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Original Article

Potential different immune phenotypes of macrophages in oral lichen planus by integrating immunofluorescence double staining and single-cell RNA sequencing

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Abstract *Background/purpose:* Oral lichen planus (OLP) is a chronic inflammatory disease with obscure etiopathogenesis. Macrophages play an important role in interaction between innate and adaptive immunity. This study aimed to investigate the macrophage phenotypes and obtain more comprehensive gene characteristics of macrophages in OLP.
Materials and methods: Double cluster of differentiation (CD) 68/CD86 and CD68/CD206 immunofluorescence staining was conducted in 11 biopsy-proven OLP tissue samples and 5 health control (HC) to represent M1 and M2 macrophages, respectively. The number of positively stained cells was manually counted, and the density was calculated. Furtherly, OLP single-cell dataset GSE211630 was downloaded from Gene Expression Omnibus. Gene characteristics and functional analysis of the macrophages were elucidated.
Results: In the OLP group, the densities of M1 ($P < 0.001$), M2 macrophages ($P < 0.001$) and M1/M2 ratio ($P = 0.001$) were significantly higher than those in HC group. Single-cell RNA sequencing revealed that proportions of CXCL10 macrophages ($P = 0.003$), IL1B/MMP19 macrophages ($P < 0.001$) were increased in OLP tissues compared with those in HC. Macrophages in

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OLP tissues had a stronger ability to cell chemotaxis, positive regulation of cell adhesion and antigen processing and presentation. Functional analysis revealed macrophages in OLP tissues could interact with multiple immune cells, and multiple signaling pathways were associated with macrophages in OLP.
Conclusion: A pro-inflammatory status of macrophages with different gene characteristics was found in the microenvironment of OLP by integrating immunofluorescence double staining and single-cell RNA sequencing, which provided a potential target for clinical treatment of OLP.

Introduction

Oral lichen planus (OLP) is a chronic inflammatory oral mucosal disease, which affects 1–2% population of the world.[1,2] Clinically, the lesions of OLP may separately or combinatorially manifest as six different forms, including reticular, plaque, papular, atrophic/erosive, ulcerative, and bullous.[3] Patients with erosive OLP usually suffer pain in the oral cavity, which affects their daily life. In addition, OLP belongs to category of oral potentially malignant disorders, with malignant transformation rate 1.4%.[4] In the development of OLP, both innate and adaptive immune responses are involved, however, the underlying mechanism remains obscure.

Macrophage is one of the cell types that play an important role in interacting between innate and adaptive immunity. As phagocytic cells, macrophages can act as the first-line defense against antigens in innate immunity.[5] Besides, macrophages are one of the antigen-presenting cells and can be recognized by T cells. Traditionally, macrophages can be differentiated into different phenotypes after exposure to different stimuli. Pro-inflammatory M1 macrophages express cluster of differentiation (CD) 68 and CD86 as surface markers while immunosuppressive M2 macrophages usually have increased expression of CD68, CD163 and CD206.[6] In the microenvironment of OLP, monocytes recruited into the lesion develop into pro-inflammatory M1 phenotype due to the high levels of granulocyte macrophage colony-stimulating factor, tumor necrosis factor-α (TNF-α) and interferon-gamma (IFN-γ).[5] However, it was indicated that no significant difference of the proportions of M1 macrophages was found between OLP and normal samples in a recent study, in which data from public databases were analyzed.[7] Moreover, the gene signature of the macrophages remain unclear.

In the past decade, single-cell RNA sequencing (scRNA-seq) has contributed to revealing the transcriptome expression profiles of various types of cells and provided a deeper understanding of inflammatory diseases with unclear pathogenesis.[8,9] In the studies on OLP, Li et al. found macrophages exhibited pro-inflammatory activity and illustrated the immune ecosystem of OLP through scRNA-seq.[10] Qing et al. revealed a novel molecular mechanism for triggering OLP erosion using scRNA-seq along with spatial transcriptomics.[11] However, the gene characteristics of macrophages in these studies had not been deeply explored.

In this study, we aimed to investigate the macrophage phenotypes in OLP using immunofluorescence double staining and obtain more abundant gene characteristics of macrophages through scRNA-seq data. Our findings might provide potential therapeutic targets for OLP.

Materials and methods

Study population

The study was independently reviewed and approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (approval ID: SH9H-2019-T174-2) and carried out following the principles of the Declaration of Helsinki. All patients provided written informed consent before their participation.

We enrolled 11 biopsy-confirmed OLP patients who came to the Department of Oral Medicine, Shanghai Ninth People's Hospital. Inclusion criteria were (1) age 18–80 years; (2) clinically diagnosed and biopsy-confirmed OLP according to the modified world health organization diagnostic criteria.[12] Exclusion criteria were (1) patients who received topical or systemic treatment for OLP within 3 months; (2) with autoimmune diseases; (3) with clear oral contact lichenoid reaction; (4) with suspected oral lichenoid drug eruptions; (5) with serious allergic diseases; (6) with serious systemic disease; (7) with a history of organ or bone marrow transplantation; (8) with infection of oral mucosa; (9) with mental and psychological illness. Lesional residual tissues after biopsy were sectioned at 4-μm thickness for immunofluorescence staining. In addition, we collected 5 normal oral mucosa tissues without infection from age- and gender-matched participants as HC.

Immunofluorescence study and evaluation of staining

Referring to previous studies,[13,14] double CD68 (Cell Signaling Technology, Danvers, MA, USA)/CD86 (Cell Signaling Technology) and CD68/CD206 (Abcam, Cambridge, MA, USA) immunofluorescence staining was performed to represent M1 and M2 macrophages, respectively. Three fields of view at superficial lamina propria, which manifested as band-like lymphocytic infiltration in OLP samples, were randomly selected in each section. Consequently, all nucleated cells with double-positive staining for the phenotype marker M1 ($CD68^+/CD86^+$) or M2 ($CD68^+/CD206^+$) in each image were counted manually by two researchers independently. When the judgments of two researchers were different, the third researcher made the

decision. Density of M1 and M2 macrophages was calculated as the number of positively stained cells per square millimeter (cells/mm^2) in the region of interest. All investigators performing measurements were blinded to patients' clinical data.

Raw data collection, processing and differential gene expression analysis

The single-cell RNA-seq data GSE211630 from 5 OLP samples and 1 normal oral mucosa sample were downloaded from the Gene Expression Omnibus database. We applied fastp with default parameter filtering the adaptor sequence and removed the low-quality reads, which included cells with less than 200 expressed genes or cells with more than 20% of mitochondrial expression, to achieve the clean data.[15]

Seurat package version 4.1.1 was used for cell normalization and regression based on the expression table according to the unique molecular identifiers counts of each sample and percent of mitochondria rate to obtain the scaled data. Principal component analysis was constructed based on the scaled data with top 2000 high variable genes, and top 10 principals were used for t-distributed stochastic neighbor embedding construction and uniform manifold approximation and projection (UMAP) construction. The marker genes and differentially expressed genes (DEGs) were identified by "FindAllMarkers" function with Wilcox rank sum test algorithm under following criteria: (1) $\log_2$ fold change $>$ 0.25; (2) Possibility (*P*) value $<$ 0.05; (3) minimal percentage $>$ 0.1.

Gene ontology (GO) and pathway analysis

To elucidate the biological implications of the DEGs and marker genes, GO analysis was performed. GO annotations were downloaded from NCBI, the Gene Ontology database and UniProt. Pathway analysis was used to explore the significant pathways of the DEGs and marker genes based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Pseudotime analysis

Single-cell trajectory analysis was performed with Monocle 2 to determine the dramatic translational relationships among cell types and clusters. Before Monocle analysis, marker genes of the Seurat clustering result and raw expression counts of the cell that passed filtering were selected. Branch expression analysis modeling was utilized for branch fate-determined gene analysis based on pseudotime analysis.

Statistical analysis

The results of continuous variables were presented in the form of means $\pm$ standard deviations or median (interquartile range, IQR) where appropriate. Chi-square or Fisher exact probability test was used for calculating the differences of clinical data of participants. Fisher's exact test was applied to identify the significant GO categories and KEGG pathways. SPSS 26.0 software (International Business Machines Corporation, Armonk, NY, USA) was used to analyze the data. Graph Prism 9 (GraphPad Software, San Diego, CA, USA) was used to illustrate graphic figures. The significance threshold was set at probability (*P*) value $<$ 0.05.

Results

Participant characteristics

The mean age of OLP patients was 52.36 $\pm$ 16.21 years, ranging from 27 to 74 years with a female predominance (63.64%, n = 7). Ten (90.91%) OLP samples were derived from buccal mucosa and 1 (9.09%) was from ventral tongue. Five participants (45.45%) manifested as erosive OLP. Demographic and clinical characteristics of OLP and HC participants are listed in Table 1.

Density of M1 and M2 macrophages between OLP and HC

Cohen's kappa value was 0.92, which represents an almost perfect level of inter-rater agreement. Representative examples of CD68$^+$/CD86$^+$ and CD68$^+$/CD206$^+$ immunofluorescence staining were shown in Fig. 1A–H. In the OLP group, the median density of CD68$^+$/CD86$^+$ M1 macrophages was 373 cells/mm^2 (IQR: 281, 710), which was significantly higher than that in HC group ($P < 0.001$, Fig. 1I). Furthermore, we observed a significantly higher density of CD68$^+$/CD206$^+$ M2 macrophages, with a mean of 223 $\pm$ 46 cells/mm^2, in OLP samples compared to that in HC (31 $\pm$ 34 cells/mm^2, $P < 0.001$, Fig. 1J). The results also showed a median M1/M2 ratio of 1.67 (IQR: 1.21, 3.41) for OLP and 0 (IQR: 0, 0.75) for the control group ($P = 0.001$, Fig. 1K).

Proportions of different cell types in OLP and normal samples through scRNA-seq

Visualization using UMAP revealed 23 distinct cell clusters (Fig. 2A) that were annotated as 11 major cell types

Table 1 Demographic and clinical characteristics of the OLP and HC participants.

	OLP (n = 11)	HC (n = 5)	*P*-value
Age at biopsy (years)			
Range	27–74	18–61	
Mean $\pm$ standard deviations	52.36 $\pm$ 16.21	38.20 $\pm$ 16.04	0.127
Gender			
Male	4	1	1.000
Female	7	4	
Smoking	1	0	1.000
Alcohol	1	0	1.000
Biopsy site			
Buccal mucosa	10	5	1.000
Tongue	1	0	

OLP, oral lichen planus; HC, health control.

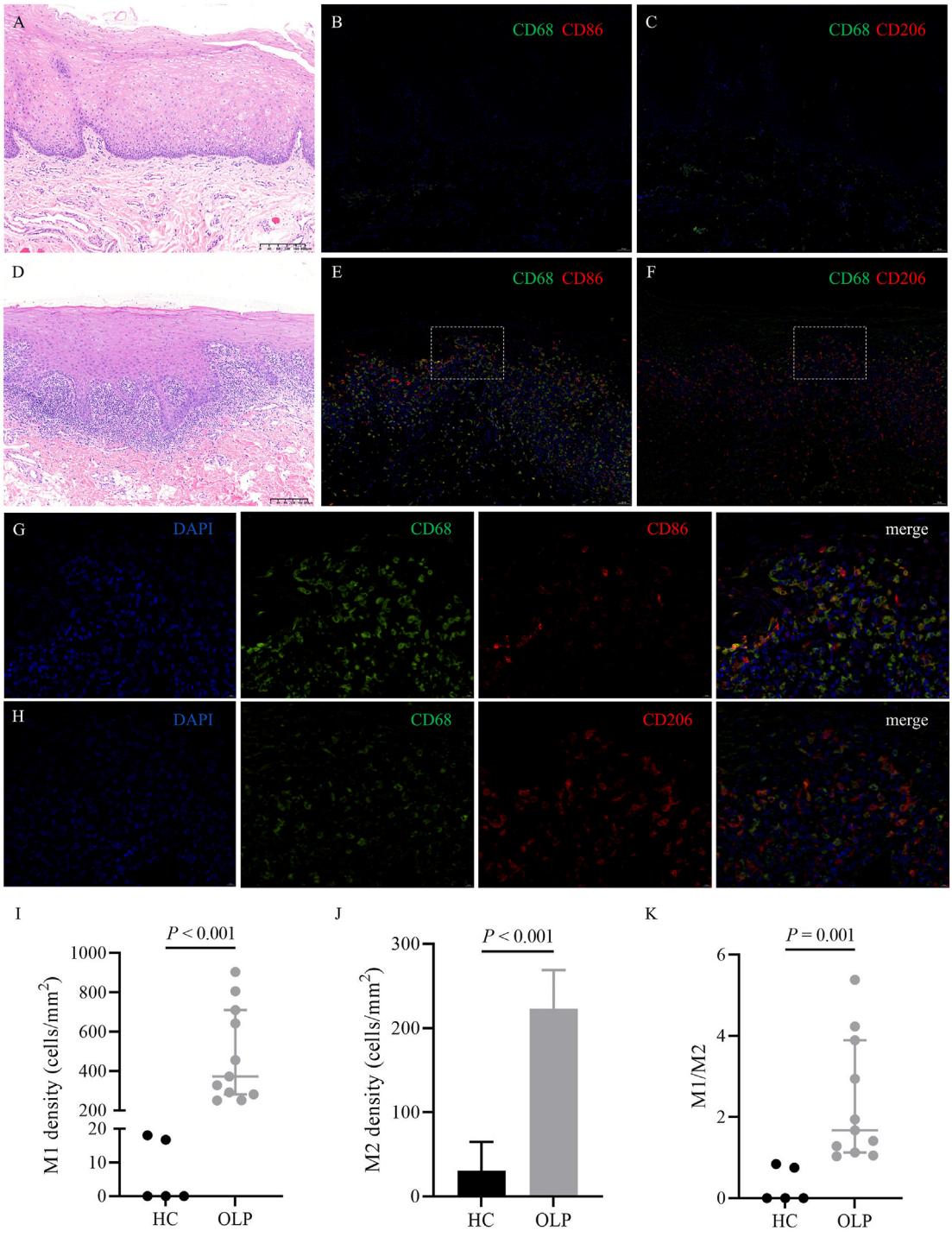

Figure 1 Identification of the density of M1 and M2 macrophages in OLP and HC tissues using immunofluorescence double staining. A – C, HC sample. A, hematoxylin and eosin staining (100 ×). B, CD68/CD86 immunofluorescence double staining (200 ×). C, CD68/CD206 immunofluorescence double staining (200 ×). D - H, OLP sample. D, hematoxylin and eosin staining (100 ×). E, CD68/CD86 immunofluorescence double staining (200 ×). F, CD68/CD206 immunofluorescence double staining (200 ×). G, enlargement of dashed box in panel E. H, enlargement of dashed box in panel F. I, comparison of M1 density in OLP and HC. J, comparison of M2 density in OLP and HC. K, comparsion of M1/M2 ratio in OLP and HC. OLP, oral lichen planus. HC, health control. CD, cluster of differentiation.

(Fig. 2B–C) based on the marker gene expression (Fig. 2D). In total, 11,446 and 40,507 cells were obtained from normal and OLP tissues, respectively. The proportions of T cells were observed to be elevated in OLP samples (63.60%) compared with those in normal tissue (22.03%, $P < 0.001$, Fig. 2E). The cell composition analysis also revealed an

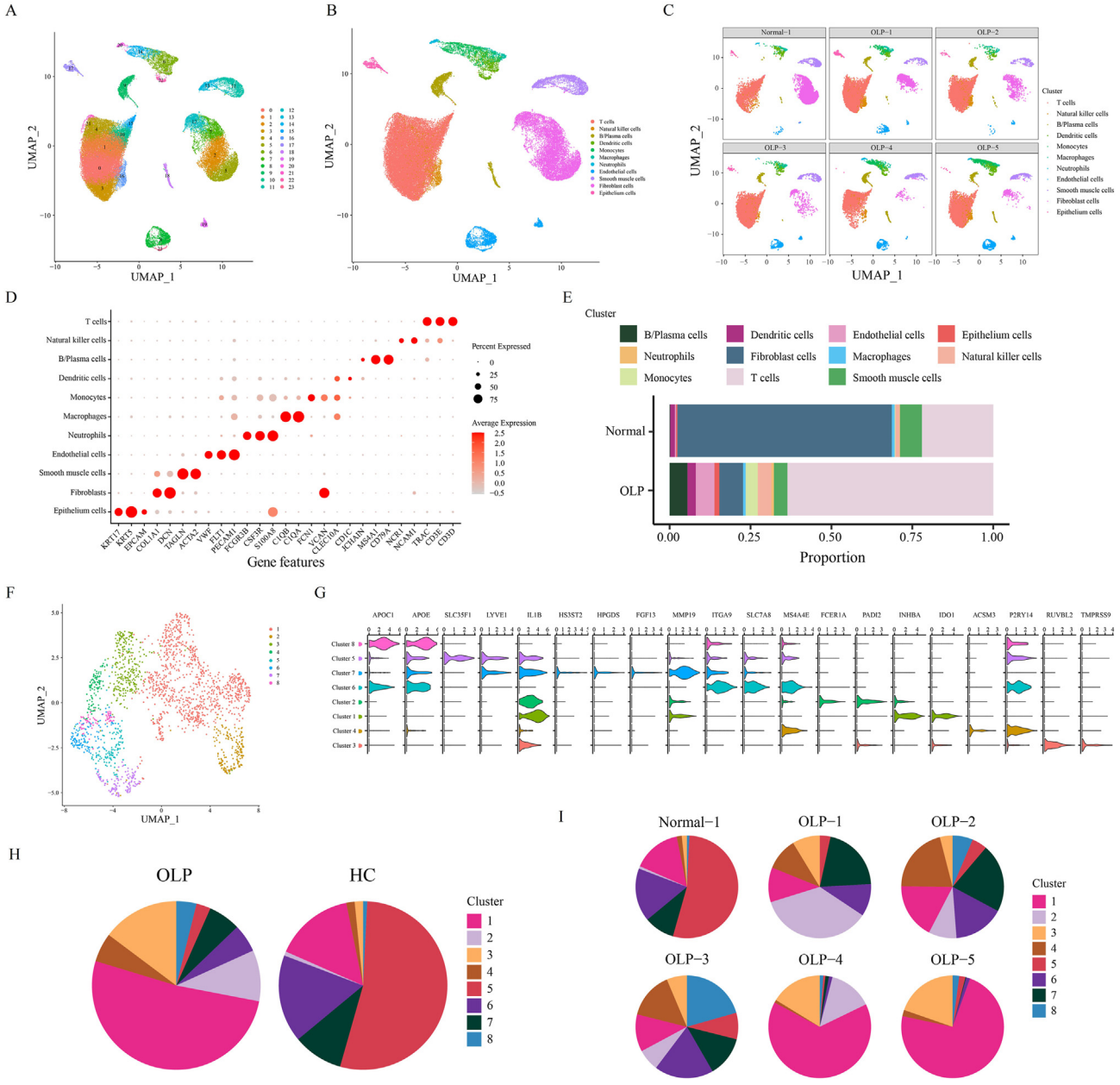

Figure 2 Screening process for monocytes and macrophages in OLP and normal samples. A, the UMAP for all cell clusters. B, the UMAP for all cell types. C, the UMAP showing cell origins from each subject. D, the dot plot showing the expression of marker genes for each cell type. E, the proportion of each cell type in OLP and normal samples. F, the UMAP for monocytes and macrophages. G, violin plots showing the expression levels of cell markers in each cluster of monocytes and macrophages. H, pie chart showing the proportion of each cluster of monocytes and macrophages in OLP and normal samples. I, pie chart showing the proportion of each cluster of monocytes and macrophages in each subject. OLP, oral lichen planus. UMAP, uniform manifold approximation and projection.

increase in the proportions of B/Plasma cells ($P < 0.001$), dendritic cells ($P < 0.001$), endothelial cells ($P < 0.001$), epithelium cells ($P < 0.001$), natural killer cells ($P < 0.001$) and monocytes ($P < 0.001$) in OLP (Fig. 2E). Even though the proportion of macrophages was not significantly different in OLP ($P = 0.620$), the number of macrophages (338 cells) in OLP tissues was more than 3-fold that in normal tissue (101 macrophages).

Screening of scRNA-seq for monocytes and macrophages in OLP and normal samples

We annotated monocytes and macrophages with marker genes, 8 cell subpopulations were described: cluster 1, interleukin 1B (IL1B) monocytes; cluster 2, CXCR4 monocytes; cluster 3, TMPRSS9 monocytes; cluster 4, MS4A4E monocytes; cluster 5, IL1B macrophages; cluster 6, MS4A4E

macrophages; cluster 7, IL1B/matrix metalloproteinase 19 (MMP19) macrophages; cluster 8, CXC-chemokine ligand 10 (CXCL10) macrophages (Fig. 2F–G). The highest proportion in normal tissue was IL1B macrophages (53.6%), while in OLP samples, the main cell subpopulation was IL1B monocytes (51.67%, Fig. 2H–I).

Proportions of subpopulations of macrophages in OLP and normal samples

Further annotations of macrophages revealed 4 cell subpopulations with markers: CXCL10 macrophages (cluster 1), IL1B macrophages (cluster 2), IL1B/MMP19 macrophages (cluster 3) and MS4A4E macrophages (cluster 4, Fig. 3A–B). Increased proportions of CXCL10 macrophages ($P = 0.003$), IL1B/MMP19 macrophages ($P < 0.001$) and decreased proportion of IL1B macrophages ($P < 0.001$) were observed in OLP. The proportion of MS4A4E macrophages ($P = 0.083$) was not found statistically different between OLP and normal tissues (Fig. 3C).

The psuedotime analysis suggested that macrophages had two different cell fates (Fig. 3D–E). A higher proportion of macrophages in OLP would enter fate 1, in which CXCL10 macrophages as well as IL1B/MMP19 macrophages were increased (Fig. 3F), indicating a pro-inflammatory condition of macrophages in OLP.

Gene characteristics and functional analysis of macrophages in OLP and normal samples

Featured markers of macrophages revealed that different markers were in different clusters. TNF was mainly expressed on cluster 3 and 4 of macrophages; Interferon regulatory factor 1 (IRF1) was expressed on all the four clusters; CXCL9 and CXCL10 were mainly expressed on cluster 1; Inhibin β-A (INHBA), a member of the transforming growth factor-β superfamily, was mainly expressed on cluster 4 (Fig. 3G). Analysis of DEGs demonstrated 1999 upregulated and 449 downregulated genes in OLP samples. The top 5 upregulated genes were CXCL9, APOE, APOC1, CCL18 and CXCL10 (Fig. 3H). In addition, macrophages in OLP tissues had a stronger ability to cell chemotaxis, positive regulation of cell adhesion and antigen processing and presentation (Fig. 3I).

Upon conducting GO analysis, we found that macrophages in OLP were significantly enriched in interaction with multiple immune cells and cytokine production (Fig. 3J). KEGG pathway analysis revealed multiple signaling pathways, including Fc gamma receptor-mediated phagocytosis, antigen processing and presentation and TNF signaling pathway, were associated with macrophages in OLP (Fig. 3K).

Discussion

In this study, we observed that M1, M2 macrophages and M1/M2 ratio were elevated in OLP tissues compared with HC. To our knowledge, it was for the first time that double immunofluorescence staining of macrophages was used in OLP samples. Furthermore, the diversity of subpopulations and gene characteristics of pro-inflammatory macrophages in OLP was uncovered at the single-cell level, which provided a potential target for clinical management of OLP.

In the previous studies, CD163 was used to represent M2 macrophage.[16,17] To reduce the non-specificity caused by single staining, we switched to double fluorescent staining to label different macrophage phenotypes in this study. As expected, we found in OLP tissues that density of pro-inflammatory M1 macrophages was increased compared with that in HC. Besides, density of M2 macrophages was also elevated in OLP tissues. Similarly, in addition to M1 macrophages, M2 macrophages also exerted pro-inflammatory functions in the process of systemic lupus erythematosus.[18] In the context of rheumatoid arthritis, M2 macrophages functionally exhibited an inflammatory response similarly to that of M1 macrophages when simultaneously exposed to specific immune complex Toll-like receptor ligands.[19] Meanwhile, increasing evidence suggested that Toll-like receptor-mediated signaling pathways influenced the inflammatory response in OLP.[20] Regardless, the ratio of M1/M2 was higher in OLP tissues than in HC tissues in our study. This finding indicates that the pro-inflammatory macrophages are still predominated in OLP.

In this study, we found that the proportion of macrophages was not elevated in OLP tissues compared with that in HC through scRNA-seq data analysis, which might be related to the small number of total cells in HC tissue and the presence of resident tissue macrophages with immunosurveillance function. Our results also demonstrated that the number of macrophages and the proportion of monocytes in OLP tissues were higher than those in HC tissues. Due to the high levels of granulocyte macrophage colony-stimulating factor, TNF-α and IFN-γ at the OLP lesion, monocytes recruited into the lesion would develop a pro-inflammatory M1 phenotype in the OLP microenvironment,[5] indicated that activation of macrophages was essential in the pathogenesis of OLP. Therefore, studies on subpopulations, gene expression and functional analysis of macrophages were furtherly carried out.

In our analysis of macrophages, increased proportions of CXCL10 macrophages and IL1B/MMP19 macrophages were observed in OLP tissues. As a T-helper 1-chemokine, CXCL10 expression was reported to be significantly increased in OLP patients.[21] The IFN-γ/CXCL10 axis was a therapeutically attractive target to reverse inflammation of lichen planus.[22] Unlike CXCL10, MMP19 has rarely been studied in OLP. Beck et al. found that MMP19 was essential for T cell development and T cell-mediated cutaneous immune responses.[23] The function of MMP19 of the macrophages in the pathogenesis of OLP needs to be furtherly studied.

In this study, we downloaded the single-cell sequencing data GSE211630 from public databases, in which 3′ end library construction strategy was used. Recently, in another published single-cell sequencing study, which included 4 paired OLP tissues and peripheral blood, 2 tissues and 3 peripheral blood samples from HC, 5′ end library construction strategy was used.[10] In addition, the reagent versions were different from the data we used in this study. Therefore, we did not combine the two datasets for analysis. The results in that study indicated the proportion of macrophages in myeloid cells was lower in OLP tissues compared with that in HC tissues. Macrophages in OLP

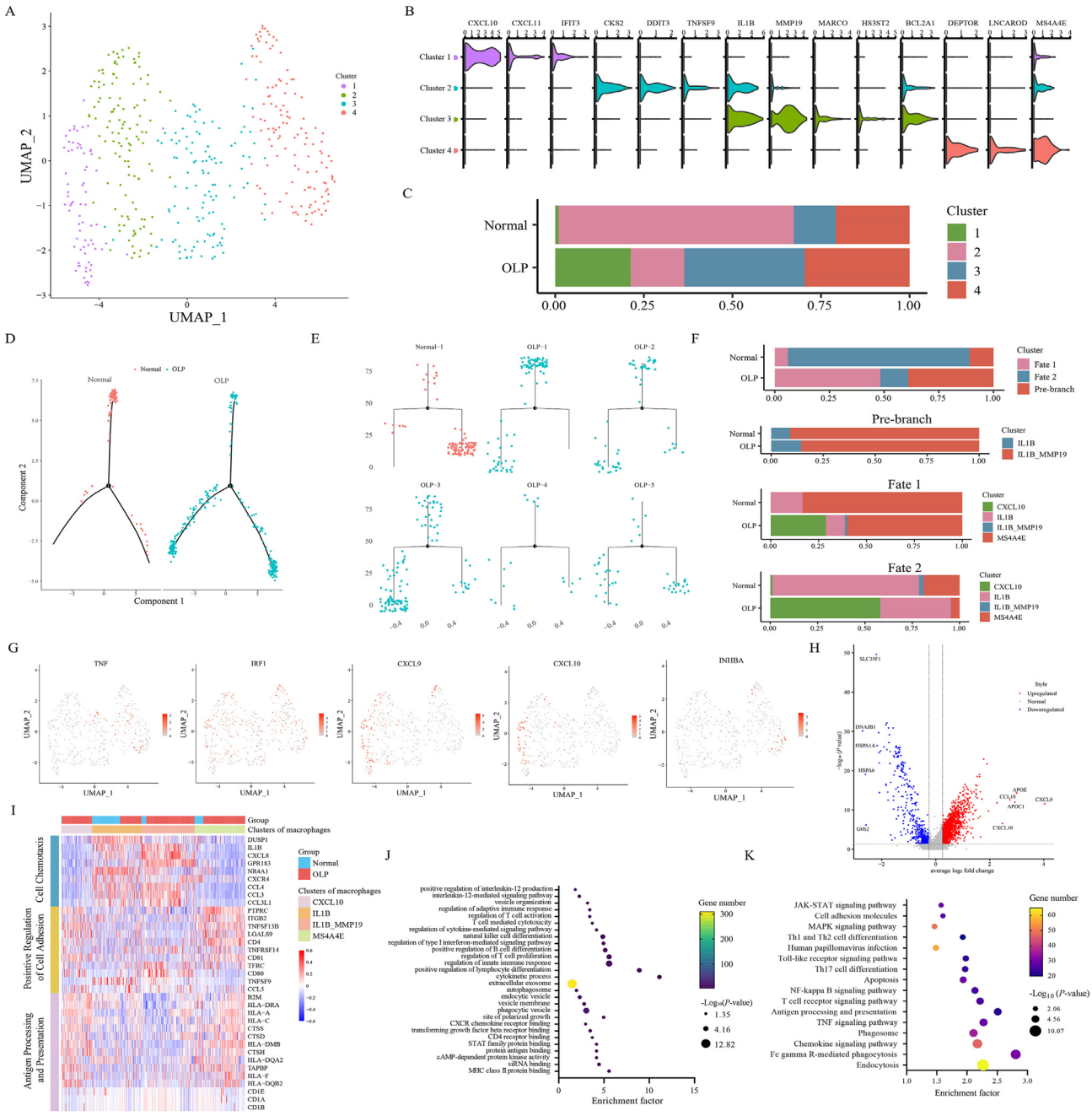

Figure 3 Gene characteristics and functional analysis of macrophage subpopulations in OLP and normal samples. A, the UMAP for macrophages. B, violin plots showing the expression levels of cell markers in macrophages. C, the proportions of clusters. D, trajectory analysis of macrophages in normal and OLP tissues. E, pseudotime tree of macrophages in each subject. F, the proportion of macrophages at different pseudotime states. G, the UMAP plot showing the expression distribution of markers. H, the volcano plot showing the DEGs between OLP and normal tissues. I, the heatmap showing expression levels of pathway-enriched genes upregulated in OLP tissues. J, the bubble plots of GO enrichment analysis of DEGs. K, the bubble plots of KEGG pathway analysis showing the macrophages-associated activated pathways in OLP tissues. OLP, oral lichen planus. UMAP, uniform manifold approximation and projection. DEGs, differentially expressed genes. GO, gene ontology. KEGG, the Kyoto Encyclopedia of Genes and Genomes. IL1B, interleukin 1B. CXCL10, CXC-chemokine ligand 10. MMP19, matrix metalloproteinase 19.

tissues also had a strong ability to positive regulation of cell adhesion and antigen processing and presentation,[10] which was consistent with the results of this study.

A few limitations exist for the current study. Firstly, despite the use of immunofluorescence double staining with CD68/CD86 to represent M1 macrophages, non-specificity was still inevitable. For instance, M2b macrophages, a type of regulatory macrophages induced by combination of immune complex with Toll-like receptor agonists or interleukin-1 receptor agonists, also expressed

CD86 and produced pro-inflammatory cytokine.[24] Secondly, only one dataset from public database was analyzed, which might result in bias. More objective and accurate data might be obtained from more published studies on OLP using scRNA-seq in future. Thirdly, scRNA-seq was just a bioinformatics method, and the gene characteristics and activated pathways of macrophages from scRNA-seq data needed to be verified *in vitro* and *in vivo*.

In conclusion, within the limitations of our study, it demonstrated a pro-inflammatory status of macrophages with different gene characteristics in the microenvironment of OLP by integrating immunofluorescence double staining and scRNA-seq, which provided a potential target for clinical treatment of OLP.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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