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ORIGINAL RESEARCH

Single-Cell RNA Sequencing Reveals Peripheral Immune Cell Senescence and Inflammatory Phenotypes in Patients with Premature Ovarian Failure

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Background: Premature Ovarian Failure (POF) is a heterogeneous syndrome characterized by ovarian dysfunction, frequently associated with autoimmune factors. The interaction between peripheral and ovarian immune signals remains unclear. Recent advancements in single-cell technology provide a unique opportunity to examine the complex peripheral immune response in POF patients at the microstructural level. This study investigates the immune microenvironment's complexity through the interaction between peripheral and ovarian local immune responses.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from three healthy individuals and four POF patients. Single-cell RNA sequencing (scRNA-seq) was used to delineate cell clusters and identify differentially expressed genes (DEGs). Enrichment, SCENIC, and pseudo-time analyses were utilized to explore cellular phenotype diversity, regulatory patterns, and evolutionary trajectories. A POF mouse model was used for validation.

Results: Seven clusters were identified and classified into two groups. POF patients exhibited increased proportions in T cells, NK cells, and B cells as well as upregulated IGLC2, GNLY, GZMB, FCGR3A, and CCL5 expressions compared to healthy controls. Monocytes, particularly non-classical monocytes, exhibited inflammatory phenotypes. $CD8^+$ Effector T cells demonstrated increased cytotoxicity and TCR clonal expansion. The trajectory of $CD8^+$ Effector T cells in POF patients involved the synchronous upregulation of cytotoxic-related genes and immune checkpoint molecules. Notably, CCL5, primarily produced by non-classical monocytes, emerged as a critical factor. Elevated levels of CCL5 in plasma and local ovaries, along with increased $CD8^+$ T cell infiltration, suggested its potential role in chemotaxis and ovarian damage in POF. Validation in the POF mouse model further supported these findings.

Conclusion: In summary, this study provides in-depth insights into the immune landscape of POF, revealing distinct cell populations, pathways, and signaling networks linked to the disease. These findings enhance our understanding of POF's immunological mechanisms, contributing to the development of potential diagnostic and therapeutic strategies.

Keywords: premature ovarian failure, immune microenvironment, single-cell RNA sequencing, monocytes, $CD8^+$ effector T cells, CCL5

Introduction

Premature Ovarian Failure (POF) represents a condition in which ovarian function prematurely declines in women under 40 years old.[1] This decline culminates in diminished ovarian hormone production, reduced ovarian follicle reserves, and

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early onset of menopause.[2] Consequently, POF can result in subfertility or infertility due to hormonal imbalances, menstrual irregularities, and challenges associated with pregnancy. Moreover, it manifests menopausal symptoms and increases the risk of long-term health complications.[3] Diagnosis is frequently delayed due to the presence of mild symptoms and limited awareness, underscoring the importance of distinguishing POF from other conditions, particularly when it transpires prior to the first menstrual period.[4] Despite recent advances, the mechanisms underlying ovarian dysfunction remain largely elusive.

POF exhibits a multifactorial etiology, encompassing a range of factors such as idiopathic causes, X-chromosome abnormalities, autosomal genetic disorders, radiation therapy, infections, immunosuppressive drugs, and autoimmune oophoritis.[1,4,5] Approximately 1–2% of women experience POF, with autoimmunity accounting for around 4–30% of POF cases. The human ovary is a frequent target of autoimmune attacks, resulting in ovarian dysfunction manifested by POF. In POF patients, evidence for autoimmune causes relates to the presence of lymphocytic oophoritis, association with other autoimmune disorders, or the detection of autoimmune antibodies. Autoimmune oophoritis can be diagnosed in women exhibiting histological inflammatory features in ovary biopsy and circulating ovarian and/or adrenal autoantibodies.[6–8] Autoimmune oophoritis is characterized by a mononuclear inflammatory cell infiltrate in the theca cells of growing follicles, with early-stage follicles lacking lymphocytic infiltration. This infiltrate comprises plasma, B, and T cells.[8]

In a mouse model of cyclophosphamide (CTX)-induced POF, a significant number of immune cells infiltrate ovarian tissue. Mesenchymal stem cells (MSCs) restore ovarian function by regulating the Th1/Th2 balance and modulating natural killer (NK) cell-mediated responses.[9] Related studies suggest that MSCs acquire immunoregulatory properties by influencing the differentiation of macrophages and T cells and controlling cytokines in POF.[10] Numerous recent investigations have demonstrated a correlation between the onset of POF and the upregulation of specific inflammatory factors. Conversely, the restoration of impaired ovarian function is accompanied by a concomitant decrease in the levels of these inflammatory factors. There is a growing consensus that inflammatory aging contributes significantly to the pathogenesis of POF. As such, managing the progression of inflammatory aging presents a potential therapeutic approach for addressing POF.[11]

Next-Generation Sequencing (NGS) has facilitated the identification of numerous candidate genes for Premature Ovarian Failure (POF). However, only a limited number of genes have been confirmed to cause POF, and no single factor has been able to fully account for the complexity of the condition.[12] In contrast to bulk sequencing, single-cell RNA sequencing (scRNA-seq) unveils distinctive transcriptional signatures within diverse cell types and subsets present in intricate tissues and peripheral blood.[13] In the realm of autoimmune disease research, this technology proves invaluable for elucidating disease-related alterations in immune cell function at a granular level. In the field of autoimmune disease research, this technology proves instrumental in elucidating disease-related alterations in immune cell function at a granular level.[14] Employing cutting-edge technology, notable changes in the composition of immune cells within the ovary as individuals age were demonstrated, indicating a shift toward adaptive immunity. Additionally, the study observed a decrease in the expression of inflammatory mediators concomitant with an increase in the expression of receptors recognizing senescent cells.[15] In a parallel investigation, the potential association between cellular immune dysfunction and idiopathic POF was confirmed by investigating the characteristics of peripheral blood mononuclear cells (PBMC) obtained from POF patients.[16] The latest study on single-cell RNA sequencing and spatial transcriptomics to characterize human ovarian aging suggests that DNA damage response in age-related genes may be a key biological pathway for oocyte aging. FOXP1 is a regulatory factor for ovarian aging, which decreases with age and inhibits the transcription of CDKN1A. Silencing FOXP1 can lead to ovarian dysfunction in mice.[17] Comprehensive research is still needed to understand the inflammatory phenotype changes exhibited in peripheral immune status and their relationship with the aging ovarian microenvironment.

Materials and Methods

Participants

Our research complies with the Declaration of Helsinki. Approval for the recruitment of participants and sample collection in our research was obtained from the institutional ethics review board of the Third Affiliated Hospital of Shenzhen University and Dongguan Maternal and Child Health Care Hospital (GDFY202041). All participants signed written informed consent forms. All participants, aged between 30 and 40 years, were recruited from the Third Affiliated

Hospital of Shenzhen University and Dongguan Maternal and Child Health Care Hospital between June 2022 and October 2023. According to the European Society of Human Reproduction and Embryology (ESHRE) and Chinese guidelines, the eligibility criteria for POF encompassed secondary amenorrhea for at least four months and serum basal Follicle Stimulating Hormone (FSH) levels exceeding 25 IU/L (on two separate occasions with an interval of more than one month) prior to the age of 40 years. Women presenting with chromosomal abnormalities, known gene mutations, a history of ovarian surgery, radiotherapy or chemotherapy, recurrent spontaneous abortion, endometriosis, autoimmune diseases, and infection within three months before enrollment were excluded. Age: Controls (mean ~34 years) vs POF (~33.5 years) – comparable. BMI: Controls (~19.6) vs POF (~19.2) – no major differences. FSH: Markedly elevated in POF (consistent with diagnosis). No significant differences in age, BMI, or lifestyle factors were observed between groups ($p > 0.05$, Student's *t*-test), except for FSH levels ($p < 0.001$), consistent with POF diagnosis. The detailed clinical characteristics of 4 patients with POF and 3 healthy controls are presented in Table 1.

Single-Cell RNA and TCR Sequencing

Single-cell suspensions with a concentration of 1×10^5 cells/mL in PBS were prepared. These suspensions were subsequently loaded onto microfluidic devices, and scRNA-seq libraries were constructed in accordance with the Singleron GEXSCOPE protocol using the GEXSCOPE Single-Cell RNA Library Kit (Singleron Biotechnologies).[18] Expression matrix files for subsequent analyses were generated based on gene counts and UMI counts.[19]

Quality Control, Dimension-Reduction and Clustering (Scanpy)

Scanpy v1.8.2 was used for quality control, dimensionality reduction and clustering under Python 3.7.[20] For each sample dataset, we filtered expression matrix by the following criteria: 1) cells with gene count less than 200 or with top 2% gene count were excluded; 2) cells with top 2% UMI count were excluded; 3) cells with mitochondrial content > 10% were excluded; 4) genes expressed in less than 5 cells were excluded.

Differentially Expressed Genes (DEGs) Analysis (Scanpy)

To identify differentially expressed genes (DEGs), we used the scanpy.tl.rank_genes_groups() function based on Wilcoxon rank sum test with default parameters, and selected the genes expressed in more than 10% of the cells in either of the compared groups of cells and with an average log (Fold Change) value greater than 1 as DEGs.[18]

Table 1 Clinical Characteristics of POF Patients and Healthy Controls

Sample Name	Age (years)	BMI	FSH (IU/L)	Menstrual History (Duration of Amenorrhea, Menstrual Cycle Regularity)	Exclusion Criteria Compliance (Absence of Autoimmune Diseases, Infections, Chemotherapy, etc.)	Lifestyle Factors (Smoking, Alcohol Consumption)	Habits (Physical Activity)
Healthy control 1	35	19.90	15.33	Regularity	Yes	No	Once a week
Healthy control 2	36	18.94	10.09	Regularity	Yes	No	Once a week
Healthy control 3	31	20.03	19.43	Regularity	Yes	No	Once a month
POF patient 1	33	18.26	71.82	Five months, irregularity	Yes	No	Once a week
POF patient 2	30	17.63	140.06	Six months, irregularity	Yes	No	Once a week
POF patient 3	32	20.08	86.48	Five months, irregularity	Yes	No	Once every ten days
POF patient 4	39	20.83	67.69	Four months, irregularity	Yes	No	Once a month

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Cell Type Annotation

Cell-ID is multivariate approach that extracts gene signatures for each individual cell and perform cell identity recognition using hypergeometric tests (HGT).[21] HGT were performed on these gene sets against ovary reference from SynEcoSys database, which contains all cell-type's featured genes in the specific organ/tissue.[22]

Pathway Enrichment Analysis

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used with the "clusterProfiler" R package v4.0.0. Pathways with p_adj value less than 0.05 were considered as significantly enriched.[18]

Cell-Cell Interaction Analysis (Cellchat)

CellChat v1.6.1 was used to analyze the intercellular communication networks from scRNA-seq data.[23] A CellChat object was created using the R package process. Cell information was added into the meta slot of the object.

Transcription Factor Regulatory Network Analysis (SCENIC)

Transcription factor network was constructed by pyscenic v1.1.2 using scRNA expression matrix and transcription factors in AnimalTFDB.[24] First, GRNBoost2 predicted a regulatory network based on the co-expression of regulators and targets. CisTarget was then applied to exclude indirect targets and to search transcription factor binding motifs. After that, AUCell was used for regulon activity quantification for every cell.

POF Mouse Model Establishment

All animal experiments were conducted under license from the Southern Medical University and were approved by the ethical committee standards at the Southern Medical University, China (L2021132). All animal experiments follow the guidelines of the Animal Welfare and Ethics Committee of Southern Medical University in China. POF mouse model was established using C57BL/6 Female Mice aged 6 to 8 weeks. These mice were treated with CTX to induce POF. The mice received an initial intraperitoneal injection of 100 mg/kg CTX (Sigma Aldrich, St. Louis, MO, USA), followed by 50 mg/kg CTX for 1 week. Control mice were injected with an equal volume of saline for the same duration. The general condition and body weight of the mice were monitored and recorded weekly.

Histology

The remaining ovary from each mouse was harvested and preserved in 4% paraformaldehyde. Following processing, the ovaries were serially sectioned. To assess ovarian reserve, one out of every six sections from the entire ovary was stained with hematoxylin and eosin (H&E), and follicles at various developmental stages were enumerated.

Flow Cytometry

Mice were anesthetized with isoflurane, and their ovaries were minced into small pieces using sterile scissors or a scalpel. The minced tissue was transferred to a 50 mL conical tube containing an enzyme solution (1 mg/mL collagenase and 0.1 mg/mL DNase I) in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS). Cells were labeled with Zombie NIR (cat. no. 423106, BioLegend) following the manufacturer's instructions, then washed in FACS Buffer (PBS + 5% newborn calf serum). They were incubated with an Fc blocking reagent (anti-mouse CD16/32, cat. no. 70–0161, Cytek), washed, and stained with a surface-staining, fluorochrome-labeled antibodies (Supplementary Table 1) for 30 minutes at 4°C in the presence of Brilliant Stain Buffer Plus (10 µL per sample; cat. no. 563795, Becton Dickinson) and CellBlox Blocking Buffer (5 µL per sample; cat. no. C001T02F01, Thermo Fisher Scientific). Cells were washed again in FACS buffer and intracellularly stained to detect transcription factor expression using the True-Nuclear Transcription Factor Buffer Set (cat. no. 424401, BioLegend) according to the manufacturer's instructions. The staining procedure concluded with cells being fixed in 2% paraformaldehyde for 5 minutes. Stained cells were acquired using a five-laser Cytek Aurora flow cytometer and analyzed with FlowJo 10.9 (Becton Dickinson).

Enzyme-Linked Immunosorbent Assay

Serum biochemical parameters, including FSH, E2, and AMH levels, were measured using an ELISA kit (FSH: E-EL-M0511, E2: E-OSEL-M0008, AMH: E-EL-M3015), following the manufacturer's instructions. Blood samples were collected from the eyeball veins and centrifuged at 3000 ×g for 20 minutes. Proteins were extracted from the ovaries, and the levels of CCL5 and CXCL16 in the ovary were determined using ELISA kits (CCL5: ab100739, CXCL16: ab197744).

Statistical Analysis

All statistical analyses were performed using SPSS v.17. All results are shown as the mean ± standard deviation (M ± SD). All statistical comparisons were performed using one-way ANOVA followed by Duncan's multiple range post hoc analysis. A P value < 0.05 was considered significant.

Results

Single-Cell Transcriptome Analysis of PBMCs in POF and HC

In this study, peripheral blood samples were collected from 4 patients diagnosed with POF and 3 healthy control (HC) subjects. We utilized single-cell transcriptome and T-cell receptor repertoire sequencing techniques to thoroughly analyze immune cells in the peripheral blood (Figure 1A). After rigorous data quality control measures, we obtained a total of over 30,000 cells. Supervised clustering techniques enabled the identification of 7 distinct cell clusters (Figure 1B). Based on each cluster's gene expression characteristics (Figure 1C), these 7 cell clusters were annotated as T cells, B cells, NK cells, neutrophils, basophils, monocytes, and red blood cells. Comparing the proportions of various immune cell clusters in the peripheral blood of POF patients and HC, we observed an increase in the proportions of T cells, NK cells, and B cells in the POF group. However, the proportion of monocytes showed no statistically significant difference, despite considerable research attention directed towards them (Figure 1D).

Regarding functionality, we compared the upregulated pathways in POF patients to the HC group and found a notable overall upregulation of adaptive immunity and cytotoxic functions in the peripheral immune profile of POF patients (Figure 1E). The heatmap clearly demonstrated the significant upregulation of transcriptional features in POF, including genes such as IGLC2, GNLY, GZMB, FCGR3A, and CCL5 (Supplementary Figure 1 and Supplementary Table 2), closely related to the functions of monocytes, T cells, and B cells. By conducting SCENIC analysis and comparing the transcriptional open activities among different immune cell clusters in the two groups (Figure 1F), several important findings emerged from the analysis, including a notable upregulation of transcription factor activity in monocytes. Specifically, ZNF658, TFEC, ENO1, and BATF3 demonstrated increased inflammatory activity.[25] In contrast, the transcriptional activity of TCF7 and LEF1 was downregulated in T cells, suggesting that T cells are in a more activated effector state.[26]

Distinct Inflammatory Phenotype in Monocytes of POF Patients' Peripheral Blood

The immune mechanisms of myeloid cells in POF has been always a central focus of attention.[27] Comparing the functional characteristics of two groups of myeloid cells, we observed a significant upregulation of genes associated with the inflammatory phenotype in the POF group, such as FCGR3A, CD36, CD52, etc. (Supplementary Figure 2). Importantly, the upregulated genes are enriched in pathways related to multiple cytokines, including MCP1 (CCL2), and are involved in functions associated with adhesion and adaptive immune activation (Figure 2A).

To further investigate the heterogeneity of myeloid cells in the peripheral blood of the POF group, we categorized them into four main cell groups (Figure 2B), annotating them as classical monocytes, non-classical monocytes, pDC, and cDC2 based on classical marker genes. The expression profiles of marker genes for each cell group are depicted in violin plots (Figure 2C), with classical monocytes constituting the majority. In the POF group, a notably higher proportion of non-classical monocytes was observed (Figure 2D), characterized by the upregulation of CD16 and complement-related genes (Figure 2E). Functionally, non-classical monocytes are closely associated with lymphocyte proliferation and T-cell activation (Supplementary Figure 3), supporting their enhanced antigen-presenting capability and increased production of

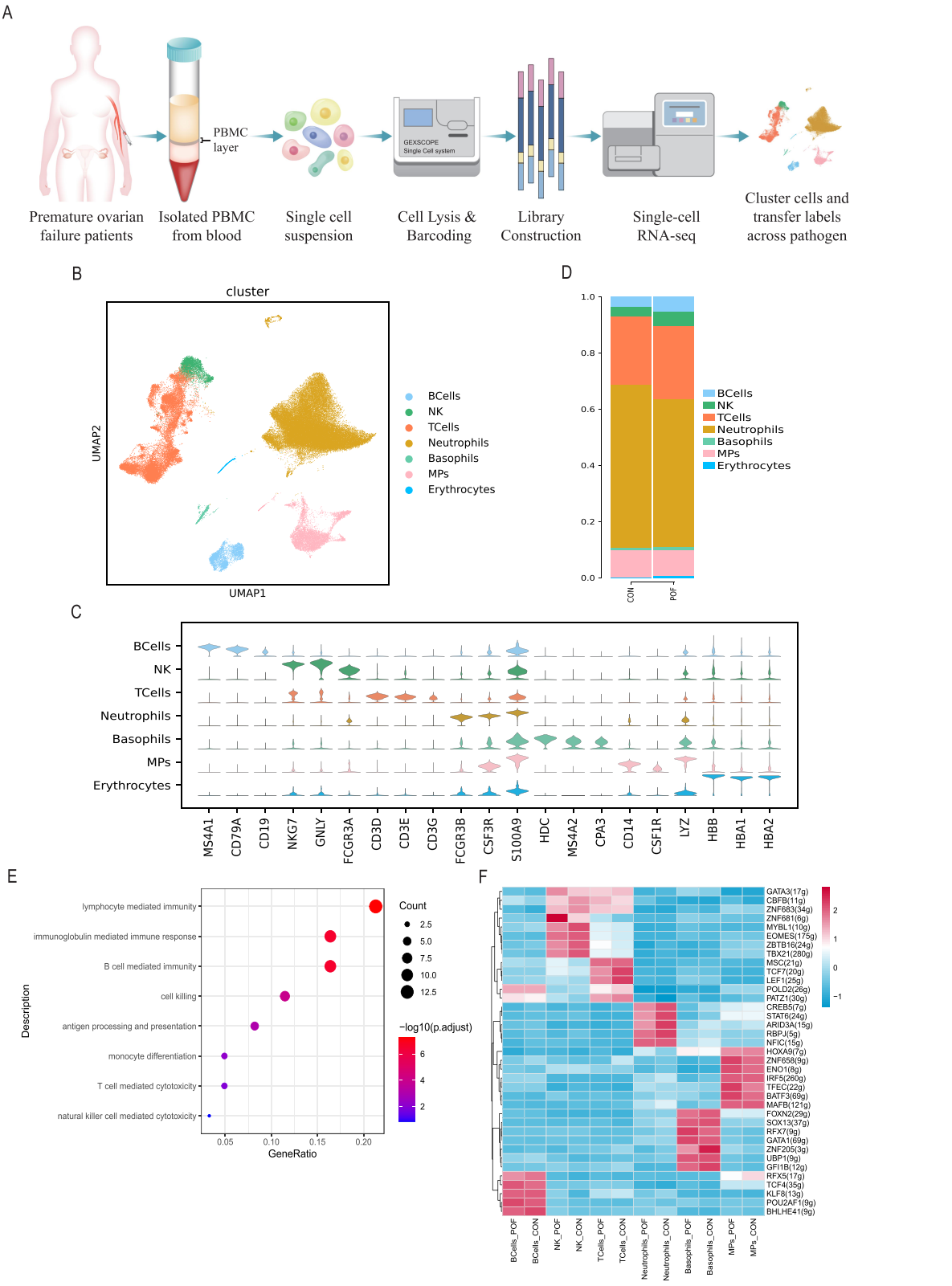

Figure 1 Single-cell Transcriptome Analysis of PBMCs in POF and CON. (**A**) Overall workflow of scRNA-seq using peripheral blood samples. (**B**) UMAP clustering by cell type coloring for all cells. (**C**) Gene expression characteristics of each cluster. (**D**) Histogram of the proportion of cell composition between the two groups. (**E**) GOBP bubble chart of differentially expressed immune genes upregulated in all seven cell clusters. The circle size in the Fig. represents the number of genes, and the color gradient from red to blue indicates the enrichment significance from high to low. (**F**) Transcription factors heatmap between two groups of different immune cell clusters.

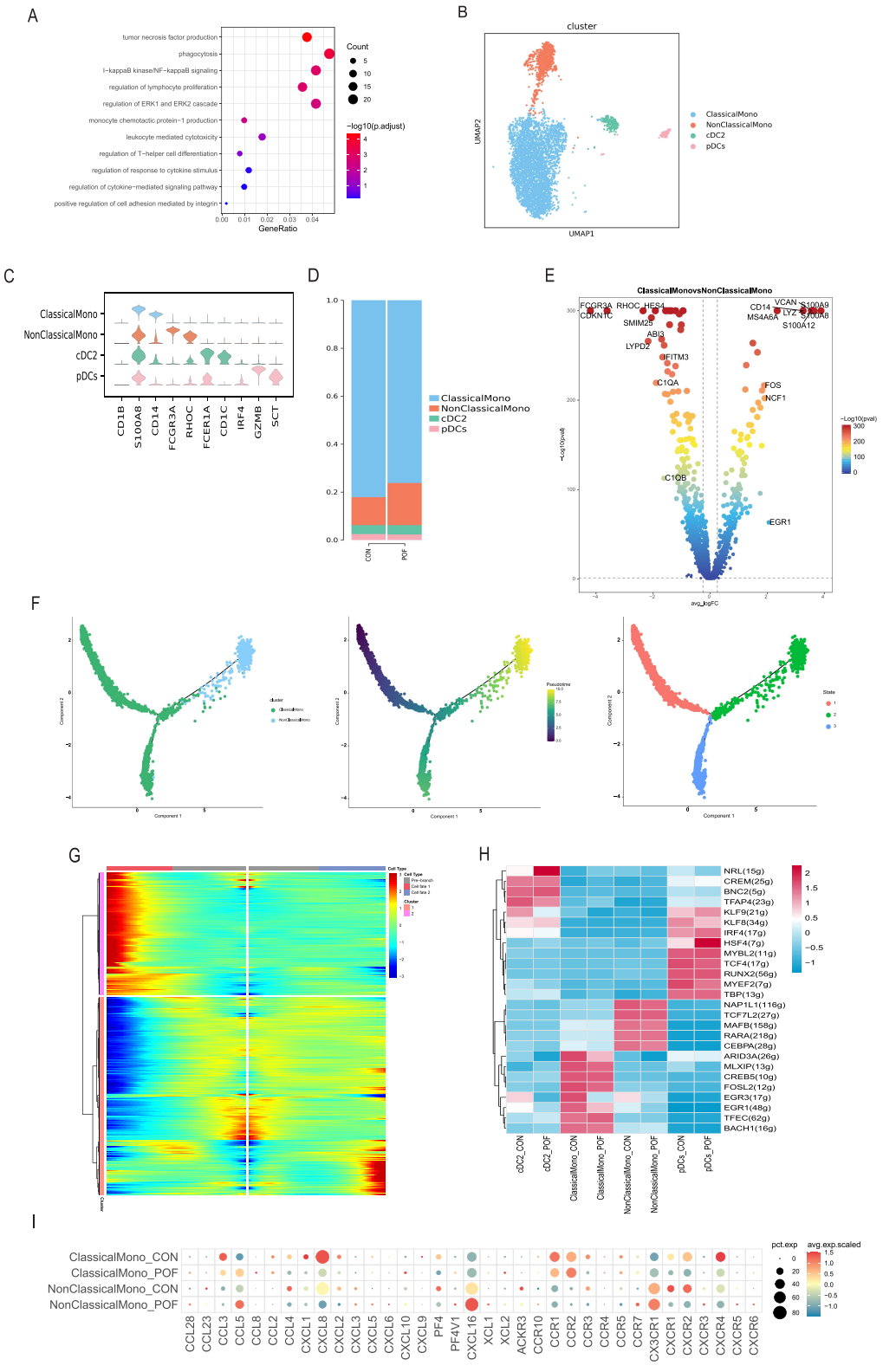

Figure 2 Distinct Inflammatory Phenotype in Monocytes of POF Patients' Peripheral Blood. (**A**) Gene Ontology(GO) enrichment dot plot of myeloid cells in POF. (**B**) UMAP clustering by cell type coloring for the heterogeneity of myeloid cells. (**C**) Gene expression characteristics based on the four myeloid cell types. (**D**) Histogram of the percentage of four myeloid cells. (**E**) Differential gene volcano map of monocytes. (**F**) Pseudotime analysis on monocytes. (**G**) Branch point gene heatmap of monocytes. (**H**) Heatmap of transcriptional regulatory patterns for each monocyte subset. (**I**) Bubble plot of chemokine expression differences between the two groups of monocytes.

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inflammatory cytokines. In addition to the high expression of CD14, VCAN, and EGR1, genes related to neutrophils, including S100A8, S100A9, and others were also upregulated in classical monocytes in POF group (Figure 2E).

The overall analysis revealed a progression from classical monocytes to non-classical monocytes. In the three stages, it was observed that non-classical monocytes were predominantly distributed in stage 2. Fate1 (spanning from stage 1 to stage 3) represents the evolution among classical monocytes (Figure 2F). Branched Expression Analysis Modeling (BEAM) analysis along the fate1 trajectory gradually upregulated genes associated with inflammation, such as CD14, CCR2, CCL2, CCL3, CCR1, IL1B (Supplementary Table 3). On the other hand, fate2 (from stage 1 to stage 2) represents the evolution from classical monocytes to non-classical monocytes, gradually upregulating FCGR3A, C1QC, CD52, CSF1R, TNF, CD83 (Figure 2G and Supplementary Table 3).

In the comparative analysis of the transcriptional regulatory patterns for each monocyte subset separately (Figure 2H), the transcriptional regulatory activity of regulons EGR1, EGR3, and ARID3A in classical monocytes of the POF group was downregulated. EGR1/EGR3 is crucial for regulating CSF1R in monocyte differentiation which suppresses inflammatory genes via the NuRD corepressor complex. This finding suggests an intricately link to abnormal inflammatory activation.[28] Conversely, the transcriptional regulatory patterns between non-classical monocytes in the two groups exhibited similarity. Under the corresponding transcriptional regulatory pattern, we identified distinct expression differences in several chemokines (Figure 2I), particularly a significant upregulation of CCL5 and CXCL16 expression in non-classical monocytes of the POF group. CCL5 serves as a specific ligand for CCR5, while CXCL16 functions as a specific ligand for CXCR6. These chemokines play crucial roles in monocyte chemotaxis to tissues and exert influence on interactions with T cells.[29,30]

Immunosenescent Phenotype in T Cell Subsets of POF Patients

The study of POF has extensively documented the role of T cells, emphasizing the importance of exploring the heterogeneity within T cell subgroups and their functions.[6,31] Comparing differentially expressed genes between the two groups, a significant upregulation of functions associated with T cell activation and cytotoxicity in the POF group was observed (Figure 3A and Supplementary Figure 4). Further subgrouping of T cells revealed eight distinct cell subgroups, encompassing three $CD4^+$ subgroups ($CD4^+$ naïve T cells, $CD4^+$ Effector memory T cells, and $CD4^+$ Tregs) and three CD8 subgroups ($CD8^+$ naïve T cells, $CD8^+$ MAIT, and $CD8^+$ Effector T cell), along with NKT and GDT (Figure 3B). Moreover, $CD8^+$ Effector T cells constitute the most concentrated cell group in terms of cytotoxic function was observed, followed by NKT (Figure 3C). The notable aspect is the overall upregulated gene features in T cells within the POF group, prominently featuring GNLY and GZMB. Additionally, GZMB exhibits specific high expression in these two subgroups (Supplementary Figure 4).

Despite observing no significant differences in the proportion of $CD8^+$ Effector T cells in the POF group (Supplementary Figure 5), noteworthy functional distinctions emerged between the two groups. A comparison of the transcriptional profiles of $CD8^+$ Effector T cells in the two groups revealed that the control group expressed higher levels of naive-related markers such as CCR7, SELL, IL7R, and TCF7. This suggests that these cells are in a functionally preliminary activated state and maintain a long-lived memory state.[32] In contrast, the POF group exhibited more signals related to cytotoxic T cells, such as PRF1 and GZMB (Figure 3D). Alongside the activation state, markers related to immune checkpoint exhaustion were also relatively upregulated in the POF group, particularly PD-1 and LAG3 (Figure 3E). Furthermore, distinctions were observed in the transcriptional regulatory networks between the two groups (Figure 3F). Within the $CD8^+$ Effector T cells, a notable upregulation was observed in the transcriptional regulons of EOMES and KLF10 in the POF disease group. Eomesodermin, encoded by the EOMES gene, has been implicated in a diverse range of T cell-specific functions.[33] Of particular interest in this context is its role in the differentiation of $CD8^+$ memory T cells.[34]

In addition to $CD8^+$ Effector T cells, NKT cells in the disease group also exhibit differences in both cell proportion and functionality (Supplementary Figure 5). This is particularly evident in the significant upregulation of cytotoxic receptors such as GZMB and GNLY. Furthermore, the inhibitory receptor KIR3DL1 was upregulated, which is commonly found on NK cell surfaces (Figure 3G and Supplementary Table 4). Within various immune checkpoints, NKT cells in the POF group display a significant upregulation of LAG3 and TIM3 (Figure 3E). Killer cell immunoglobulin-like receptors (KIRs) play a crucial role in negatively regulating the activation status of natural killer T (NKT) cells in conjunction with immune checkpoint molecules such as TIM3 and LAG3. They often appear concomitantly with the activation state of immune cells.[35,36] However, in the progression of many autoimmune diseases, the malfunction of this regulatory brake often occurs, leading to downstream damage to self-

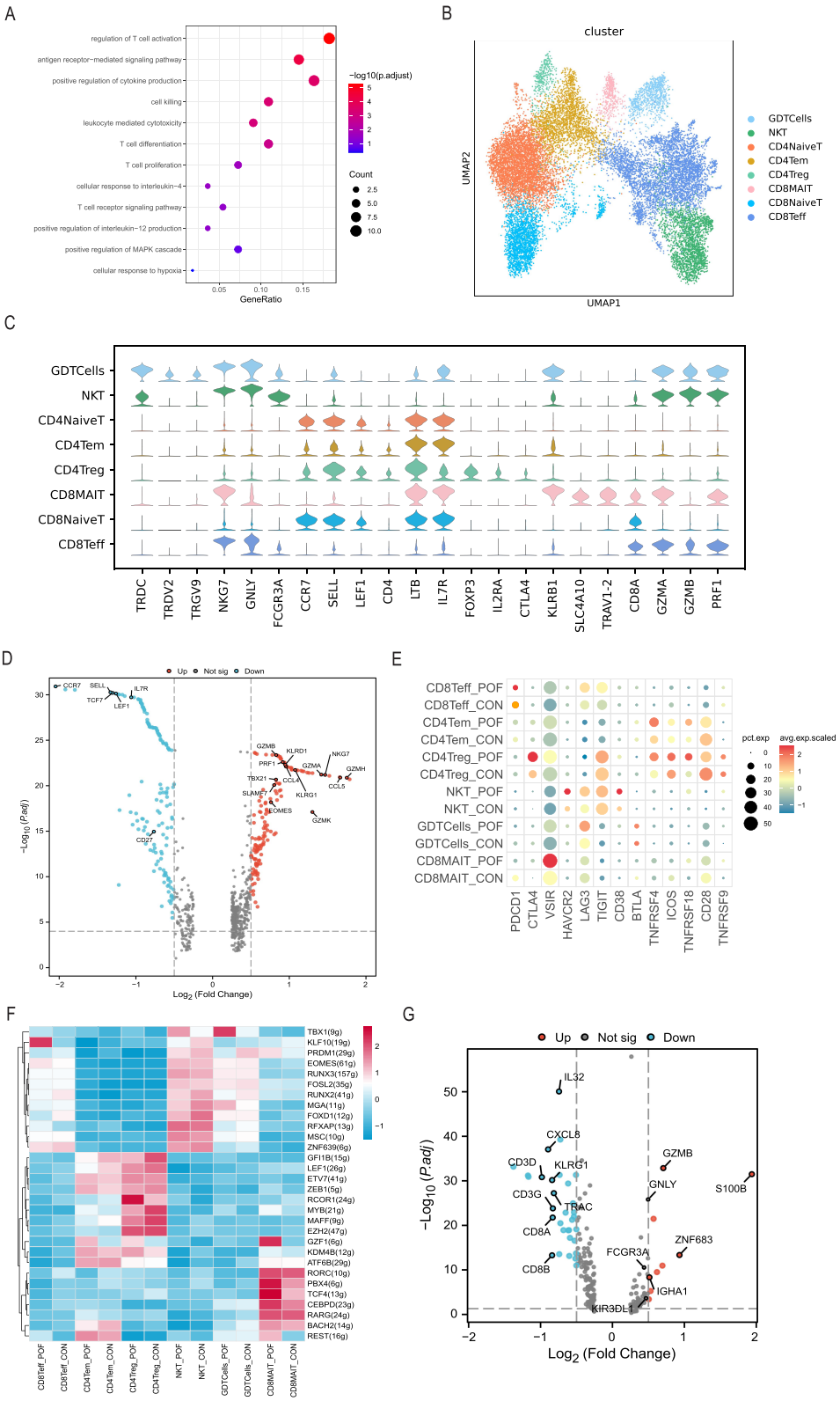

Figure 3 Immunosenescent Phenotype in T Cell Subsets of POF Patients. (**A**) Gene Ontology(GO) enrichment dot plot of T cells in POF. (**B**) UMAP clustering by cell type coloring for T Cell Subsets of POF Patients. (**C**) Gene expression characteristics of each T cell subset. (**D**) Volcano plot of differential genes in CD8$^+$ Effector T cells between the two groups. (**E**) Bubble plot of markers associated with immune checkpoint dysfunction in T cells from both groups. (**F**) Heatmap of transcription factors in T cells from the two groups. (**G**) Volcano plot of differential genes between NKT cells from the two groups.

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tissues and the progression of inflammation.[37] The transcriptional regulatory regulon specific to NKT cells is akin to $CD8^+$ Effector T cells, and notably, only the transcription factor TBX1's regulatory network is specifically upregulated in the POF disease group, with no significant differences observed in other regulons in terms of transcriptional activity (Figure 3F).

Expansion of TCR Clonal Groups in $CD8^+$ Effector T Cells of POF Patients

Large clone populations, consisting of more than 10 cells, were predominantly present in $CD8^+$ Effector T cells, with over half of the clonally expanded cell proportion in the POF group (Figure 4A). Upon ranking clonotypes from most abundant to least, the top ten clonotypes were primarily distributed in $CD8^+$ Effector T cells (Figures 4B and 3B), displaying a relatively concentrated pattern, indicating similar transcriptional characteristics and functional states. Except the third, fourth, and tenth clonotypes, the remaining seven originated from the POF disease group (Figure 4C).

The evolution from $CD8^+$ naïve T to $CD8^+$ Effector T cells could be bifurcated into two distinct trajectories (Figure 4D and Supplementary Figures 6 and 7). Stage 3 cells were predominantly derived from the POF group, while stage 2 encompassed cell populations from both the POF and CON groups (Figure 4E). The major large clonotypes were concentrated in stage 3 (Figure 4F). To identify the specific transcriptional regulatory factors that steer the evolution of $CD8^+$ naïve T cells through stage 3, we performed BEAM. In cell fate1, representing the transition from stage 1 to stage 3, we observed the upregulation of various activated functional genes and transcription factors, including EOMES, SLAMF7, and other activation markers such as GZMB, PRF1, KLRB1, KLRG1, TIGIT, and several chemokine receptor genes like CCR5 and CXCR6. Notably, these receptors correspond to ligands, including CCL5 and CXCL16, found to be upregulated in non-classical monocytes in the POF group (Figure 2I). Conversely, cell fate2 predominantly involved genes associated with a long-lived memory phenotype, such as TCF7, CD27, and IL7R, corresponding to genes differentially upregulated in $CD8^+$ Effector T cells in the control group (Figure 4G and Supplementary Table 5). This evolutionary pathway is more closely linked to the long-lived central memory T cell phenotype.[23]

Profound Peripheral Signaling Specificity in POF Patients

The differences in communication signal networks between the two groups were not significant, with only a few changes in communication signals reflecting enhanced input signals in MAIT and NKT cells in the POF group. Nevertheless, it is noteworthy that the two monocyte cell groups consistently emerged as crucial sources of output signals, while $CD8^+$ T cells, encompassing both naïve $CD8^+$ T and effector $CD8^+$ T cells, were identified as the primary recipients of signals. This implies that the signaling network between these two cell groups is the most active (Figure 5A).

Although no significant differences were observed in overall strength, we noted an upregulation of multiple signaling pathways, including CCL, CXCL, and TNF, in the POF group in terms of relative signal strength (Figure 5B). Upon closer examination, a significant upregulation of the CXCL and CCL pathways in the output signals of non-classical monocytes in the POF group was observed, particularly directed towards T cells, including $CD8^+$ MAIT and effector $CD8^+$ T cells (Figure 5C). Additionally, classical monocytes received more chemotactic signals such as CXCL and CCL from non-classical monocytes (Figure 5C). This result aligned with our earlier observations and speculations regarding the interaction of CXCL16-CXCR6 and CCL5-CCR5 between monocytes and $CD8^+$ Effector T cells, once again underscoring the importance of this signaling axis in studying the unique functional phenotypes of peripheral monocytes and T cells in the POF disease state. Therefore, we will conduct animal experiments to verify the interaction between monocytes and CD8+T cells. Furthermore, despite the significant upregulation of inflammatory phenotypes in multiple cell groups in the POF group in our previous analyses, no significant differences were observed in the TNF signaling pathway, except for a slightly higher TNF output signal in non-classical monocytes in the POF group (Supplementary Figure 8).

Non Classical Monocytes Activate $CD8^+$ T Cell Clone Amplification Through CCL5-CCR5 Pathway

In addition to the interactions between circulating immune cells, chemokines play a crucial role as communication signals between immune cells and tissues, possessing the ability to induce directed chemotaxis of nearby responsive cells.[38] However, as the tissue microenvironment of patients with POF cannot be directly observed, we utilized a cyclophosphamide (CTX) induced POF model.

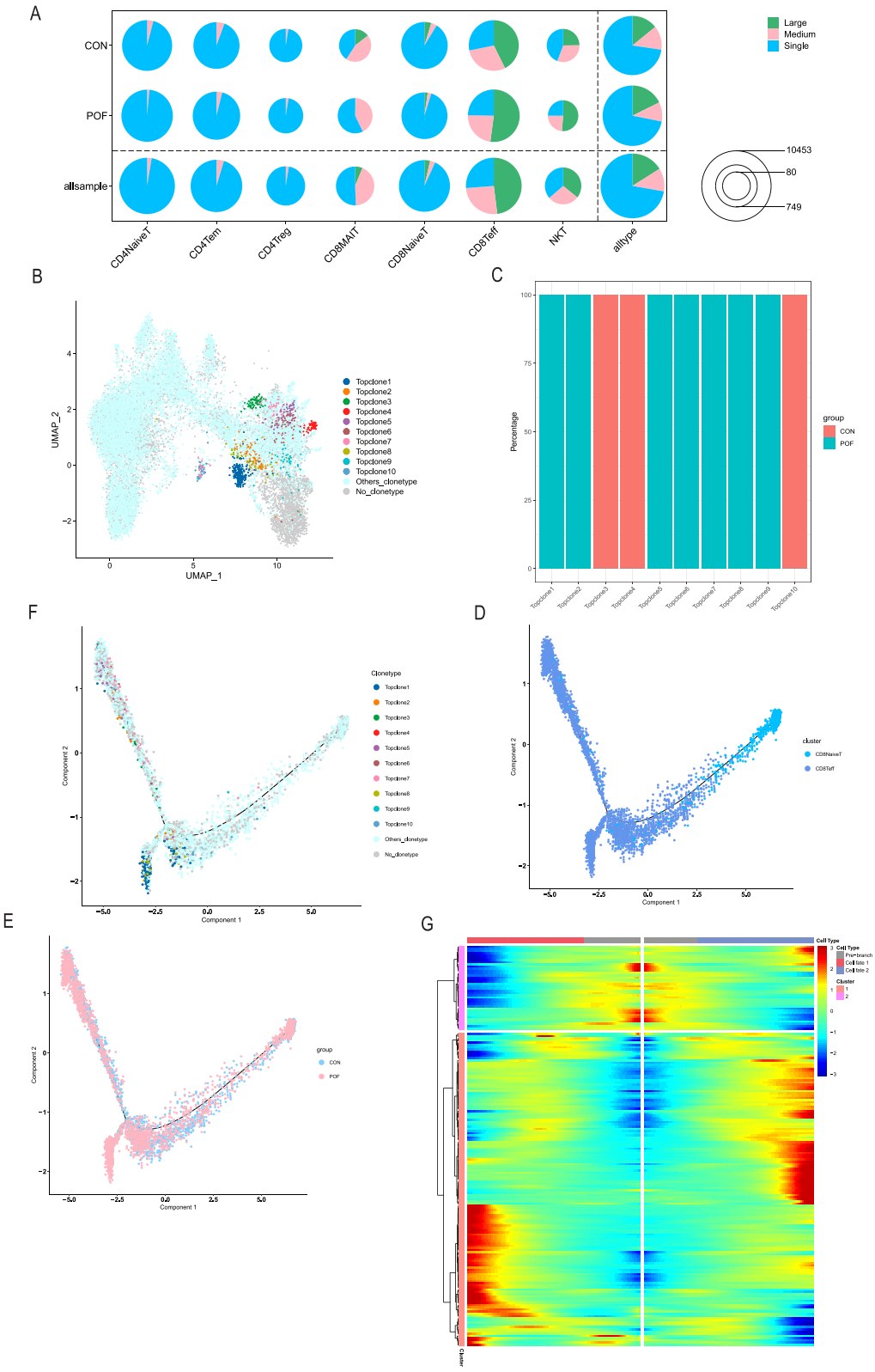

Figure 4 Expansion of TCR Clonal Groups in CD8$^+$ Effector T Cells of POF Patients. (**A**) Expanded cell proportion of CD8$^+$ Effector T cells in the POF group. (**B**) Distribution of clonotypes in T cells. (**C**) Histogram of the distribution of the top 10 clonotypes between the two groups. (**D**) Pseudotime analysis on CD8$^+$ naïve T and CD8$^+$ Effector T cells. (**E**) Pseudotime analysis of cell populations from both the POF and CON groups. (**F**) Distribution of CD8$^+$ Effector T cell clonotypes in three stages. (**G**) Heatmap of expression patterns of key genes at CD8 T cell branching points.

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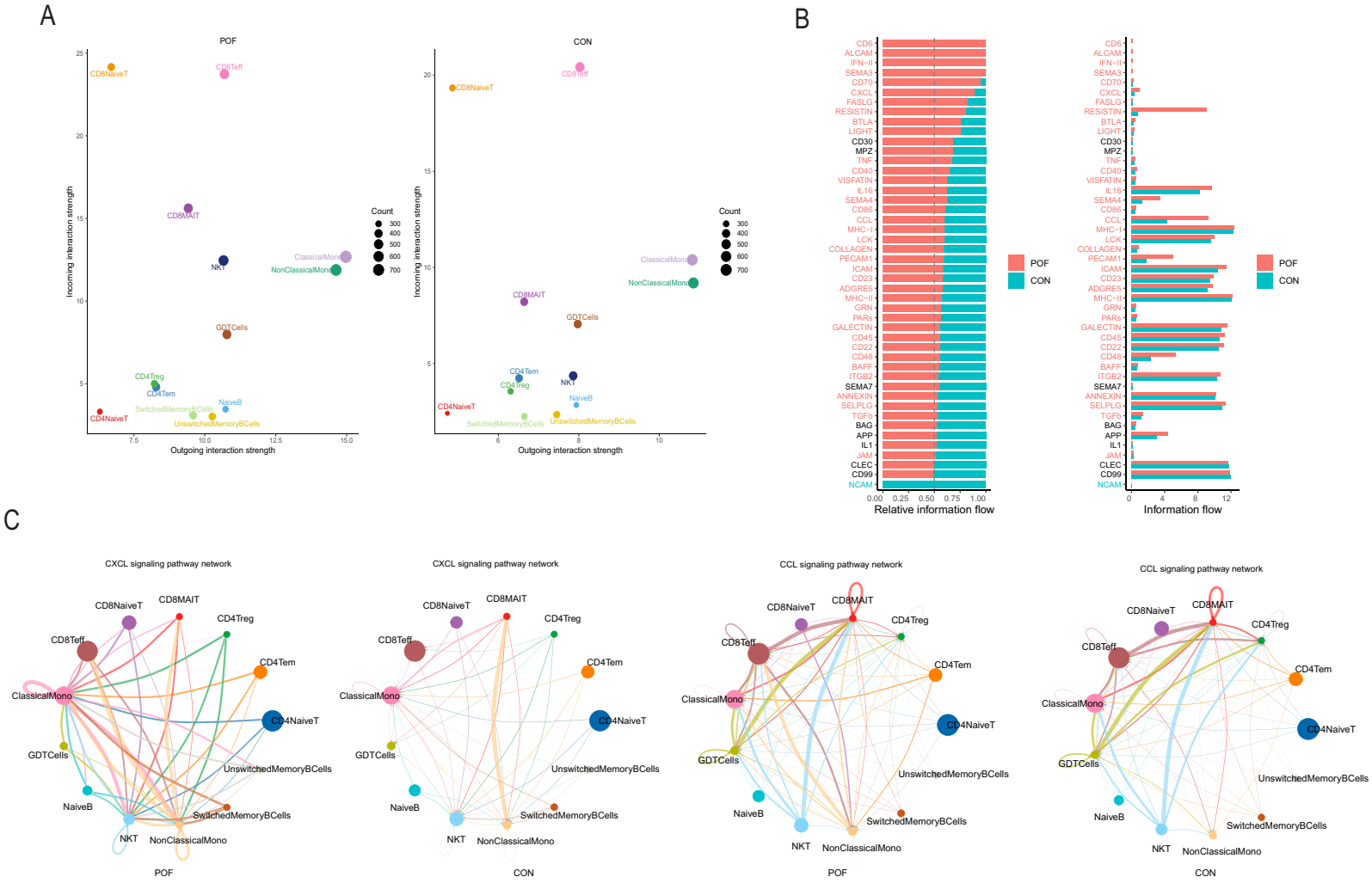

Figure 5 Profound Peripheral Signaling Specificity in POF Patients. (**A**) Diagram of cellular communication signals between immune cell populations in the two groups. (**B**) Pathway diagram of relative signal strengths in the two sets. (**C**) Interaction network diagram between all cell types, where network nodes represent cell types, network edge thickness represents the total number of ligand-receptor pairs, and line color consistent with the ligand cell type.

The morphology of the CTX induced POF model revealed a significant reduced the number of follicles (Supplementary Figure 9). Additionally, CTX induced POF model showed disturbed estrous cycle (Supplementary Figure 10) and reduced Anti-Müllerian Hormone (AMH), E2 and increased FSH levels (Supplementary Figure 11). Additionally, CD3 T cells and CD8 T cells were more abundant in the ovaries of aging mice compared to normal mice (Figure 6A), suggesting a higher infiltration of $CD8^+$ T cells in the aging ovaries. The expression levels of CCL5 and CXCL16 were also significantly increased in the ovaries of POF (Figure 6B). Our current data demonstrate a correlation between elevated CCL5/CXCL16 levels and $CD8^+$ T cells infiltration. This infiltration ultimately contributes to damage and aging of the ovarian tissue.

Discussion

POF is a complex and multifactorial syndrome, encompassing autoimmune, genetic, and environmental factors. Autoimmune oophoritis, marked by immune cell infiltration, is implicated in 4–30% of cases.[4,5,12] Emerging studies underscore the pivotal role of the immune system in POI, highlighting the human ovary as a frequent target for autoimmune attacks.[7,8]

In the comprehensive analysis of transcriptional features, notable upregulation of functional genes (IGLC2, GNLY, GZMB, FCGR3A, and CCL5) were observed in POF patients which aligns with the study by Zhang et al.[16]

$CD14^-CD16^+$ monocytes are known to produce higher levels of TNFα and IL-1b, indicating an enhanced proinflammatory function and their roles in the proliferation and stimulation of $CD4^+$ T cells.[39–41] In POF groups, the trajectory from $CD8^+$ naïve T cells to $CD8^+$ Teff involved the simultaneous upregulation of functional genes (GZMB, PRF1) and immune checkpoint molecules (LAG3, PD1). Additionally, we observed the upregulation of two transcription factors (EOMES, KLR10) and heightened regulatory activities, potentially playing crucial roles in the regulatory network of $CD8^+$ T cells in POF groups.

$CD8^+$ Effector T cells from the POF group exhibited raised levels of cytotoxic-related molecules and immune checkpoints, along with a decrease in markers related to long-lived memory T cells, such as CD127 and CD27. This condition, similar to chronic infection, indicates ongoing antigen stimulation and cellular functional activation. Nevertheless, the clonal expansion triggered by this stimulation results in a decrease in naïve $CD8^+$ T cells without a corresponding rise in the number of $CD8^+$ Effector T cells. This reduction in T cell reserve capacity, along with a decreased diversity in effector memory cells, corresponds with key features of T cell aging. These characteristics comprise a diminished TCR repertoire, an imbalance between naïve and memory cells, T cell senescence, and a lack of effector plasticity.[42,43] The demand for extensive clonal expansion and extreme proliferative pressure in these specific clone groups may be attributed to continuous exposure to autoantigens. Simultaneously, T-cell aging induced by autoantigen stimulation also contributes to the onset and progression of autoimmune conditions.[44]

Previous studies have indicated a correlation between the progression of ovarian dysfunction and immune dysregulation, marked by abnormal $CD8^+$ T cell proportions and upregulation of CCL5 and IFNγ.[15,16,45] In this study, we specifically identified non-classical monocytes as the primary source of CCL5 at single-cell resolution. As a secreted inflammatory chemokine, CCL5 binds to receptors including CCR1, CCR3, and CCR5.[26] Previous research has suggested an upregulation of CCL5 expression in aging ovaries, potentially originating from peripheral CCL5 deposition.[15] Our immunohistochemistry results confirmed a significant increase in the local expression levels of CCL5 and CXCL16 in the ovaries of aging mice. CCL5 in ovaries has been reported to directly induce apoptosis in granulosa cells and theca cells.[46]

The animal model adopted in the study was the cyclophosphamide-induced premature ovarian failure model. The cyclophosphamide-induced POF model was chosen primarily for its well-characterized mechanism of inducing ovarian failure through DNA damage and apoptosis, which is a common pathway leading to follicular depletion and ovarian dysfunction. This model is widely used in the field, providing a robust and reproducible platform for studying ovarian failure and testing potential therapeutic strategies. While it is true that autoimmune mechanisms are a significant cause of POF, our focus was on understanding the broader aspects of ovarian failure that might share common pathways with chemotherapy-induced damage. The insights gained from this model can still be relevant to other forms of POF by highlighting fundamental biological processes involved in ovarian follicle depletion and dysfunction. In future studies, we plan to explore additional models, including those that mimic autoimmune POF, to validate and extend our findings. This will help ensure that our results are applicable to a wider range of POF etiologies and provide a more comprehensive understanding of the condition.

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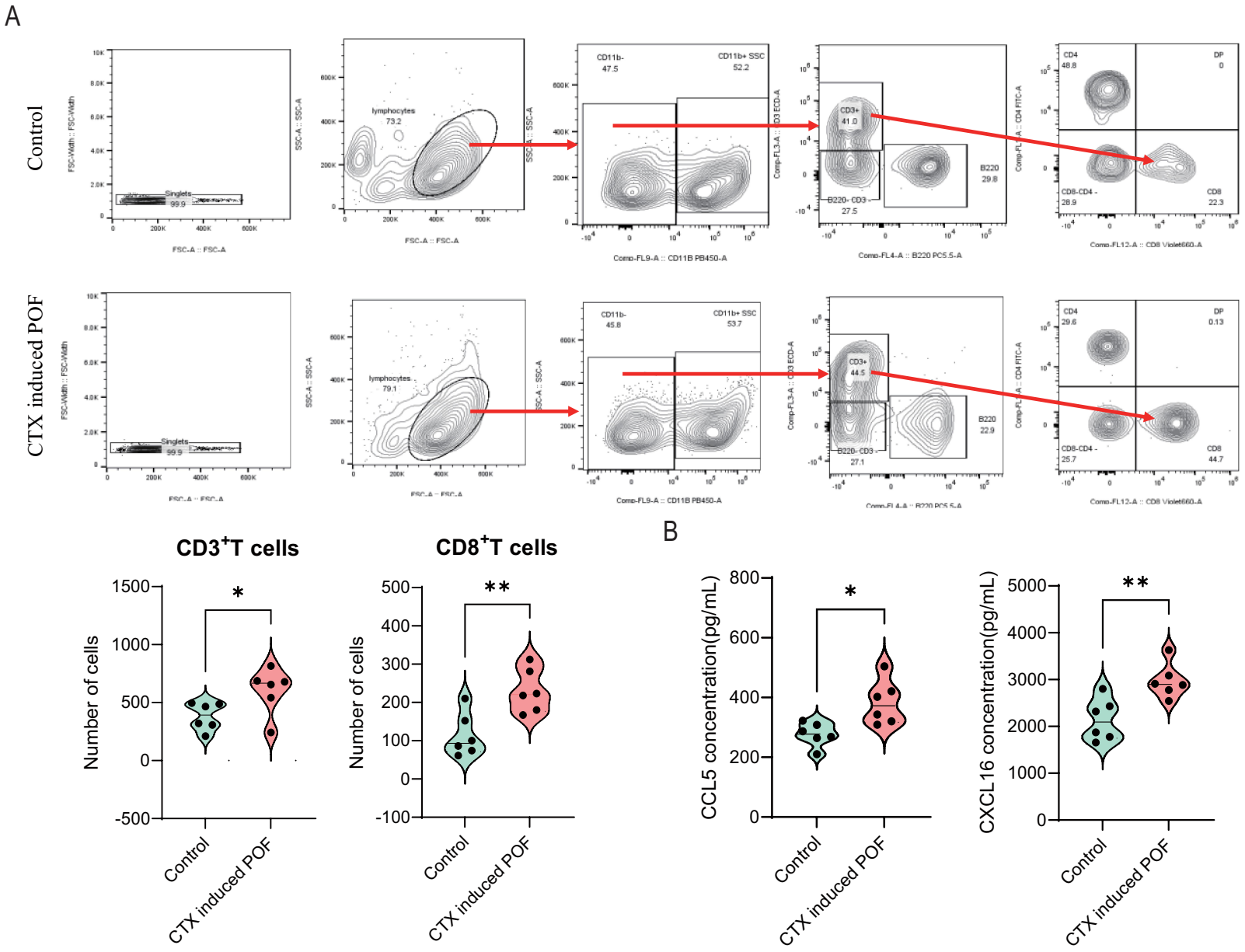

Figure 6 Non classical monocytes activate CD8$^+$ T cell clone amplification through CCL5-CCR5 pathway. (**A**) Total levels of CD3$^+$ T cells and CD8$^+$ Effector T cell subpopulations were analyzed by flow cytometry (N=6). (**B**) The concentration of CCL5 and CXCL16 in ovaries was measured by Enzyme-linked immunosorbent assay(ELISA)(N=6). *P<0.05. **P<0.01.

However, it must be acknowledged that our research has limitations. The sample size for single-cell sequencing is relatively small, and further validation with a larger queue is needed to enhance the robustness of our research results. In addition, dissecting the ovarian immune microenvironment of POF patients is crucial, and using a mouse model of ovarian aging as a substitute may not fully address this gap. Furthermore, future research should address the need for mechanism validation, which remains another limitation of our current work. Inhibition of CCL5/CXCL16 in CTX-induced mouse models of POF (eg, using neutralizing antibodies or receptor antagonists) to assess a reduction in $CD8^+$ T cells infiltration. Chemotactic assay was used to determine whether CCL5/CXCL16 directly promoted $CD8^+$ T cells migration. Spatial transcriptomics or multiple immunohistochemistry mapping of CCL5/CXCL16 expression and $CD8^+$ T cells localization in human ovarian tissue.

Conclusion

In Conclusion, our study holds promise for clinical applications, provides new insights into diagnosis for POF, deepens our understanding of the molecular mechanisms underlying the pathogenesis of POF, and lays the foundation for further research. In order to comprehensively understand the immune regulatory mechanism and clinical significance of POF, future research requires more sample size and experimental validation.

Data Sharing Statement

The sequencing data utilized in this study are accessible through online repositories named NCBI GEO, with the accession number PRJNA1091456. Any other data underlying this study will be provided by the corresponding authors upon reasonable request.

Ethics Approval Statement

Ethical approval and consent to participate were obtained.

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We are indebted to all the patients who participated in present study.

Author Contributions

The reported work has been significantly contributed to by all the authors. Their contributions encompass a range of areas, such as conceptualization, study design, data acquisition, execution, analysis, and interpretation. Moreover, drafting, revising, and critically reviewing the article involved the participation of all authors. They have provided their final approval for the version intended for publication and have reached a consensus on the target journal. Additionally, all authors acknowledge their responsibility for every aspect of the work.

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Disclosure

All authors declare no conflicts of interest in this work.

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