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Single-cell transcriptomic analysis uncovers heterogeneity in the labial gland microenvironment of primary Sjögren's syndrome

Jun Huang [1], Jia Tang [1], Chen Zhang, Tingting Liu, Zhiyong Deng [**], Lei Liu [*]

Department of Rheumatology and Immunology, The First People's Hospital of Kunshan, Jiangsu, Suzhou, 215300, China

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ABSTRACT

Objective: Primary Sjögren's syndrome (pSS) is a systemic autoimmune disorder with an unclear pathogenetic mechanism in the labial gland. This study aims to investigate the cellular and molecular mechanisms contributing to the development of this disease.

Methods: Single-cell RNA sequencing (scRNA-seq) was performed on 32,337 cells of labial glands from three pSS patients and three healthy individuals. We analyzed all cell subsets implicated in pSS pathogenesis.

Results: Our research revealed diminished differentiation among epithelial cells, concomitant with an enhancement of interferons (IFNs)-mediated signaling pathways. This indicates a cellular functional shift in reaction to inflammatory triggers. Moreover, we observed an augmentation in the population of myofibroblasts and endothelial cells, likely due to the intensified IFNs signaling, suggesting a possible reconfiguration of tissue structure and vascular networks in the impacted regions. Within the immune landscape, there was an apparent increase in immunosuppressive macrophages and dendritic cells (DCs), pointing to an adaptive immune mechanism aimed at modulating inflammation and averting excessive tissue harm. Elevated activation levels of $CD4^{+}$T cells, along with a rise in regulatory T (Treg) cells, were noted, indicating a nuanced immune interplay designed to manage the inflammatory response. In the $CD8^{+}$T cell subsests, we detected a notable increase in cells expressing granzyme K (GZMK), signaling an intensified cytotoxic activity. Additionally, the escalated presence of T cells with high levels of heat shock proteins (HSPs) suggests a cellular stress condition, possibly associated with persistent low-grade inflammation, mirroring the chronic aspect of the condition.

Conclusions: Our research identified distinct stromal and immune cell populations linked to pSS, revealing new potential targets for its management. The activation of myeloid, B, and T cells could contribute to pSS pathogenesis, providing important guidance for therapeutic approaches.

1. Introduction

Sjögren's syndrome (SS) is characterized as an autoimmune disorder predominantly manifested by chronic dryness, occurring either as primary SS or concomitantly with other autoimmune conditions in its secondary form. The trajectory of research is swiftly advancing, with a focused lens on the etiology, pathogenesis, diagnostic modalities, and therapeutic avenues [1,2]. Investigative efforts are concentrated on deciphering the complex interrelations between autoimmune responses, genetic predispositions, and environmental triggers [3]. Diagnostic advancements, including salivary gland imaging techniques and the development of innovative biomarkers, are significantly refining the accuracy of disease detection [4,5]. Therapeutically, the research agenda is pivoting towards the discovery of more potent interventions, notably biologics and small molecule inhibitors [6]. Concurrently, there is a growing prioritization of enhancing patient quality of life, facilitated by holistic disease management and tailored treatment regimens, with the ultimate goal of deepening the comprehension of SS and ameliorating patient health outcomes.

Single-cell RNA sequencing (scRNA-seq) intricately delineates cellular diversity and gene expression nuances in Sjögren's syndrome, shedding light on its complex pathophysiology. This technique not only facilitates the identification of novel biomarkers and therapeutic avenues but also deepens our understanding of the disease's pathogenic

* Corresponding author. Department of Rheumatology and Immunology, the First People's Hospital of Kunshan, Jiangsu, Suzhou, 215300, China.
** Corresponding author. Department of Rheumatology and Immunology, the First People's Hospital of Kunshan, Jiangsu, Suzhou, 215300, China.
E-mail addresses: yichun1988@yeah.net (Z. Deng), ksliulei@126.com (L. Liu).
[1] Jun Huang and Jia Tang have contributed equally to this work.

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underpinnings by dissecting the cellular heterogeneity in the peripheral blood of patients. Specifically, individuals with primary Sjögren's syndrome (pSS) show significant variations in naïve and memory B cell ratios, along with a conspicuous upregulation of interferon-stimulated genes within B cell populations, pinpointing potential therapeutic targets [7]. The expansion of $CD4^+$ cytotoxic T lymphocytes (CTLs) in pSS patients hints at their involvement in disease pathogenesis, guiding therapeutic development [8]. Moreover, pSS patients exhibit a higher ratio of $CD56^-CD16^+FCER1G^+/CD56^-CD16^+FCER1G^-$ natural killer (NK) cells than healthy individuals, emphasizing the immunological intricacy in peripheral blood mononuclear cells (PBMCs) in pSS [9]. Additionally, a prior study illustrates abnormal monocyte subset distribution and transcriptomic changes in Sjögren's syndrome, identifying $TNFSF10^{high/+}$ monocytes as pivotal in its pathogenesis and as a viable therapeutic target [10]. Extensive RNA-seq analysis associates the presence of pro-inflammatory M1 macrophages, other immune cells, a senescence-associated secretory phenotype, and significant metabolic reprogramming with the disease [11]. scRNA-seq has unveiled cellular heterogeneity in primary Sjögren's syndrome (pSS), revealing altered cell populations and signaling pathways in the salivary glands and peripheral blood, thereby underscoring potential therapeutic targets, particularly in epithelial regeneration and immune cell migration [12].

While single-cell transcriptomic sequencing has significantly enhanced our comprehension of the pathophysiological mechanisms in the peripheral blood and salivary glands of Sjögren's syndrome patients, investigations into the microenvironmental pathogenesis within the labial glands are notably unclear. This study aims to bridge this gap by collecting labial gland tissues from both Sjögren's syndrome patients and healthy controls, employing single-cell transcriptomic sequencing to meticulously analyze the alterations within their microenvironment.

2. Materials and methods

2.1. Patients and tissue specimens

In this investigation, labial gland tissues were obtained from three individuals diagnosed with pSS and three healthy donors. All participants were recruited from the Rheumatology and Immunology Department of Kunshan First People's Hospital during 2022. All participants with pSS evaluated in this study met the 2016 American College of Rheumatology/European League Against Rheumatism classification criteria [13]. All participants gave written informed consent prior to the investigations. The use of whole labial gland tissue samples for further studies beyond routine diagnosis was approved by the Ethics Committee of Kunshan First People's Hospital, China (2024-03-014-K01).

2.2. Single-cell RNA sequencing cell capture and cDNA synthesis

Using the BD Rhapsody™ Cartridge Reagent Kit (BD, 633,731) and BD Rhapsody™ Cartridge Kit (BD, 633,733), we processed a cell suspension containing 300–600 viable cells per microliter, as quantified by Count Star. The cells were loaded onto the BD Rhapsody™ Cartridge to facilitate the generation of single-cell magnetic beads within the microwells, adhering to the manufacturer's guidelines. In brief, we suspended single cells in the provided sample buffer and introduced approximately 6000 cells into each channel, aiming to recover an estimated 3000 cells. The captured cells underwent lysis, releasing RNA that was subsequently barcoded during reverse transcription within individual microwells. We conducted reverse transcription using a ThermoMixer® C (Eppendorf) set at 1200 rpm and 37 °C for 45 min. This process yielded cDNA, which was amplified and quality-checked using an Agilent 4200, a service provided by CapitalBio Technology, Beijing.

For library preparation, we adhered to the manufacturer's guidelines to construct single-cell RNA-seq libraries using the BD Rhapsody™ WTA Amplification Kit (BD, 633,801). CapitalBio Technology, Beijing, sequenced the libraries on an Illumina NovaSeq 6000 platform, achieving a sequencing depth of at least 50,000 reads per cell with a paired-end 150 bp (PE150) strategy.

2.3. scRNA-seq data analysis

Utilizing the BD Rhapsody™ sequencing pipelines, we generated expression matrices for further analysis. These matrices were then converted into Seurat objects, adhering to the protocols outlined by the Seurat package [14]. Subsequently, we quantified the proportions of mitochondrial genes using the PercentageFeatureSet function and normalized the data with the NormalizeData function. Identification of highly variable genes, crucial for downstream analysis, was achieved through the DUBStepR function within the DUBStepR package [15]. Data centering was accomplished using the ScaleData function. Principal Component Analysis (PCA) was executed using the RunPCA function, and the top 30 principal components were selected for UMAP (Uniform Manifold Approximation and Projection) to facilitate dimensionality reduction and clustering analysis. Clusters were meticulously annotated based on their gene expression profiles, and the visualization was conducted using the integrated tools within the Seurat package.

2.4. Transcriptional regulatory network analysis

Datasets on motif scores for regions 500 bp proximal to transcription start sites and within a 10 kb surrounding area were sourced from cisTarget databases, enabling initial Regulon scoring. The SCENIC package [16] facilitated the removal of low-expression genes from expression matrices, with the exportsForArboreto function exporting these refined matrices and transcription factors. The GRNBoost2 algorithm in pySCENIC package was used to construct co-expression networks, offering improved efficiency over the GENIE3 package in R. These networks were imported into R using importArboreto for subsequent analysis. Regulon scoring and activity assessment across cell types were performed using the RcisTarget and AUCell packages, respectively. Finally, the Seurat package's DoHeatmap function graphically represented the AUC values of Regulons, illustrating their expression profiles.

2.5. Analysis of cell-cell interactions

To elucidate the influence of epithelial cells on stromal cells, we harnessed the nichenetr package [17]. Initially, our methodology involved the isolation of ligand genes actively expressed in epithelial cells, in conjunction with the identification of genes differentially expressed in stromal cells, adhering to the guidelines of the nichenetr package's protocol. We then employed the FindAllMarkers function to detect genes with elevated expression in epithelial cells, and intersected these with the previously identified ligand genes to confirm their predominant expression in epithelial cells compared to other cell types. Subsequently, we computed potential receptor genes in epithelial cells following the nichenetr package's protocol. The visualization was used the make_heatmap_ggplot function.

To thoroughly investigate the interactions and communications between cellular clusters, we employed the CellChat package for analysis [18]. We extracted normalized gene expression data and cellular annotations from the Seurat object and constructed a CellChat object following the CellChat protocol, selecting CellChatDB.human as the communication database appropriate for our human-derived samples. After establishing the initial setup, our analysis, following CellChat's standardized methods, included the calculation of intercellular communication probabilities, identification of significant communication pathways, and revelation of complex cellular communication networks through network analysis. All visualizations were used the functions provided by CellChat package.

2.6. Statistical analysis

All bioinformatics analyses were conducted using R version 4.2.3, RStudio, and Python 3.8. No new algorithms were generated during these bioinformatics analyses. All figures were created using Adobe Illustrator 2023 for scientific visualization. scRNA-seq data were analyzed using the Seurat package, while SCENIC analysis was carried out with the pyscenic package in Python 3.8. For specific details on statistical tests, please refer to the legends associated with the figures.

3. Results

3.1. scRNA-seq analysis of labial gland tissues from pSS patients and normal donors

We procured labial gland tissues from three individuals diagnosed with pSS and three healthy donors, subjecting these six samples to scRNA-seq (Supplemental Fig. 1 and Table 1). Utilizing the Seurat toolkit's official guidelines, we conducted a basic filtration on the acquired scRNA-seq data. During this procedure, we noted that partial cells exhibited elevated levels of mitochondrial gene expression. To improve the data quality, we carried out a filtering process based on the mitochondrial gene expression scores within the cellular populations (Supplemental Figs. 2A–2C). Consequently, we obtained 32,337 cells for further analysis.

By utilizing UMAP for dimensionality reduction and clustering analysis, these cells were classified into seven distinct populations: Epithelial cells, Stromal cells, Mast cells, Myeloid cells, T cells, B cells and Plasma cells (Fig. 1A and B). Notably, epithelial cells accounted for approximately 50 % of the total cells (Fig. 1C). Comparing to normal tissues, the proportion of epithelial cells in pSS was decreased, whereas the proportions of stromal cells, myeloid cells, and plasma cells was increased. Additionally, there was also a slight increase in the T cell proportion (Fig. 1D). Due to mast cells accounting for fewer cells, these cells were excluded from further analysis.

Based on prior researches that has extensively characterized the roles of B cells and plasma cells in pSS, our scRNA-seq data further confirmed an increased presence of plasma cells in pSS (Fig. 1D). Nonetheless, the alterations in epithelial and stromal cells, along with their interactions with immune cells in pSS, have not been exhaustively elucidated. Therefore, our investigation will focus on uncovering the underlying pathogenic mechanisms in pSS, with a specific interest in these cell populations.

3.2. Analysis of epithelial cell subsets

The decrease in epithelial cells observed in patients with pSS was predominantly attributed to immune-mediated targeting, aligning with findings from our scRNA-seq data. Given the distinctive structural features of labial gland tissue, we categorized epithelial cells into three main subgroups: serous acinar cells, mucous acinar cells, and ductal cells (Fig. 2A and B). GSEA for each subgroup revealed that serous acinar cells mainly engage in processes such as DNA repair, protein secretion, and xenobiotic metabolism. Mucous acinar cells are closely associated with estrogen response and glycolysis, whereas ductal cells are significantly involved in TNF-α signaling, apoptosis, and TGF-β signaling pathways (Fig. 2C). Statistical analysis of the proportions within these subgroups revealed no significant differences (Fig. 2D and E), suggesting a uniform reduction of epithelial cells across all subgroups in patients with pSS throughout the disease's progresses.

To investigate transcription factors (TFs) involved in the altered transcriptional landscape characteristic of disease in epithelial cells, we utilized SCENIC analysis to infer the regulatory activities of TFs in epithelial cells from patients with pSS. In comparison to epithelial cells from healthy donors, we observed a notable decrease in the expression and regulatory activities of *EHF*, *TGIF1*, and *SOX9* in pSS patients (Fig. 2F and G). It has been established that SOX9 plays a role in suppressing the differentiation of epithelial cells [19]. EHF, a member of the ETS family, negatively regulates the AP-1 family, which are key downstream mediators of the MAPK signaling pathway [20]. Furthermore, TGIF1 has been recognized as a repressor of the TGF-β signaling pathway [21]. Collectively, these TFs collectively contribute to the

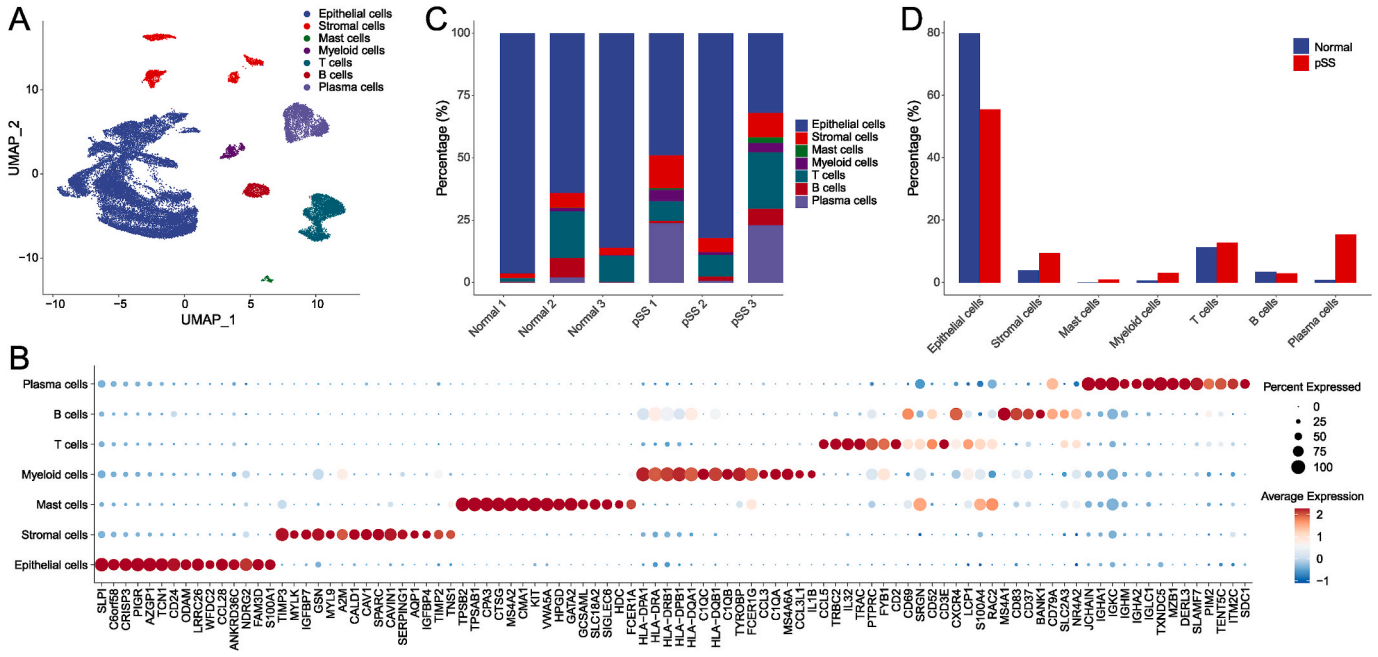

Fig. 1. Dimensionality reduction and clustering analysis of scRNA-seq data
(A) Two-dimensional UMAP visualization of 32,337 cells from healthy donors (Control) (n = 3) and patients with pSS (n = 3).
(B) Dot plot displaying top 15 marker genes of each cluster. Circle size representing expressed percentage; circle color representing average expression.
(C) Bar plot showing proportions of each cluster across each sample, color representing cell type.
(D) Bar plot comparing proportions of each cluster between control and pSS, color representing different group.

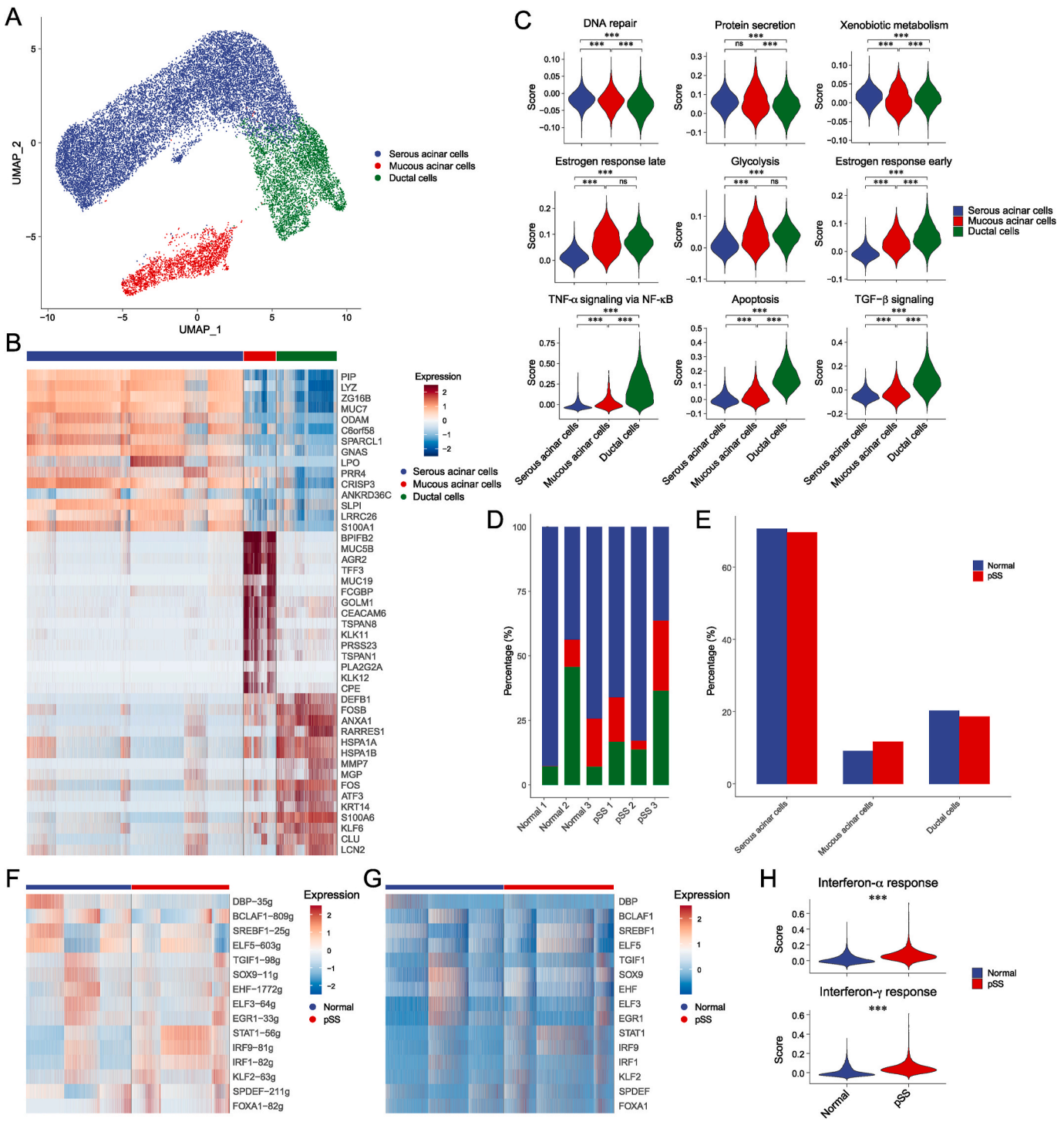

Fig. 2. Analysis of epithelial cell subpopulations

(A) Two-dimensional UMAP visualization of 21,294 epithelial cells.

(B) Heat map displaying top 15 marker genes of each subcluster.

(C) Violin plot showing enriched pathways among different subclusters.

(D) Bar plot showing proportions of each cluster across each sample, color representing cell type.

(E) Bar plot comparing proportions of each cluster between control and pSS, color representing different group. (F) Heat map displaying different regulons between control and pSS.

(G) Heat map displaying expressions of TFs in (F).

(H) Violin plot showing enriched pathways associated IFNs between control and pSS.

Statistical analyses in (C) were performed using the Kruskal-Wallis multiple-comparison test, and (H) were performed using an unpaired, two-tailed Student's *t*-test. ***$P < 0.001$.

inhibition of epithelial cell differentiation in the context of pSS. Additionally, an enhanced expression and regulatory activity of IFNs-related transcription factors, such as *STAT1*, *IRF1*, and *IRF9*, were observed in pSS patients (Fig. 2F and G), which was further substantiated by scoring the activity of IFNs-related signaling pathways (Fig. 2H). These findings suggest a downregulation of epithelial cell differentiation and an upregulation of immune-associated pathways in the epithelial cells of pSS patients.

3.3. Analysis of stromal cell subsets

In patients with pSS, we observed an upregulation in the proportion of stromal cells. To delineate this alteration, we performed a detailed subgroup analysis of the stromal cells, segregating them into four distinct populations based on their characteristic gene expression profiles: smooth muscle cells, inflammatory fibroblasts, myofibroblasts, and endothelial cells (Fig. 3A and B). Statistical analysis of the proportions within these subgroups revealed a notable increase in the myofibroblast and endothelial cell populations, accompanied by a reduction in smooth muscle cells (Fig. 3C).

SCENIC analysis was utilized to explore the TFs implicated that are pivotal in shaping the altered transcriptional landscape characteristic of the disease in stromal cells. This analysis uncovered that the regulatory activities of TFs associated with cell differentiation, such as *MEF2C*,

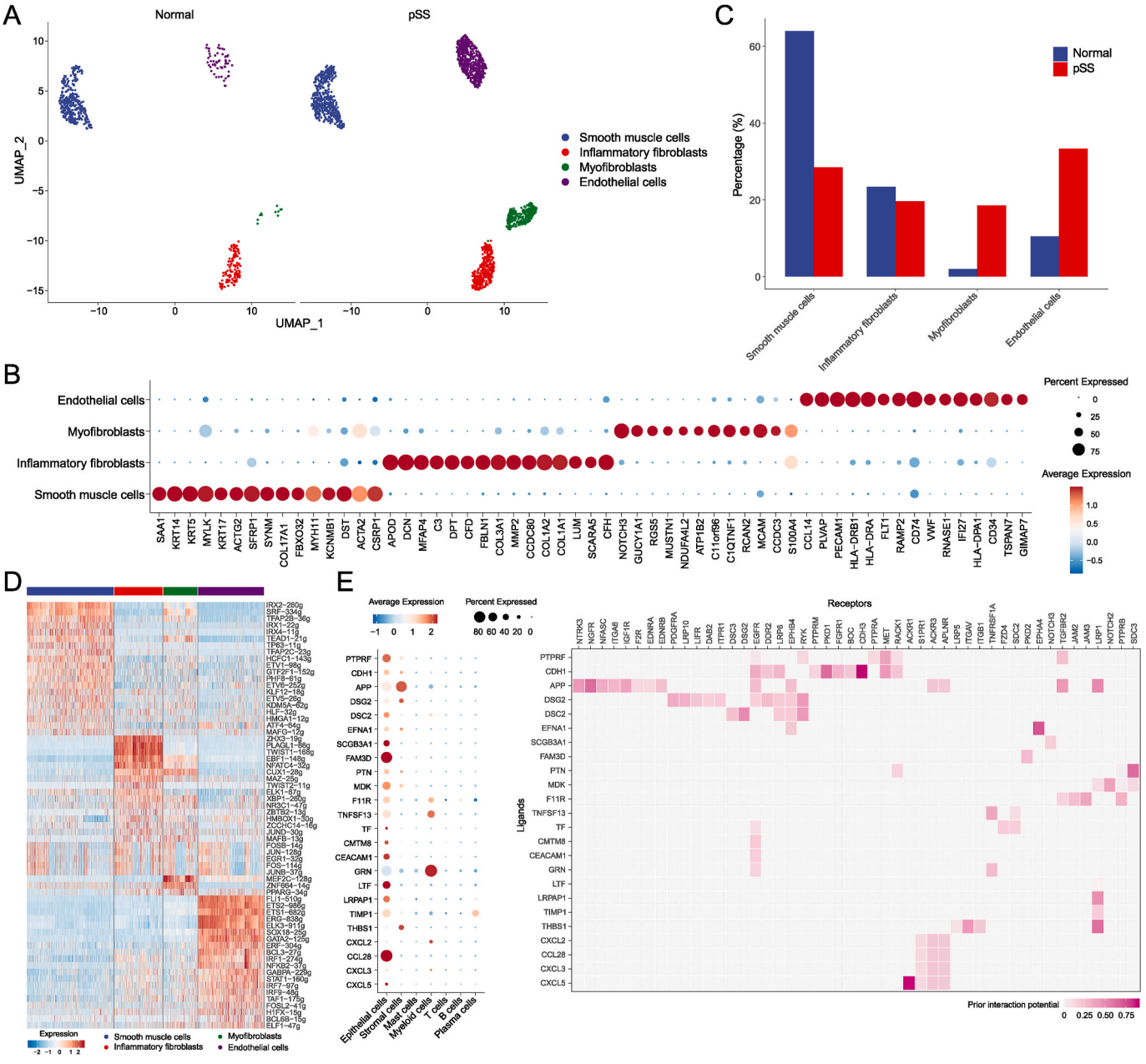

Fig. 3. Analysis of stromal cell subpopulations
(A) Two-dimensional UMAP visualization of 2302 stromal cells.
(B) Dot plot displaying top 15 marker genes of each subcluster. Circle size representing expressed percentage; circle color representing average expression.
(C) Bar plot comparing proportions of each cluster between control and pSS, color representing different group.
(D) Heat map displaying different regulons among subclusters.
(E) Nichenet analyzed the regulatory interactions between epithelial cells and stromal cells. Dot plot illustrated the expression levels of selected ligands. Heat map displayed the ligands sourced from epithelial cells interacted with receptors expressed on stromal cells.

ZNF664, and *PPARG*, were significantly enhanced in myofibroblasts. Moreover, the regulatory activities of interferon-related transcription factors, specifically *STAT1* and *IRF*, were found to be more pronounced in endothelial cells (Fig. 3D).

To further explore the influence of epithelial cells on stromal cells, we utilized the nichenetr package [17] for a comprehensive analysis, focusing on how gene expression in stromal cells is modulated by molecules secreted from epithelial cells in pSS patients (Fig. 3E). Our analysis revealed that epithelial-derived molecules, including *PTPRF*, *CDH1*, *APP*, *DSG2*, and *DSC2*, played a crucial role in promoting endothelial cells production. Notably, in pSS patients, molecules related to the Notch signaling pathway, such as *NOTCH2/3*, appeared to be influenced by epithelial-derived *MDK* and *SCGB3A1*, potentially contributing to increased myofibroblasts. Furthermore, our findings underscored the critical role of chemokines like *CXCL2*, *CCL28*, *CXCL3*, and *CXCL5*. These molecules not only actively participated in this regulatory mechanism but also exhibited significant interactions with *ACKR1/3* and *S1PR1*, highlighting their substantial contribution to interactions between epithelial and stromal cells. Collectively, our study revealed significant alterations in stromal cells in pSS patients, potentially influenced by epithelial cells.

3.4. Analysis of myeloid cell subsets

In pSS patients, a marked increase in myeloid cell populations has been observed, necessitating detailed subgroup analysis. Through transcriptome expression profiling, these myeloid cells were classified into four main subgroups: Macrophage 1, Macrophage 2, Dendritic Cells (DCs), and Proliferating macrophages (Fig. 4A and B). Notably, the Macrophage 2 and DCs subgroups were significantly more prevalent in pSS patients (Fig. 4C).

SCENIC analysis revealed enhanced regulatory activity in transcription factors downstream of the MAPK pathway, specifically *FOS*, *FOSB*, *FOSL2*, *JUN*, *JUNB*, and *JUND*. Additionally, elements associated with the NF-κB pathway (*NFKB1/2* and *REL*), along with key components of the IFNs signaling pathway, including *STAT1* and *IRF1/9*, showed increased activity in pSS patients (Fig. 4D). This complex regulatory network sheds light on the intricate interplay and convergence of critical signaling pathways within pSS.

Additionally, the significant overexpression of key transcription

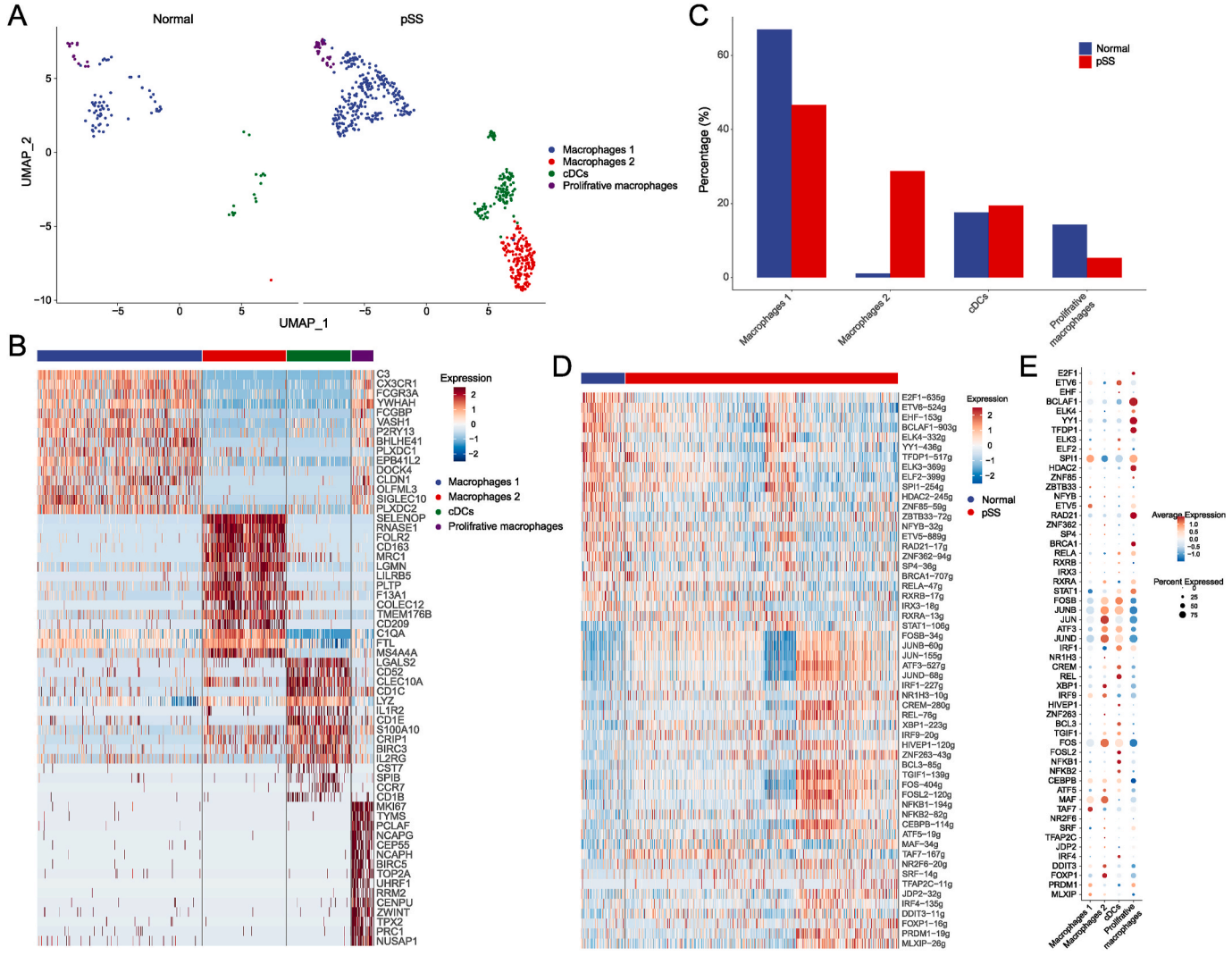

Fig. 4. Analysis of myeloid cell subpopulations
(A) Two-dimensional UMAP visualization of 662 myeloid cells.
(B) Heat map displaying top 15 marker genes of each subcluster.
(C) Bar plot comparing proportions of each cluster between control and pSS, color representing different group.
(D) Heat map displaying different regulons between control and pSS.
(E) Heat map displaying expressions of TFs in (D).

factors in the Macrophage 2 and DCs subsets underscores the activation of the MAPK and NF-κB pathways (Fig. 4E). This dysregulated activation, together with the pronounced impact of the IFNs pathway, played a critical role in the pathogenesis of pSS.

3.5. Analysis of T cell subsets

Our comprehensive gene expression profiling of T cell subsets identified 10 distinct groups, uncovering an increased prevalence of conventional T (Tconv) cells, regulatory T (Treg) cells, CD8-GZMK cells, and T-HSP (T cells expressing heat shock proteins) in pSS patients (Fig. 5A to C). This observation is consistent with prior research, underscoring the pivotal roles of Tconv and Treg cells in pSS. The presence of granzyme K (GZMK) in $CD8^{+}$T cells signifies their cytotoxic potential [22], whereas T cells with HSP play a role in chronic inflammation modulation [23].

SCENIC analysis revealed a reduction in *TCF7* regulatory activity in pSS patients, alongside an upregulation of AP-1 transcription factors, indicating an intensified T cell receptor (TCR) signaling pathway activation (Fig. 5D and E). This analysis also showed a notable increase in *FOXP3* expression in Treg cells, consistent with their expanded numbers (Fig. 5D and E). Enhanced transcriptional regulatory activity and gene expression of *EOMES* in GZMK $^{+}$ $CD8^{+}$T cells were also observed, confirming EOMES in regulating *GZMK* expression (Fig. 5D and E). Gene Set Enrichment Analysis (GSEA) validated the amplified TCR signaling pathway in this subgroup (Fig. 5F). These data collectively demonstrate that T cells in pSS patients exhibit increased activation and enhanced effector functions, which are fundamental to the pathobiology of the disease.

3.6. Analysis of cell-cell interactions revealed the altered pathways in pSS patients

To deepen our understanding of the complex interactions among various cell populations, our study conducted an extensive analysis of the intercellular communications between these cell types. We discovered that epithelial and myeloid cells were exceptionally active in outgoing signals, highlighting their essential role in the networks of cellular communication. Conversely, myeloid, B, and T cells exhibited increased sensitivity to incoming signals, emphasizing their critical positions in the cellular response hierarchy (Fig. 6A and B).

Exploring the cellular mechanisms further, we found that myeloid cells play a pivotal role in regulatory functions, primarily through the

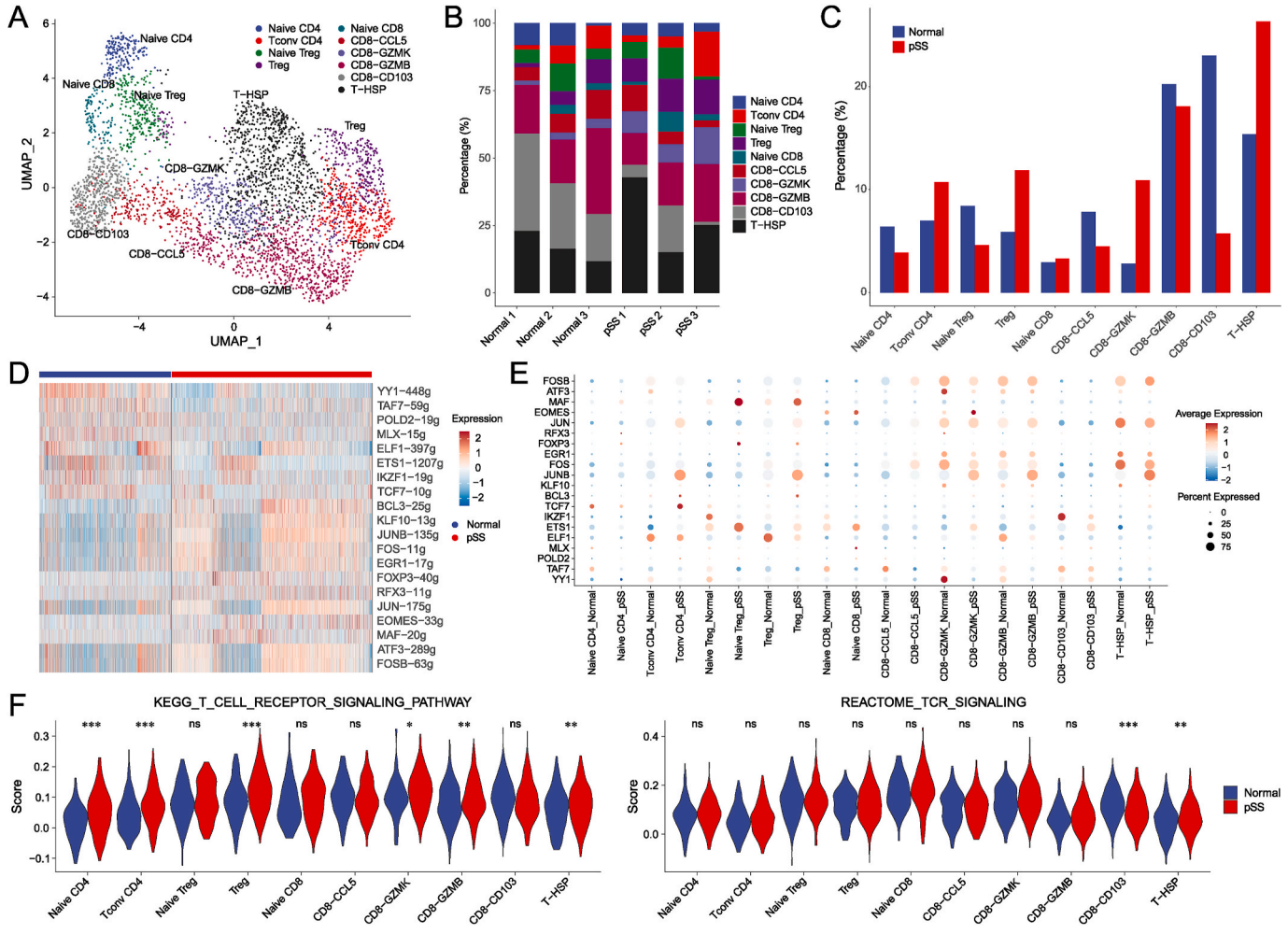

Fig. 5. Analysis of T cell subpopulations
(A) Two-dimensional UMAP visualization of 3924 T cells.
(B) Bar plot showing proportions of each cluster across each sample, color representing cell type.
(C) Bar plot comparing proportions of each cluster between control and pSS, color representing different group.
(D) Heat map displaying different regulons between control and pSS.
(E) Heat map displaying expressions of TFs in (D).
(F) Violin plot showing enriched pathways of TCR signaling among different subclusters.
Statistical analyses in (F) were performed using an unpaired, two-tailed Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

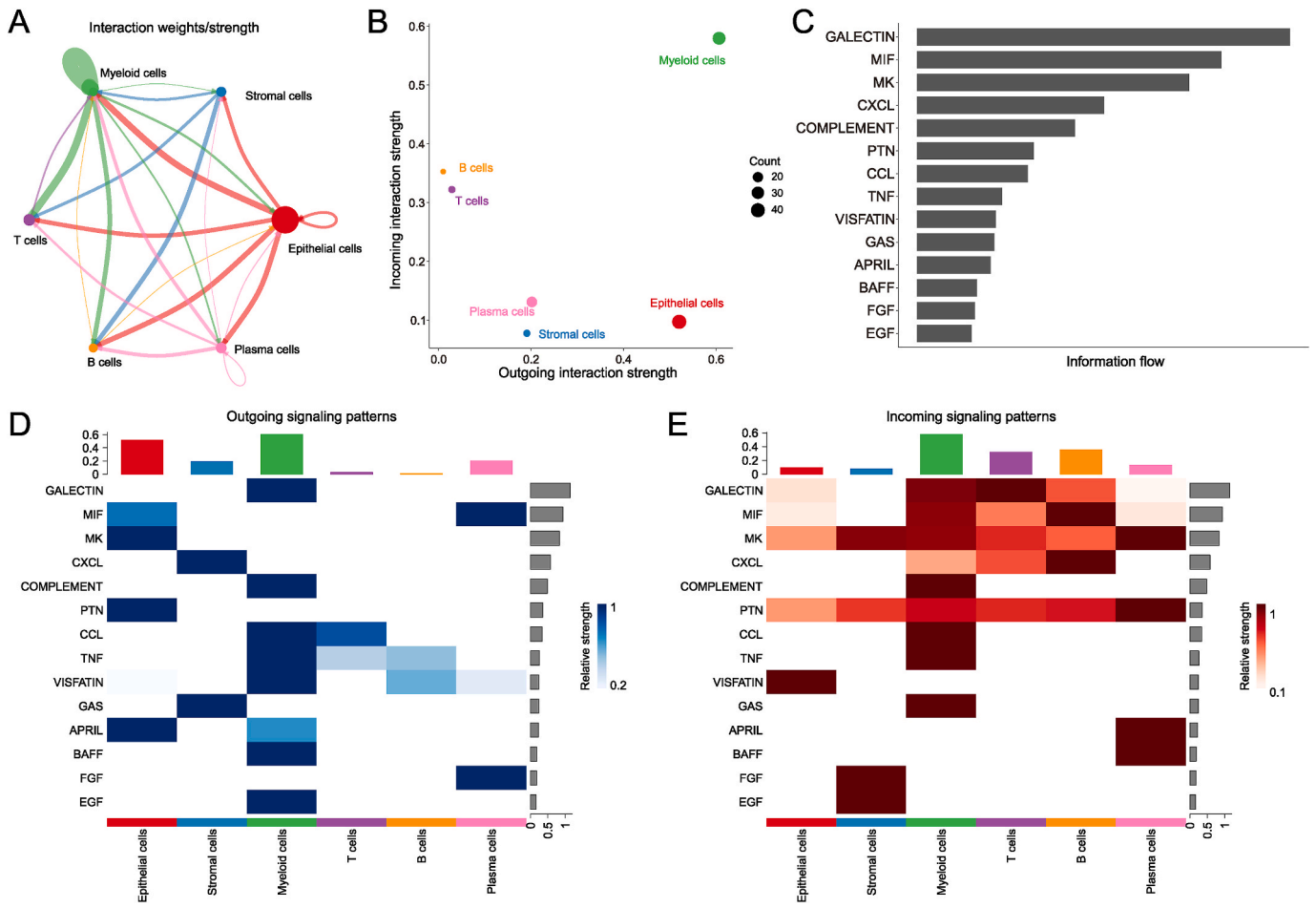

Fig. 6. Cell-cell communication among global populations
(A) Circle plot showing interactions among different clusters.
(B) Scatter plot displaying incoming and outgoing interaction strength among different clusters.
(C) Bar plot showing enriched signaling patterns.
(D) Heat map illustrating enriched outgoing patterns in different clusters.
(E) Heat map illustrating enriched incoming patterns in different clusters.

expression of Galectin, marking a vital pathway in cellular regulation (Fig. 6C and D). Additionally, epithelial cells were observed to exert a significant influence on various cellular functions, mainly through the secretion of proteins PTN, MIF, and MDK (Fig. 6C and D, Supplemental Figs. 3A and 3B). This suggests the existence of a complex network of signaling pathways orchestrating cellular activities.

Our study illuminated the biological significance of CXCL12 from stromal cells in amplifying chemotactic responses towards T and B cells, accentuating the influence of stromal-derived factors on immune cell migration (Fig. 6C to E, Supplemental Figs. 3C and 3D). Additionally, the roles of CCL5 from T cells and CCL3 from myeloid cells in facilitating myeloid cell aggregation were recognized, underscoring the collaborative nature of cellular interactions in modulating the immune response (Fig. 6C to E, Supplemental Figs. 3E and 3F).

Within the context of pSS, our research identified substantial changes in the interactions among cell populations, leading to a reorganized microenvironment. These alterations were associated with accelerated disease progression, emphasizing the biological consequences of these changed cellular interactions on the course of the disease. This underscores the critical need to understand these mechanisms in pathological conditions.

4. Discussion

Recent research has underscored the pivotal involvement of immune and stromal cells in the pathogenesis of pSS. Although scRNA-seq of PBMCs from pSS patients has delineated alterations in specific immune cell subsets, and recent single-cell transcriptomic explorations of salivary glands have enriched our understanding of pSS's evolution [12], investigations into labial gland tissues are comparatively sparse. This study employed single-cell transcriptomics on labial gland samples from three pSS patients and matched controls, elucidating the cellular dynamics within the pSS immune microenvironment. The analysis revealed epithelial cell dedifferentiation and significant activation of the IFNs pathway. Notably, an increase in myofibroblasts and endothelial cells in pSS patients was observed, implying an enhanced capability for mononuclear cell migration. Additionally, an elevation in immunosuppressive myeloid and dendritic cells was recorded. Enhanced $CD4^{+}$T cell activation and Treg cell proliferation were evident, suggesting intricate immune regulatory processes. The $CD8^{+}$T cell subset exhibited increased GZMK expression, indicative of heightened cytotoxic activity. The presence of T cells with elevated HSP levels suggested a stress response, likely related to continuous low-grade inflammation, characteristic of the disease's chronic nature. The findings also corroborated previous observations of increased B cell proportions in peripheral blood.

B cells are pivotal in the etiology and immune dysregulation of Sjögren's syndrome, with studies revealing distinct anomalies in B cell subsets among SS patients [24]. These include an expansion of atypical memory B cells, a decline in IgM memory B cells, and shifts in naive B cell populations. Furthermore, SS patients frequently demonstrate signs of B cell hyperactivation, evidenced by elevated numbers of circulating plasmablasts and transitional B cells [25]. The perturbation of B cells in SS correlates with the disease's clinical features, activity, and autoantibody profiles, shedding light on the diversity and molecular attributes of B cells in SS [26]. Elucidating these B cell abnormalities could enhance patient stratification, prognosis accuracy, and the crafting of bespoke therapeutic approaches for SS.

In Sjögren's syndrome, immune system dysfunction is epitomized by the aberrant activities of $CD4^+$T cells, particularly in the subsets of Treg cells and Th1 (T helper 1) cells. These cells are pivotal in producing IFN-γ, implicated in triggering ferroptosis in the salivary gland epithelial cells [27]. Prior research in pSS has underscored a pronounced hyperactivation of $CD4^+$T cells, as evidenced by the upregulated expression of chemokine receptors and activation markers such as CCR9, CXCR5, PD-1, and ICOS [28]. These molecules are integral to T cell migration, localization, and function, and their heightened levels reflect a persistent, robust immune response in pSS, contributing to the chronic inflammation and tissue damage characteristic of this disease. Our study supports the notion of increased $CD4^+$T cell activation in Sjögren's syndrome, consistent with previous observations. However, we noted a significant expansion in the Treg cell subset, a deviation from earlier reports that indicated a decrease or no change [29]. The discrepancy in Treg cell data may stem from our study's limited sample size. Therefore, further research is necessary to explore the changes in the Treg cell population and their significance in the context of Sjögren's syndrome.

In patients with pSS, our comprehensive analysis reveals a significant hyperactivation of $CD8^+$T cells, which plays a pivotal role in intensifying disease severity and contributing to tissue damage. Notably, this pronounced activation of $CD8^+$T cells was observed in both the bloodstream and glandular areas, establishing a clear link with glandular dysfunction and functional impairment [30]. Interestingly, while $CCR9^+CD8^+$T cells secreting CCL5 were suspected to contribute to the immunopathology of pSS [31], our findings reveal no significant change in the proportion of the CD8-CCL5 subset. This suggests that other mechanisms might be at play in mediating the disease's pathogenesis. Our data demonstrate a notable increase in $CD8^+$T cell activation, which is further characterized by an elevated expression of GZMK. Prior studies have highlighted that GZMK-expressing $CD8^+$T cells in pSS possess cytotoxic capabilities and interact extensively with activated epithelial cells in the affected glands, contributing to the pathological process [32]. Moreover, another study of T-cell receptor (TCR) clonality unveiled a striking similarity between peripheral blood $GZMK^+CXCR6^+CD8^+$T cells and labial gland $CD69^+CD103^-CD8^+$ tissue-resident memory (Trm) cells [22]. This resemblance suggests a shared cytotoxic profile and potential functional overlap between these cell populations, further underscoring the role of $CD8^+$T cells in the pathogenesis of pSS.

Type I interferons (IFN–I) play a critical role in the pathogenesis of pSS, driving innate immune responses and boosting interferon-stimulated gene expression [33–35]. Elevated IFN-I levels in the peripheral blood cells and exocrine glands of pSS patients underscore its significant influence on the disease trajectory [36]. In these patients, increased expression of STAT1, STAT2, and IRF9 in B cells reflects transcriptional changes closely tied to the clinical and serological manifestations of SS [37]. Additionally, the association between serum IFNα2 levels and the expression of interferon-stimulated genes in SS, systemic lupus erythematosus, and systemic sclerosis highlights the potential of IFN-centric therapeutic approaches for patient stratification and comprehensive retrospective analyses. This evidence reinforces the crucial role of Type I IFNs in SS pathophysiology. While Type II interferons (IFN-γ) have received less focus in SS research, with the spotlight on Type I IFNs, our data reveal enhanced IFN signaling in the epithelial and stromal cells of pSS patients and activation of $CD4^+$T and $CD8^+$T cells, which may be associated with IFNs regulation. However, the precise role of Type II IFNs in SS is still unclear, calling for more research to elucidate their possible influence on the disease.

Chemokines have emerged as significant diagnostic biomarkers due to their strong correlation with disease activity and glandular damage. Due to their ability to modulate inflammatory responses by inhibiting specific pathways, they are promising therapeutic targets. In pSS patients, the serum levels of key chemokines such as CXCL13, CXCL9, CXCL10, CXCL11, and CCL5, are markedly elevated, indicating their crucial role in the disease's progression and pathophysiology [31, 38–40]. Interestingly, our scRNA-seq data revealed a decrease in the proportion of $CCL5^+$T cells, which contrasts with the increased serum concentration of CCL5. Additionally, our research identified a significant increase in CXCL12 expression derived from stromal cells. CXCL12 likely affects the circulation of T and B cells through its receptors, CXCR4, thereby influencing immune cell migration and localization. This discovery underscores the critical role of stromal cells in regulating immune responses and suggests that they may become important targets for future therapeutic interventions.

This investigation delineated the functional roles of major immune cell populations within the labial gland, delineating the pathophysiological distinctions within pSS cohorts. Our research provides a preliminary cellular atlas for the labial gland for the pSS, providing novel avenues for therapeutic exploration and intervention in this disease.

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Ethics approval and consent to participate

The authors are accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved.

CRediT authorship contribution statement

Jun Huang: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Jia Tang:** Writing – original draft, Formal analysis, Data curation. **Chen Zhang:** Writing – original draft, Visualization, Formal analysis. **Tingting Liu:** Writing – original draft, Software, Formal analysis, Data curation. **Zhiyong Deng:** Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization. **Lei Liu:** Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2024.100248.

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