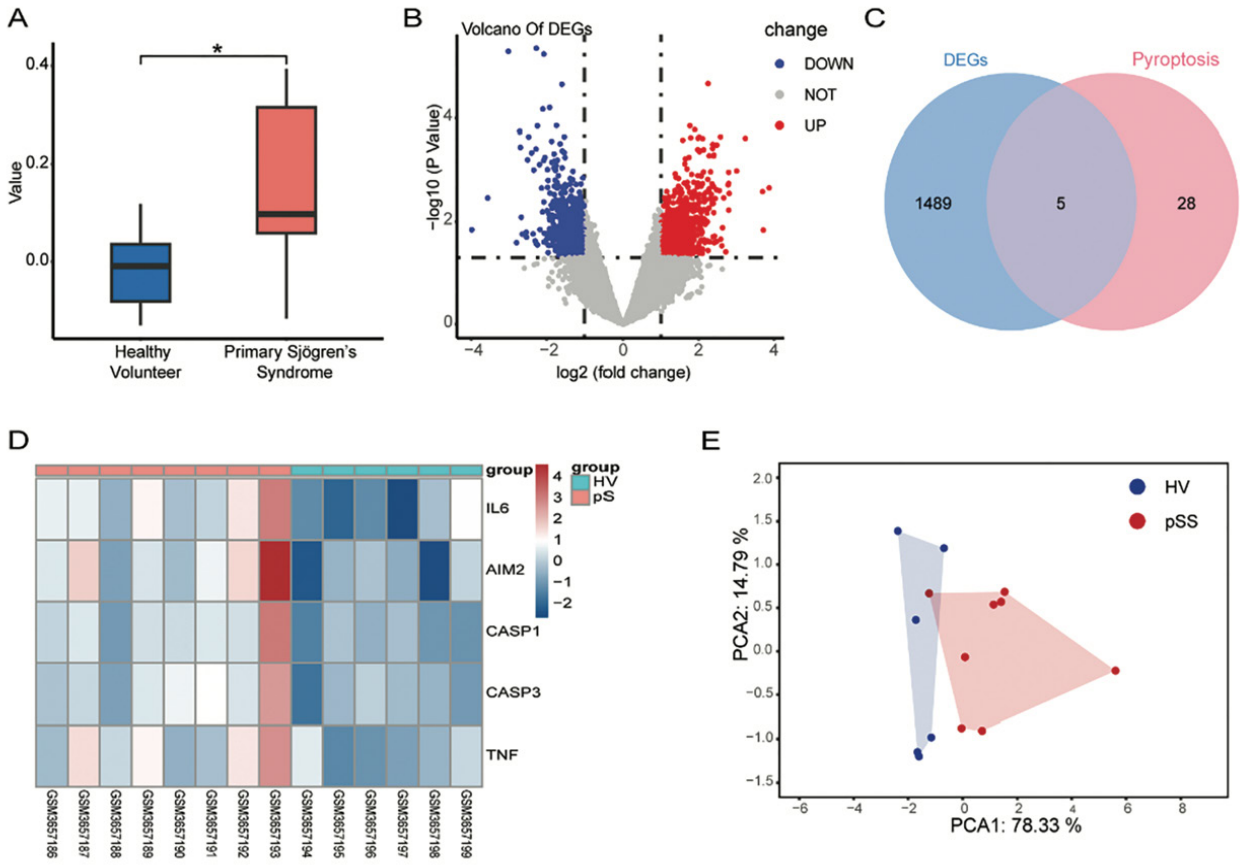

Fig. 1. Identification of pyroptosis-related hub genes in pSS. **A**: Boxplot showed relative levels of pyroptosis in pSS and HV. *$p < 0.05$. **B**: Volcano plot the significant DEGs between pSS and controls. **C**: Venn diagram showed the number of DEGs for the dataset and pyroptosis-related genes, and the number of genes that overlapped between them. **D**: The heat map showed five pyroptosis-related hub genes (AIM2, CASP1, CASP3, IL6, TNF) in the dataset. **E**: Principal component analysis (PCA) visualised the distribution of two groups.

sections were incubated in the dark for 60 min. After washing with PBS, the slides were mounted with or without 4'6-diamidino-2-phenylindole (DAPI). Aipathwell (Servicebio) was used to scan the slides and quantify the positive cells density.

Statistical analysis

Quantitative results were presented as means ± standard error (SE). Statistical analyses were performed using GraphPad Prism software. Depending on distribution and experimental design, Student's t-test was used. Statistical significance was set at $p<0.05$.

Results

Identification of pyroptosis-related hub genes in pSS

Increasing evidence has shown that pyroptosis could play a role in autoimmune diseases; thereby, to explore the effect of pyroptosis in pSS, we compared the relative levels of pyroptosis pathway and identified pyroptosis-related hub genes between pSS and healthy volunteers. Firstly, we quantified the relative levels of pyroptosis using the ssGSEA algorithm with the publicly available database GSE127952. The results indicated higher pyroptosis activity in pSS patients compared to healthy volunteers (*t*-test, $p<0.05$) (Fig. 1A), suggesting a potential role of pyroptosis in pSS. Then, to obtain pyroptosis-related hub genes, we calculated 1494 DEGs between pSS patients and healthy volunteers and identified five genes that overlapped between the DEGs and 33 pyroptosis-related genes. The volcano plot showed the significant DEGs in pSS patients ($p <0.05$) (Fig. 1B), and the Venn diagram illustrated the overlap between these DEGs and pyroptosis-related genes (Fig. 1C), leading to the identification of five pyroptosis-related hub genes (AIM2, CASP1, CASP3, IL6, TNF). Additionally, we investigated the expression levels of these five hub genes, revealing a clear distinction between pSS patients and healthy volunteers in a heatmap (Fig. 1D). Moreover, Principal component analysis (PCA) highlighted the hub gene expression differences among groups in GSE127952, emphasising the role of hub genes (Fig. 1E). Collectively, these findings emphasised the potential involvement of pyroptosis in pSS.

Functional enrichment analysis of DEGs

To delve into the functionality of the identified 1494 DEGs, we performed GO and KEGG pathway enrichment analysis using the R package clusterProfiler. The results showed that the top 10 enriched BP terms included positive regulation of cytokine production, activation of immune response, cytokine-mediated signalling pathway, immune

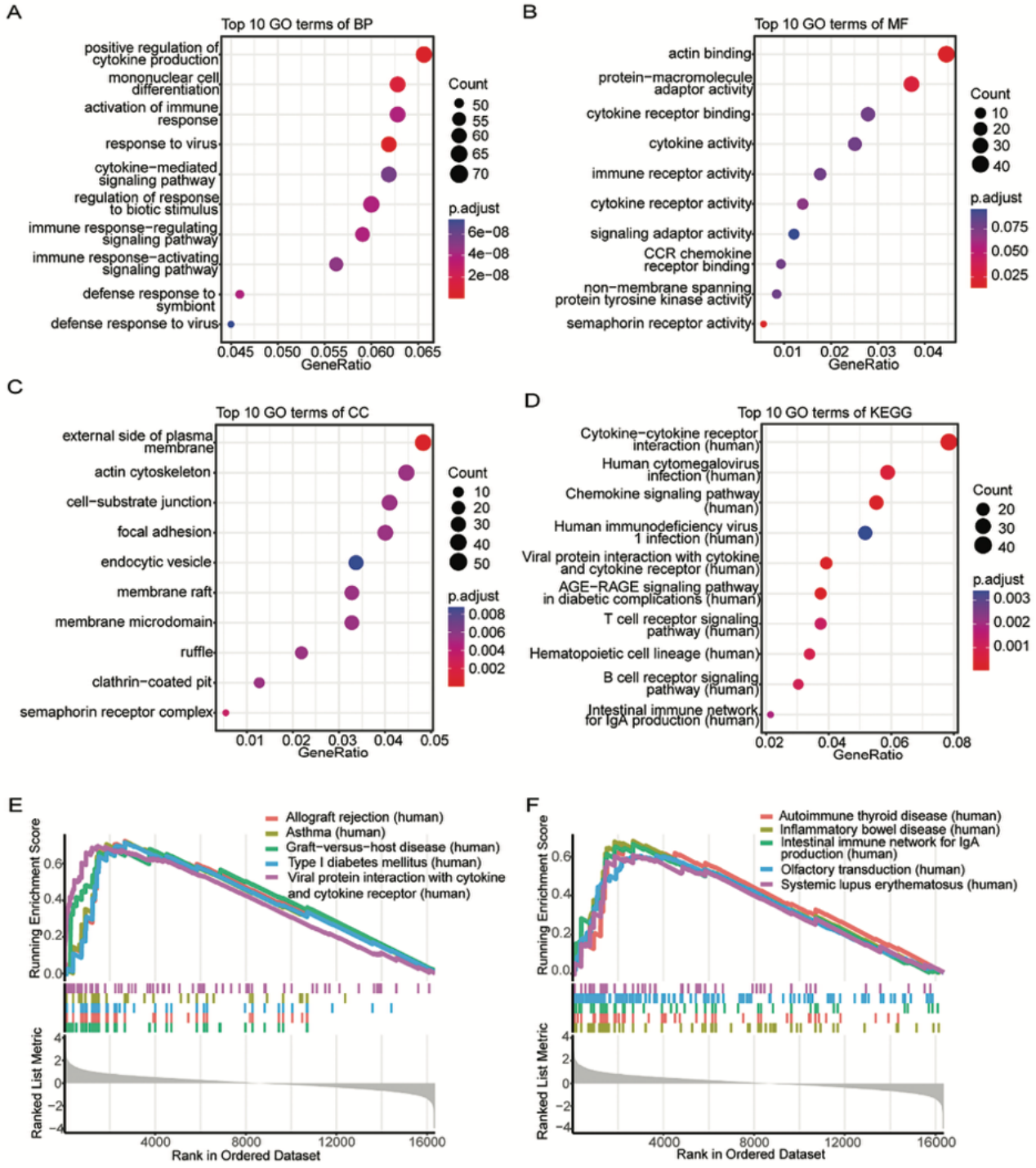

Fig. 2. Functional enrichment analysis of DEGs between pSS and healthy volunteers.
A-B-C: Top 10 BPs, CCs and MFs in GO enrichment analysis. **D**: The KEGG pathway analyses of DEGs. **E-F**: Gene set enrichment analysis (GSEA) of top 1-5 and top 6-10 KEGG pathway enrichment for the ranked differential expressed genes.
BP: biological process; CC: cellular component; MF: molecular function.

response-regulating signalling pathway, and immune response-activating signalling pathway (Fig. 2A). For molecular function (MF), the findings showed the top 10 enriched terms consisted of actin binding, cytokine receptor binding, cytokine activity, immune receptor activity, and cytokine receptor activity (Fig. 2B). In terms of cellular component (CC), the DEGs were mainly enriched in the plasma membrane, actin cytoskeleton, cell-substrate junction, focal adhesion, and endocytic vesicle (Fig. 2C). For KEGG pathway analysis, the top 10 significantly enriched pathways of DEGs were shown which included cytokine-cytokine receptor interaction, chemokine signalling pathway, T cell

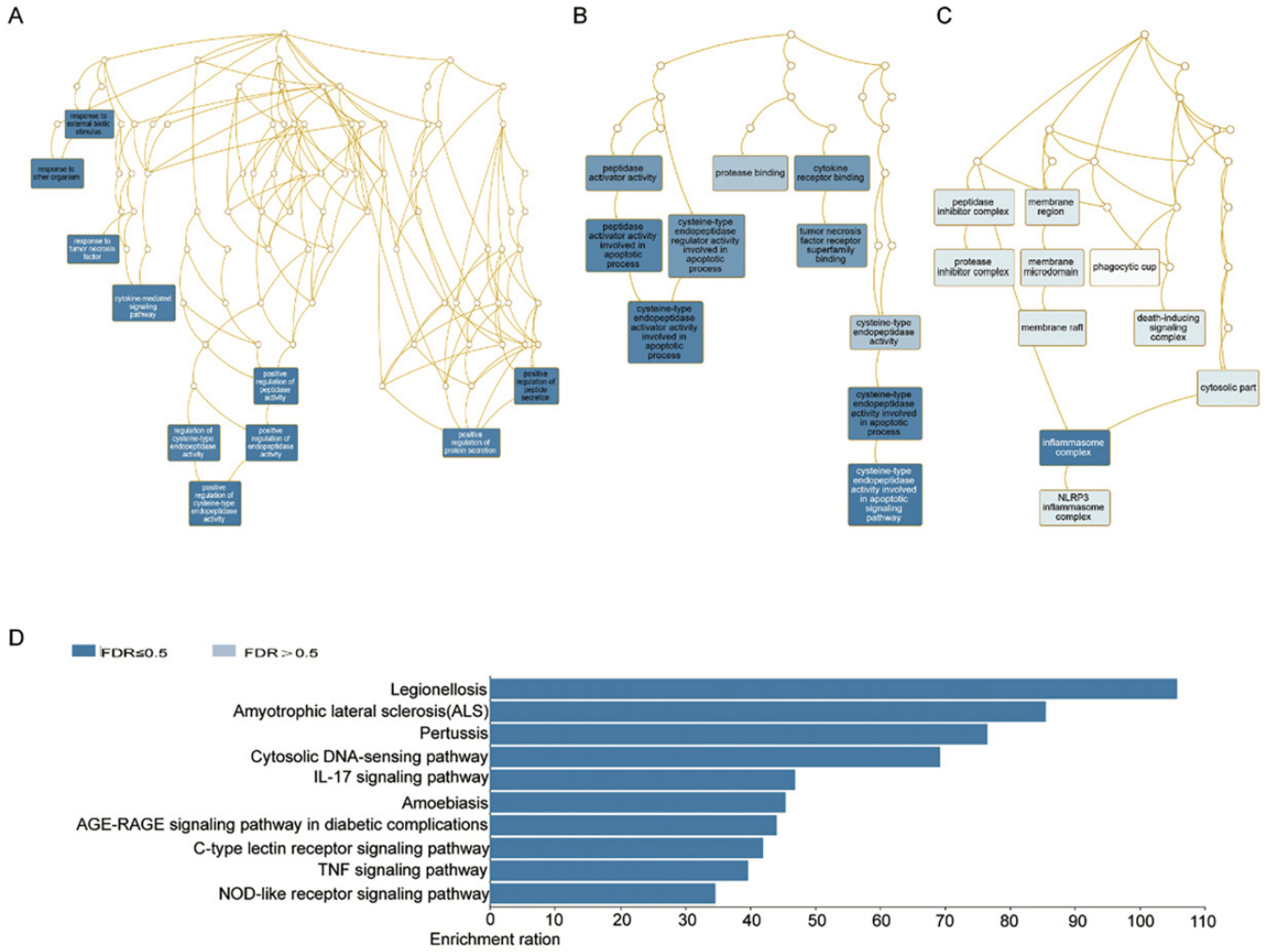

Fig. 3. Functional annotation of pyroptosis-related hub genes using the WebGestalt database. **A**: Biological process, **B**: molecular function, **C**: cellular component, **D**: KEGG pathways for the five hub genes.

receptor signalling pathway, B cell receptor signalling pathway and intestinal immune network for IgA production (Fig. 2D). Above results showed that prominent BP, MF, CC, and KEGG pathways associated with immunity, indicating that the function of DEGs was of relativity with immunity.

To validate these findings, we performed GSEA using the ranked differentially expressed genes for KEGG pathways. Consistently, the results highlighted the immunity-related terms, such as cytokine and cytokine receptor, autoimmune thyroid disease, intestinal immune network for IgA production, and systemic lupus erythematosus (p<0.1) (Fig. 2E). Together, these findings consistently underscored the strong connection between the identified DEGs and immune processes, substantiating the pivotal role of immunity in pSS.

Functional annotation of pyroptosis-related hub genes

Since the DEGs exhibited an involvement with immunity, we wondered whether the pyroptosis-related hub genes were associated with immunity. we proceeded to perform the GO and KEGG pathway enrichment analysis for the five hub genes through the online tool WebGestalt. The analysis demonstrated significant enrichment of hub genes in biological processes (BP) including positive regulation of cysteine-type endopeptidase activity, positive regulation of endopeptidase activity, positive regulation of peptidase activity, regulation of cysteine-type endopeptidase activity, positive regulation of protein secretion, response to tumour necrosis factor, and cytokine-mediated signalling pathway (Fig. 3A). Molecular function (MF) analysis indicated their roles in tumour necrosis factor receptor superfamily binding, cytokine receptor binding and activities involved in apoptotic process like cysteine-type endopeptidase activity and peptidase activator activity (Fig. 3B). Notably, the hub genes were found to be enriched in the peptidase inhibitor complex, phagocytic cup, death-inducing signalling complex, and inflammasome complex in cellular components (CC) (Fig. 3C). KEGG pathway analysis highlighted pathways like cytosolic DNA-sensing pathway, IL-17 signalling pathway, C-type lectin receptor signalling pathway, TNF signalling pathway, and NOD-like receptor signalling pathway (Fig. 3D), further supporting their immune-related functions. Altogether, the pyroptosis-related hub genes exhibited an involvement in immune processes.

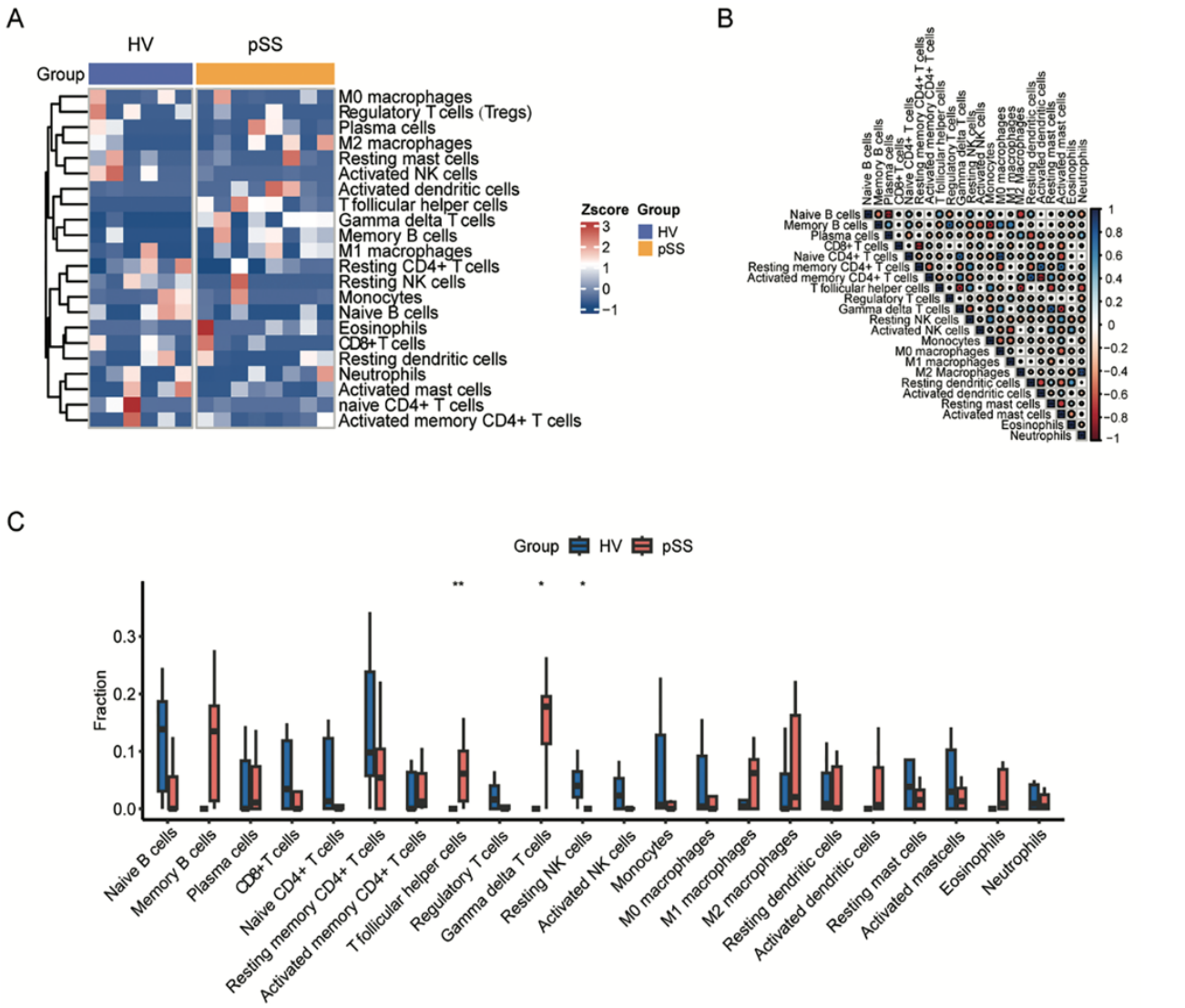

Fig. 4. Characterisation of immune infiltration in pSS. **A**: The heatmap depicted the proportion of 22 immune cells in pSS and healthy volunteers. **B**: Spearman correlation of 22 immune cells with each other in pSS. (*C*) Comparison of 24 immune cell infiltration for pSS *versus* healthy volunteers.

Investigation of immune cell infiltration

Considering that immunity played a central role in the development of pSS, we assessed the changes of 22 immune cells infiltration levels in pSS using CIBERSORTx. The heatmap depicted the proportion of 22 immune cells in pSS and healthy volunteers, presenting that activated dendritic cells, T follicular helper cells, delta gamma T cells, memory B cells, and M1 macrophages were dominated in pSS patients compared with healthy volunteers (Fig. 4A). According to the relative levels of immune cell infiltration, we then calculated the correlation of 22 immune cells with each other in pSS. The results showed that activated dendritic cells was positively correlated with resting memory CD4 T cells, while it was negatively correlated with activated memory CD4 T cells; delta gamma T cells was positively correlated with resting mast cells, and negatively correlated with activated mast cells and T follicular helper cells; memory B cells have a positive correlation with Tregs, and a negative correlation with monocytes (Fig. 4B). Additionally, we compared the signature scores of 22 immune cells using ssGSEA in patients with pSS *versus* healthy volunteers. The findings revealed that T follicular helper cells and delta gamma T cells were significantly higher in pSS, while resting NK cells were significantly lower in pSS (Wilcoxon-test, $p<0.05$) (Fig. 4C). Collectively, these results underscored the role of various immune cells in pSS, particularly T follicular helper cells and delta gamma T cells.

Relationship between pyroptosis and immune infiltration in pSS

Considering immune cells' potential role in pSS, we next explored the interplay between the hub genes and immune cells. Boxplot analysis indicated that the hub genes were upregulated in patients with pSS compared to healthy volunteers (Fig. 5A, Wilcoxon-test, $p<0.05$). Then we assessed the correlation values between the pyroptosis pathway and immune cells in pSS using the "ggpubr" R package. The scatter plot depicted that the pyroptosis was associated with immune cell infiltration like gamma delta

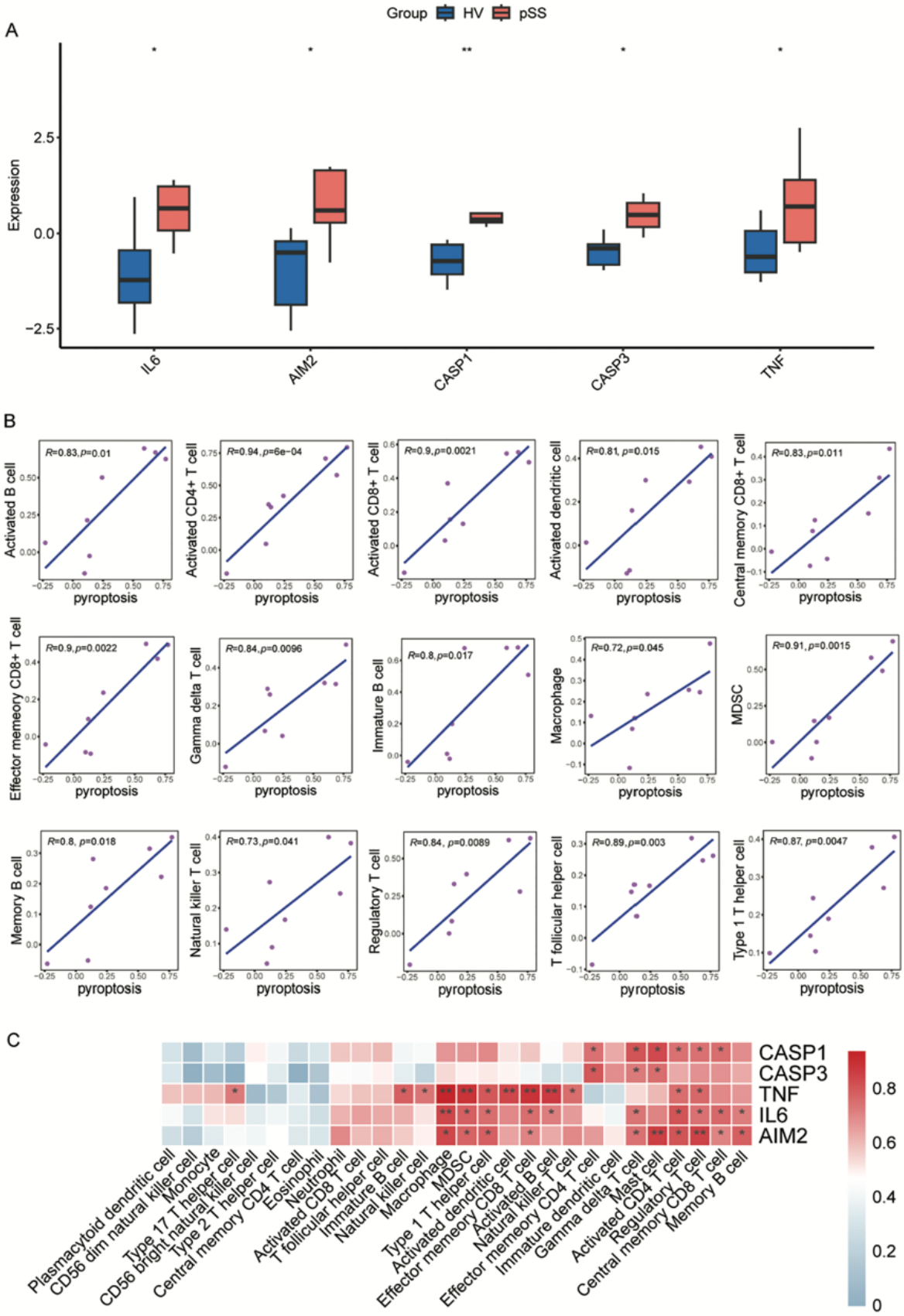

Fig. 5. Relationship between pyroptosis and immune infiltration in pSS.
A: Boxplot showed expressions of the hub genes in patients with pSS compared to healthy volunteers.
B: Scatter plot depicted the correlation between immune cells and the pyroptosis pathway in pSS.
C: Heatmap showed correlations between hub genes and immune cell.

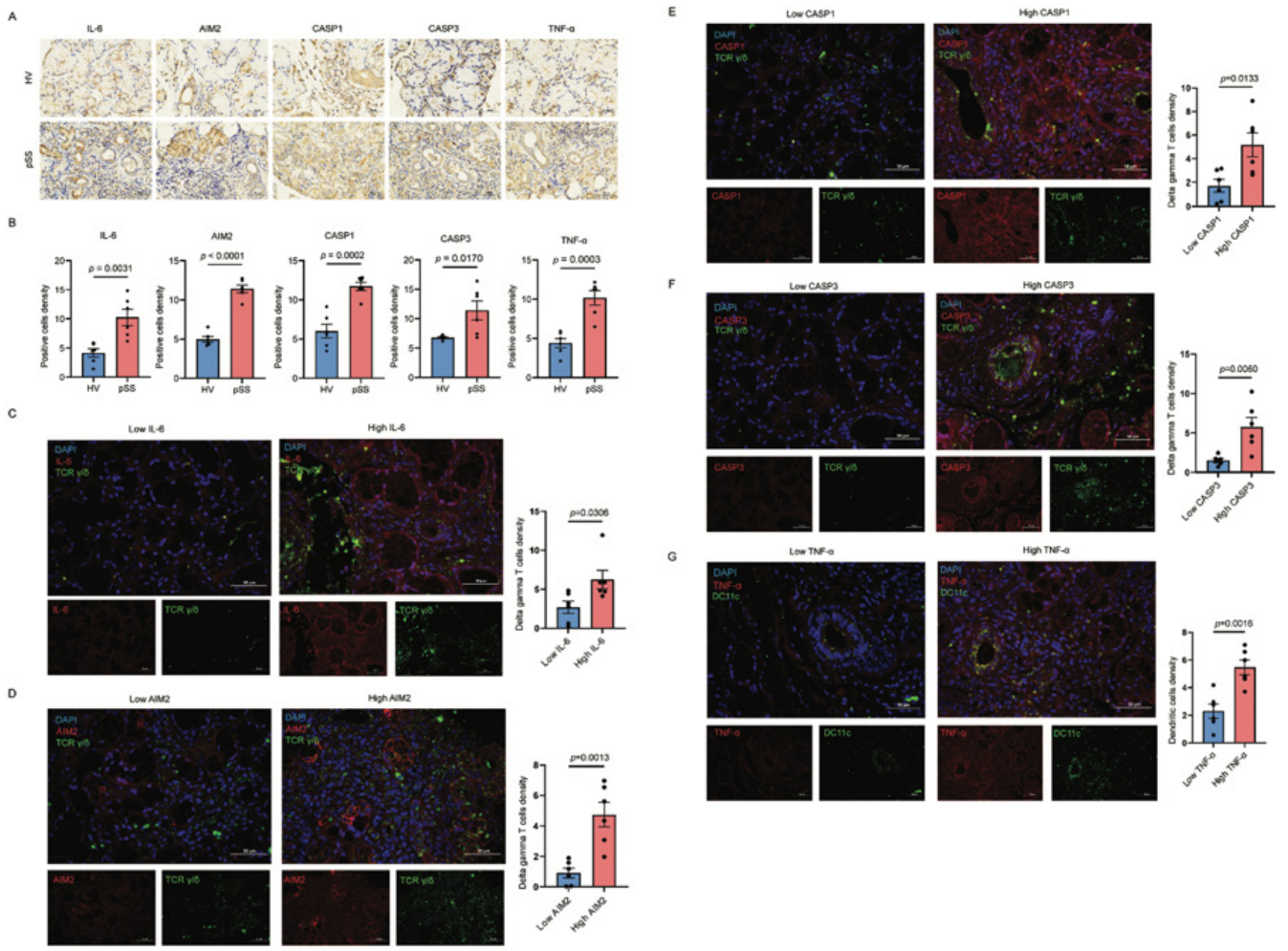

Fig. 6. Validation of pyroptosis-related hub genes and the immune microenvironment by immunohistochemical staining and double labelling immunofluorescence. Validation of pyroptosis-related hub genes by immunohistochemical staining.
A: Immunohistochemical staining for proteins of the hub genes in labial minor salivary gland specimens. Scale bar=50μm.
B: Boxplots showed the positive cells density of five hub genes in pSS and healthy volunteers. Double labelling immunofluorescence and its cells density comparation between two groups for (**C**) IL-6 and delta gamma T cells, (**D**) AIM2 and delta gamma T cells, (**E**) CASP1 and delta gamma T cells, (**F**) CASP3 and delta gamma T cells, and (**G**) TNF and dendritic cells. Scale bar = 50μm.

T cells ($p<0.05$) (Fig. 5B). Moreover, a heatmap illustrated correlations between hub genes and immune cells ($p<0.05$) (Fig. 5C). The finding suggested that CASP1 was related to delta gamma T cells and Tregs; CASP3 was related to delta gamma T cells; TNF was related to activated dendritic cells and Tregs; IL-6 and AIM2 were both related to delta gamma T cells, Tregs, and memory B cells. Overall, these findings suggested a correlation between pyroptosis and the immune microenvironment in pSS.

Validation of pyroptosis-related hub genes and the immune micro-environment by immunohistochemical staining and double labelling immunofluorescence

Since these five pyroptosis-related hub genes demonstrated a potential role in the development of pSS and a relationship with immune cells, we sought to further verify its expression levels of these genes in pSS and the correlation with immune cells. And thus, we collected labial minor salivary gland biopsy samples from six untreated pSS patients and six healthy controls and performed immunohistochemical staining to investigate their protein levels. Consistent with the previous results, we found that all the five hub genes presented a significant increased expression level in the labial minor salivary gland of pSS patients compared with healthy volunteers (Fig. 6A-B), further highlighting their role in pSS. Then we conducted double labelling immunofluorescence in salivary gland biopsy samples from 12 pSS patients. Samples were sorted into two groups according to the expression levels of genes. The findings presented that the group with high expression levels of IL-6, AIM2, CASP1, and CASP3 exhibited elevated levels of delta gamma T cells, while the group with high expression levels of TNF-α showed increased levels of dendritic cells (Fig. 6C-G).

Discussion

pSS is an inflammatory systemic autoimmune disease primarily affecting secretory glands, while the role and mechanisms of pyroptosis in pSS remain largely undefined. The findings in this study provide unique insights into the role and mechanisms of pyroptosis in the pathogenesis of pSS. In our

study, we identified 1494 DEGs between pSS patients and healthy volunteers from the GSE127952 datasets and obtained five pyroptosis-related hub genes by intersecting these DEGs and 33 pyroptosis-related genes. According to GO and KEGG pathway enrichment analyses, we found that immune-related signalling pathways were significantly enriched, including activation of the immune response, immune response-regulating signalling pathway, and immune response-activating signalling pathway. By investigating immune microenvironment in pSS, we found that various immune cells participating in pSS. Among them, delta gamma T cells were significantly positively correlated with CASP3.

By comparing the relative levels of pyroptosis of pSS and healthy volunteers from GSE127952 datasets, we found that pSS patients showed a higher level of pyroptosis. Currently, some researches indicated that pyroptosis may play a role in the pathogenesis or progression of SS through Caspase-1-Mediated Canonical Pathway and Caspase-4/5/11-Mediated Noncanonical Pathway (21, 22). However, the study of pyroptosis in pSS was still limited. The pathway of pyroptosis in other autoimmune diseases still needs to be verified in SS, such as the caspase-3-dependent and the caspase-free pathways.

The absence of melanoma 2 (AIM2) was one of the canonical inflammasomes that enable the induction of pyroptosis (23). AIM2 belonging to the PYHIN (pyrin and HIN domain-containing) protein family, was composed of a C-terminal HIN-200 domain and an N-terminal pyrin domain (PYD) (24). The AIM2 inflammasome functioned as a sensor for cytoplasmic DNA and was established to detect and respond to DNA originating from diverse sources (25). Recent study found that the ductal epithelial cells in SS patients display significant activation of the AIM2 (10), and another study showed that the mRNA expressions of CASP1 and AIM2 in minor salivary gland samples were markedly elevated in SS patients in contrast to sicca controls (11). These findings were consisted with our result. But the research on AIM2 in lacrimal gland epithelial cells was limited, more *in vivo* and *in vitro* experiments were still needed in the future.

Interleukin-6 (IL-6) was a proinflammatory cytokine, which took part in B-cell proliferation and differentiation (26). The production of IL-6 could be regulated directly or indirectly by CASP-1 activation (27). Tumour necrosis factor (TNF) was a cytokine predominantly produced by activated leukocytes and was able to induce cell death. TNF was one of the first step signalling for activation of the NLRP3 inflammasome in pyroptosis (28). Researchers found that the expressions of TNF and IL-6 were elevated in both salivary glands and serum (29-31). Our results were consistent with these researches showing that IL-6 and TNF were involved in the pathogenesis of pSS. More details about the relationship between these cytokines and the pyroptosis in pSS were needed.

Caspase-1 is first recognised as a protease for converting the inactive precursors of interleukin-1β (IL-1β) and IL-18 into their active cytokine forms (32). The study found that individuals with pSS had higher levels of active caspase-1 in their saliva and salivary gland cells, including both leukocytes and gland epithelial cells, when compared to controls (11). In our finding, both bioinformatic and immunohistochemical results presented that patients with pSS exhibited an increase in caspase-1 activity which supported the previous study.

Caspase-3 is commonly identified as an executioner caspase in apoptosis and is known to cleave gasdermin-E (GSDME), releasing the gasdermin N-terminal fragment that can trigger pyroptosis (33). A study had found that activated caspase-3 were expressed in ductal and acinar cells in pSS salivary glands showing the importance of caspase-3 in the salivary dysfunction of pSS (34). Unfortunately, there was no study reporting that the caspase-3-mediated emerging pathway participating in pSS. In our results, we also discovered an increased expression of CASP3 in the salivary gland samples of pSS patients indicating that the caspase-3-mediated emerging pathway might play a role in the pathogenesis or progression of pSS. To ascertain the precise mechanisms, further experimental investigations were necessary.

An increasing number of studies revealed that pyroptosis can link the innate and adaptive immunity (35). During pyroptosis, the maturation and release of interleukin-1β (IL-1β) gave the signal to macrophages to be activated and recruited by upregulating selective adhesion molecules on circulating cells (36). As for $CD4^+$ T cells, T cell-intrinsic signalling downstream of IL-1 and IL-18 played a critical role not only in the initial activation but also in sustaining the immune response (37). By stimulate the production of IL-6, IL-1 could indirectly promote B cell proliferation, differentiation, and antibody production (36). These studies tied well with our results wherein the various immune cells like T helper cells, memory B cells, and M1 Macrophage took part in the process of pyroptosis of pSS. However, no generalisation can be made on the exact relationship between pyroptosis and those immune cells in pSS, more evidence was still waiting to be discovered.

Notably, there were a few limitations in this study. Firstly, the sample size of our data was relatively small, which might limit the statistical power and generalisability of our findings. Future studies with larger sample sizes are needed to validate and further elucidate our findings. Furthermore, while our results had been validated by histological staining, further lab studies were still warranted to expand our findings to clinical utility. Moreover, the inherent cellular heterogeneity within salivary gland tissues poses an additional challenge; our gene expression analyses were conducted on mixed cell populations, providing a composite rather than individual cellular contributions to the overall expression profile. Thereby, the specific cellular origins of the dysregulated pathways in pSS remained to be precisely defined.

Conclusion

By performing a comprehensive analysis of gene expression profiles between pSS patients and healthy controls, we

revealed the involvement of pyroptosis in pSS and identified five pyroptosis-related hub genes as a potential link between pyroptosis and the immune microenvironment in pSS. These findings could pave a way for further research and clinical treatment of pSS.

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