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ORIGINAL RESEARCH

APOE and *CCR2*: Potential Macrophage-Specific Biomarkers in the Rheumatoid Arthritis Synovial Microenvironment Identified by Bioinformatics and Experimental Verification in Murine Models

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Background: Rheumatoid arthritis (RA) is a chronic inflammatory joint disorder in which macrophages play crucial roles. Given macrophage heterogeneity, novel biomarkers are needed for timely diagnosis and severity assessment. This study aimed to identify macrophage-specific hub genes in RA and investigate their biological functions.

Methods: Bulk and single-cell RNA-seq datasets were downloaded from the Gene Expression Omnibus (GEO). Differentially expressed genes (DEGs) in RA synovial macrophages were identified from the GSE97779 dataset using the Limma R package. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to determine the biological processes and pathways associated with the DEGs, followed by Gene Set Enrichment Analysis (GSEA) for further validation. Hub genes were identified using the STRING database and Cytoscape. Based on the single-cell dataset GSE192504, cell clusters were annotated with Seurat to determine macrophage-specific hub genes, whose associated biological processes were explored via gene set variation analysis (GSVA). Further sub-clustering revealed distinct macrophage subtypes. Finally, immunofluorescence staining was performed to identify molecular markers of macrophage subtypes, while RT-qPCR and ELISA were used to validate the mRNA and protein expression of macrophage-specific hub genes in in vitro experiments.

Results: We identified 334 DEGs enriched in immune-related pathways. Ten hub genes (*FN1*, *CXCL10*, *FOS*, *CCR2*, *GZMB*, *CD69*, *CXCL9*, *FCGR1A*, *APOE*, *IGF1*) were identified, with *APOE* and *CCR2* specifically expressed in macrophages and strongly associated with inflammatory response. As expected, IL-4 stimulation upregulated *APOE* in RAW264.7 cells, while LPS/IFN-γ increased *CCR2* expression. Additionally, six macrophage subsets were found, with an expanded $APOE^+$ subset following IL-4 exposure.

Conclusion: *APOE* and *CCR2* are specifically highly expressed in synovial macrophages and correlate with inflammatory responses, highlighting their potential as biomarkers for disease progression and promising therapeutic targets in RA. Further investigation is required to elucidate the underlying mechanisms.

Keywords: rheumatoid arthritis, macrophages, immune infiltration, APOE, CCR2, biomarkers

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease pathologically characterized by inflammatory cell infiltration and pannus formation in synovial tissues, leading to progressive joint dysfunction and pain.[1] Current anti-rheumatic therapies are limited by suboptimal efficacy and an increased risk of opportunistic infections.[2] Therefore, there is a clear need to identify reliable biomarkers that can not only facilitate timely assessment of disease severity but also hold potential as therapeutic targets.

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Substantial evidence supports the role of autoantibody-mediated dysregulated immunity in the pathophysiology of RA, where genetic factors contribute to a cascade of events culminating in synovitis.[3,4] Notably, macrophages, as heterogeneous and multifunctional immune cells, play vital roles in immune dysregulation and tissue homeostasis in RA. Recent studies demonstrate that embryo-derived tissue-resident macrophages form a protective immunological barrier around intra-articular structures, whereas monocyte-derived macrophages recruited to the joint actively promote inflammatory response.[5–7] Furthermore, changes in the local microenvironment can drive macrophage phenotype switching along a dynamic continuum, classically polarized toward two extremes: the pro-inflammatory M1 phenotype and the tissue-remodeling M2 phenotype.[8] Although interest in discovering novel macrophage subsets and effective biomarkers is growing, the functional roles of intermediate macrophage subsets and their surface markers remain poorly understood, largely due to the complexity of macrophage biology. This underscores the need for further exploration of macrophage heterogeneity and the identification of candidate biomarkers.

The growing abundance of data in public bioinformatics repositories offers an opportunity to analyze accumulated genomic datasets from freely accessible databases. In this study, we identified hub genes specifically expressed in synovial macrophages and investigated their associated biological processes in RA synovial inflammation by analyzing microarray data from the Gene Expression Omnibus (GEO) public database. We also identified macrophage clusters in synovial tissue through hierarchical clustering and annotation, and validated our findings with a series of in vitro experiments. The study findings may provide potential biomarkers and therapeutic targets for the early diagnosis and timely treatment of RA.

Materials and Methods

Data Collection

Microarray data comprising 9 RA synovial macrophages and 5 human primary monocyte-derived macrophages from healthy subjects were retrieved from the GEO dataset (GSE97779) using the GEO query R package. Additionally, the GSE48780 dataset, which includes 83 synovial samples from RA patients, was also publicly obtained from the GEO database. Single-cell RNA-seq data of synovial cells from healthy and collagen-induced arthritis (CIA) mice were acquired from the GEO dataset (GSE192504) (Table 1).

Differentially Expressed Gene Acquisition

Differentially expressed genes (DEGs) in RA synovial macrophages were identified using an empirical Bayes test implemented in the Limma R package, and the Top 50 DEGs were displayed in a heat map. Up- and down-regulated genes were defined as those with adj.*P*.value < 0.05 and Log2 |fold change (FC)| >3, and were subsequently visualized using a volcano plot. A stringent threshold of Log2|FC| > 3 was used to identify genes with substantial expression changes, and the Benjamini-Hochberg procedure was applied to control the false discovery rate (FDR < 0.05).

Table 1 Summary of the GEO Datasets

Dataset Accession	Platform	Species	Sample Source/Type	Groups (Sample Size)	Total Samples/ Cells
GSE97779	GPL16791 (Illumina HiSeq 2500)	Human	Synovial macrophages; Primary monocyte-derived macrophages	RA synovial macrophages (n=9); Healthy control (n=5)	14 samples
GSE48780	GPL6480 (Agilent-014850 Whole Human Genome)	Human	Synovial tissue	RA patients (n=83)	83 samples
GSE192504	GPL24247 (Illumina NovaSeq 6000)	Mouse	Synovial cells (single-cell RNA-seq)	Collagen-Induced Arthritis (CIA) model (n=3); Healthy normal (n=9)	12 samples

Functional Enrichment Analysis and Gene Set Enrichment Analysis (GSEA)

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of the differentially expressed genes were performed using the ClusterProfiler package. The top 10 most significant terms, ranked by P-value, were respectively displayed using scatter plots and a bar chart. GSEA was subsequently employed to further validate these findings.

Construction and Analysis of Protein-Protein Interaction (PPI) Network

The PPI network of DEGs was constructed using the online Search Tool for the Retrieval of Interacting Genes (STRING) (https://www.string-db.org) with a medium confidence level of 0.40. STRING interaction data was subsequently imported into Cytoscape 3.9.1 for visualization. Submodule analysis was then performed using the Molecular Complex Detection (MCODE) plug-in with default parameters (degree cutoff ≥2, node score cutoff ≥2, k-core ≥2, and max depth=100). Finally, the top ten genes with the highest in-degree connectivity were identified as hub genes using the CytoHubba plugin.

Single-Cell Analysis

Following quality control and normalization, highly variable expressed genes were identified across cells in the GSE192504 dataset. Gene clusters were generated dataset through principal component analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) using the Seurat tool. Cell clusters were manually annotated based on the expression of canonical cell-type markers. Specifically, the "Macrophage" cluster was defined by high expression of *Adgre1* (*F4/80*); the "Monocyte" cluster was defined by high expression of *Ly6c2* (*Ly6C*). Automated annotation results from the SingleR package were used as a reference and were consistent with this marker-based approach.Data from the CIA group were subsequently extracted using the tidyverse package, and the expression of hub genes was examined across different synovial cell types. Macrophage subsets were then isolated from the CIA group and subjected to the same procedure, followed by single-cell trajectory inference using the Monocle algorithm.

Gene Set Variation Analysis (GSVA)

The gene sets obtained from the GO site (https://amigo.geneontology.org/amigo) were utilized for GSVA. Enrichment scores were calculated for synovial samples from RA patients in the GSE48780 dataset and visualized using a heatmap. Spearman correlation analysis was then performed to evaluate the association between hub genes and biological processes.

Cell Culture and Processing

RAW 264.7 macrophage cells were supplied by the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. To induce polarization, RAW 264.7 cells were stimulated for 24 h with either 100 ng/mL lipopolysaccharide (LPS) plus 20 ng/mL interferon (IFN)-γ to promote a pro-inflammatory phenotype or with 20 ng/mL interleukin (IL)-4 to induce an anti-inflammatory phenotype, which respectively modulated inflammatory and reparative responses.

Immunofluorescence Staining

The obtained specimens were fixed with paraformaldehyde. The sections were then blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, followed by overnight incubation at 4 °C with the following primary antibodies (1:100 dilution): anti-apolipoprotein E (APOE) (rabbit monoclonal antibody, Proteintech Group, Inc., China), anti-placenta specific 8 (PLAC8) (rabbit monoclonal antibody, Proteintech Group, Inc., China), anti-CD74 (mouse monoclonal antibody, Proteintech Group, Inc., China), anti-podoplanin (PDPN) (mouse monoclonal antibody, Proteintech Group, Inc., China). After washing three times with PBS containing 0.35% Tween-20 (PBST), the sections were incubated with species-compatible fluorescent secondary antibodies at a dilution of 1:400 for 1 h. After staining

Table 2 The Primer Sequences for RT-qPCR

Target Gene	Forward Sequence	Reverse Sequence
APOE	5′-CTCCCAAGTCACACAAGAACTG-3′	5′-CCAGCTCCTTTTTGTAAGCCTTT-3′
CCR2	5′-TGTGATTGACAAGCACTTAGACC-3′	5′-TGGAGAGATACCTTCGGAACTT-3′

with DAPI for 10 min, the results were observed with a fluorescent microscope and quantified using ImageJ software. Experiments were repeated independently three times (biological replicates).

Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the harvested macrophages using RNAiso Plus reagent. Following the manufacturer's instructions, cDNA was synthesized from the extracted RNA using the Prime Script™ RT reagent Kit with gDNA Eraser. RT-qPCR was performed with SYBR Premix Ex Taq^{TM} II (RR820A, TaKaRa, Japan), and relative gene expression was calculated using the $2{-}\Delta\Delta Ct$ method. GAPDH was used as an endogenous control. The specific gene primer sequences are detailed in Table 2. Experiments were repeated independently three times (biological replicates).

Enzyme-Linked Immunosorbent Assay (ELISA)

The total proteins of APOE and C-C motif chemokine receptor 2 (CCR2) in LPS/ IFN-γ or IL-4-induced macrophages were quantified using a BCA protein assay kit (Beyotime Biotechnology, China). Absorbance was measured at 450 nm using an ELISA plate reader. All experiments were performed in triplicate. Experiments were repeated independently three times (biological replicates).

Statistical Analysis

Datasets obtained from the GEO database were analyzed by R software (version 4.2.3), while experimental data were analyzed using GraphPad Prism 8.4.0. Multiple group comparisons were assessed by one-way analysis of variance (ANOVA) followed by the post hoc Tukey's post hoc test. A *P*-value of less than 0.05 was considered statistically significant.

Results

The Overall Process of Experimental Design

We began by screening DEGs in RA synovial macrophages from the microarray dataset GSE97779, followed by GO and KEGG analyses to uncover enriched molecular pathways and functional categories (Figure 1). Ten hub genes were subsequently identified through Molecular Complex Detection (MCODE) in Cytoscape. Using single-

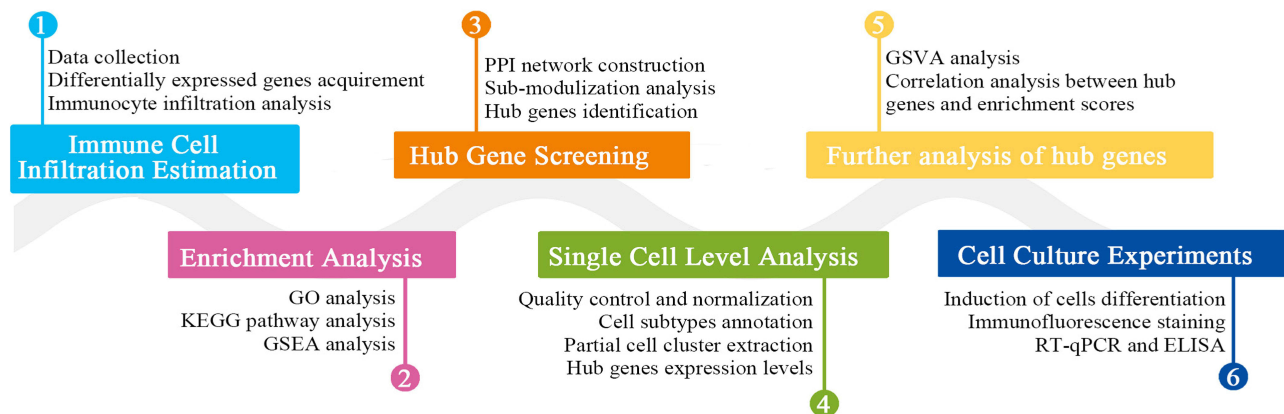


Figure 1 A flowchart showing work process.

cell RNA-Seq data from GSE192504, we estimated the composition of immune cell populations in the knee joints of CIA mice and pinpointed hub genes with specific expression in macrophages. The macrophage cluster was then extracted and further subdivided into distinct subtypes, followed by an analysis of hub gene expression across these subpopulations. We also computed the correlation between two macrophage-specific hub genes and immune-related biological processes by calculating Gene Set Variation Analysis (GSVA) enrichment scores using dataset GSE48780. Finally, through in vitro experiments, we examined alterations in macrophage subpopulations and the expression of two macrophage-enriched hub genes under different polarizing conditions.

Differentially Expressed Genes

To screen differentially expressed genes between the RA synovial macrophages and the primary human monocyte-derived macrophages (control), we analyzed microarray data from the GSE97779 dataset by calculating Log_2 fold change (FC) values and applying quality filters. A total of 334 genes met the criteria for differential expression ($|log_2FC| > 3$ and P-value < 0.05), comprising 222 upregulated and 112 downregulated genes (Supplementary Table S1). The results were visualized using a volcano plot (Figure 2B), and a two-dimensional heatmap was generated to show the Top50 DEGs (Figure 2A).

Functional Enrichment Analysis Based on DEGs

To better understand the biological processes and signaling pathways involved in DEGs between the RA and the control group, we conducted functional and pathway enrichment analyses. Gene Ontology (GO) analysis revealed that biological processes were significantly enriched in cytokine-mediated signaling pathways, cell migration (leukocyte, mononuclear cell), cell chemotaxis (leukocyte, neutrophil), and inflammatory response (Figure 3A). Cellular components were mainly the external side of the plasma membrane (Figure 3B). Molecular functions were primarily associated with cytokine and immune receptor (cytokine receptor, chemokine receptor, signaling receptor) binding and activity (Figure 3C). KEGG analysis showed that upregulated genes were significantly enriched in cytokine-cytokine receptor interaction, infection, TH17 cell differentiation, osteoclast differentiation, the Janus kinase/signal transduction and activator of transcription (JAK-STAT) signaling pathway, and IL-17 signaling pathway (Figure 3D). Subsequent GSEA further identified the above-mentioned biological processes and signal pathways (Figure 3E and F).

PPI Network Construction and Hub Gene Screening

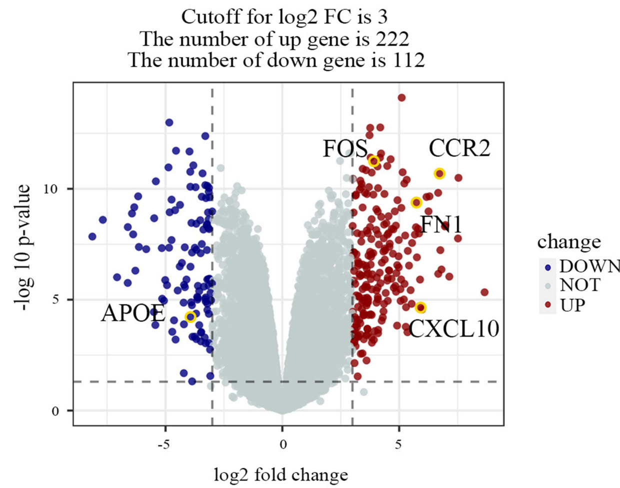
We constructed a PPI network of the DEGs using the STRING online database, which contained 252 nodes and 799 edges. The top four significant clusters within the network were identified and visualized using the MCODE plug-in (Figure 4A and B). The top 10 nodes with higher degrees were identified as the hub genes, including eight upregulated genes (*FN1*, *CXCL10*, *FOS*, *CCR2*, *GZMB*, *CD69*, *CXCL9*, *FCGR1A*) and two downregulated genes (*APOE*, *IGF1*) compared to the control group (Table 3 and Figure 4C).

High Immune Cell Heterogeneity in CIA Mice Synovial Tissue in scRNA-Seq Database

To assess the cellular heterogeneity in RA synovial tissue and examine the expression of hub genes across different immune cell types, we analyzed the knee synovial tissues of normal and CIA mice in the single-cell RNA-Seq database GSE192504. Following data preprocessing and quality control, 20 principal components were obtained (Figure 5A and B). Cells from synovial tissues of both groups were clustered into eight distinct populations via PCA and t-SNE analyses and subsequently annotated manually. Compared with the normal group, the CIA group exhibited a significant increase in the proportion and abundance of monocytes, macrophages, and fibroblasts (Figure 5C and D). We next extracted and analyzed the data from the CIA group to determine whether hub genes were specifically expressed in the macrophages of CIA mice's synovial tissue (Figure 5E). As a result, only the expression of *APOE* and *CCR2* was specifically increased in macrophages, as shown in Figure 5F.

Macrophage Clusters in CIA Mice's Synovial Tissue in the scRNA-Seq Database

Macrophages of different phenotypes exhibit distinct functional properties and play diverse roles in joint inflammatory responses. Re-clustering of macrophages extracted from the CIA group identified six distinct subtypes. Cells in clusters 0 and 2 were mainly located in the root of the phylogenetic tree, whereas clusters 3 and 4 were located



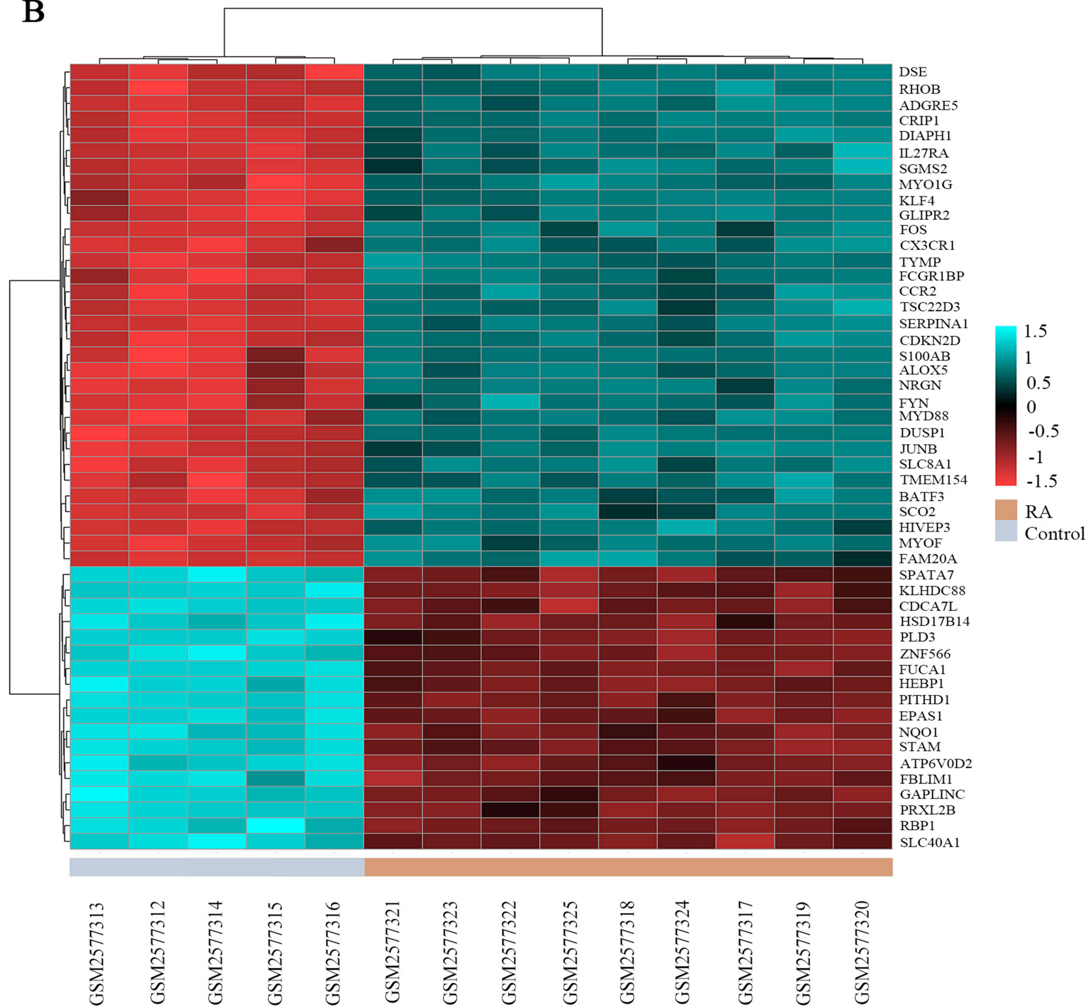


Figure 2 The differentially expressed genes (DEGs) and immune infiltration landscape in rheumatoid arthritis (RA) synovial macrophages group compared with the primary human monocyte-derived macrophages (control) group. (**A**) Hierarchical clustering heatmap of the top 50 DEGs in GSE97779. Blue, black, and red colors indicated high, intermediate, and low relative expression, respectively. (**B**) Volcano plot of 334 DEGs. Red, grey, and blue colors indicated upregulated, nonsignificant, and downregulated genes, respectively.

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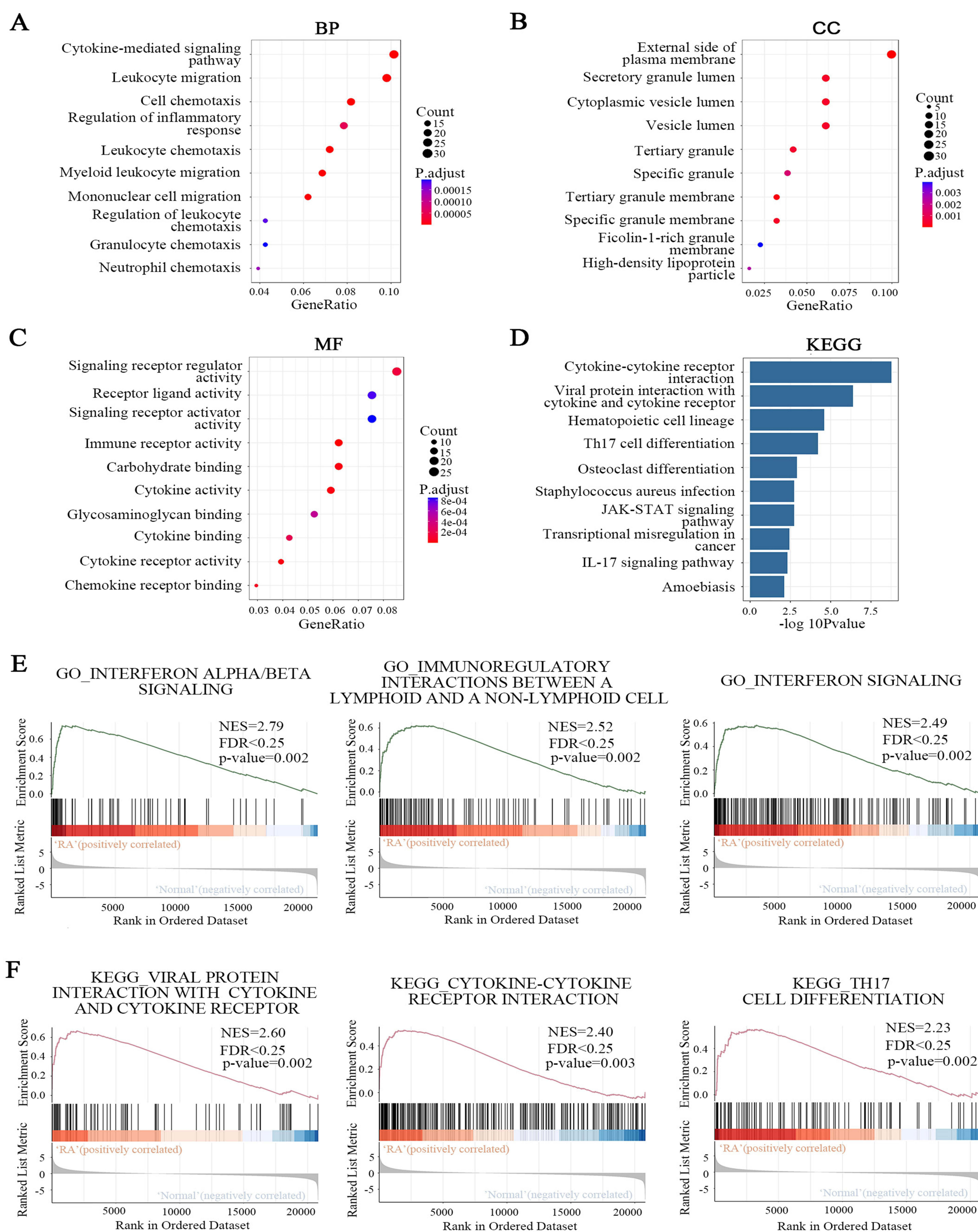

Figure 3 Functional and pathway enrichment analysis of DEGs in RA and Control group. (**A**–**C**) The Gene Ontology (GO) enrichment analysis covered three categories: biological process (BP), cellular component (CC), and molecular function (MF). (**D**) The top 10 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. (**E** and **F**) Functionally related gene sets identified by gene set enrichment analysis (GSEA).

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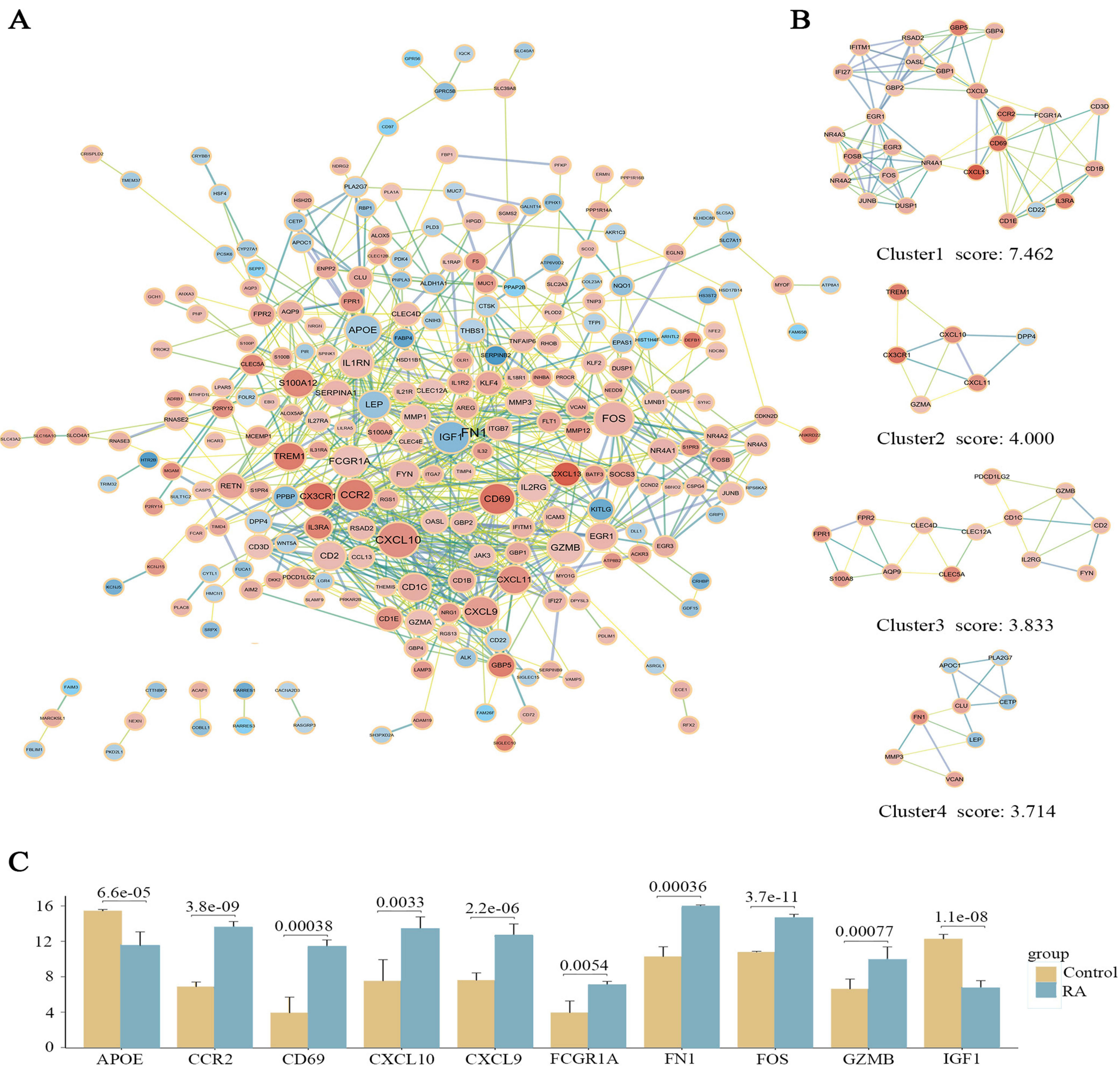

Figure 4 Protein-protein interaction (PPI) network and hub genes in RA synovial macrophages. (**A**) PPI network of DEGs determined by online STRING and Cytoscape 3.9.1. The interaction network consists of 252 nodes and 799 edges. Nodes represented proteins, while edges represented protein-protein associations. Blue and red circular nodes represented downregulated and upregulated genes, respectively. The higher the degree was, the larger the shape size. Yellow and blue edges represented low and high combined scores, respectively. (**B**) The top 4 cluster modules identified by the MCODE plugin. (**C**) Hub gene expression levels between RA and Control group.

along the lower branch, and cluster 1 along the right branches (Figure 6A and B). Interestingly, most genes enriched in cluster 1—including *SELENOP*, *C1QC*, *C1QA*, and *APOE*—were also expressed in cluster 0, though at moderately lower levels (Figure 6D and Supplementary Table S2). Cluster 2 had high expressions of *PDPN*, *CD36*, and *SPP1*, which are elevated in pro-inflammatory macrophages. Cluster 3 was regarded as the antigen-presenting cell with the enriched expression of the major histocompatibility complex II gene expression, like *H2-Aa*, *H2-Ab1*, and *CD74*, rather than falling into the M1/M2 classification of macrophage phenotype. The high expressions of *PLAC8* and *LY6C2* in cluster 4 indicated that this cluster might originate from monocytes. A set of genes closely related to osteoclasts, including *SLC9B2*, *CTSK*, and *ACP5*, was expressed in cluster 5. In addition, we also found that cluster 0 possessed the highest expression of *FOS* and *APOE* genes, while the highest upregulated genes in cluster 2 and cluster 4 were *FN1* and *CCR2*, respectively (Figure 6C).

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Table 3 10 Hub Genes Identified with CytoHubba

Gene Symbol	Description	logFC	Q Value	Regulation
FN1	Fibronectin-1	5.74	1.14e-07	Up
CXCL10	C-X-C motif chemokine ligand 10	5.92	0.0003	Up
FOS	FBJ murine osteosarcoma viral oncogene homolog	3.92	7.42e-09	Up
CCR2	C-C motif chemokine receptor 2	6.73	1.54e-08	Up
IGF1	Insulin-like growth factor 1	−5.49	3.48e-07	Down
GZMB	Granzyme B	3.34	0.0028	Up
APOE	Apolipoprotein E	−3.93	0.0007	Down
CD69	Cluster of differentiation 69	7.52	1.44e-06	Up
CXCL9	C-X-C motif chemokine ligand 9	5.08	3.30e-05	Up
FCGR1A	High-affinity immunoglobulin gamma Fc receptor I	3.17	0.0001	Up

The Correlation Between Hub Genes and Key Mechanisms Underlying Synovitis

Immune-mediated synovitis, characterized by inflammatory cell infiltration, angiogenesis, and synoviocyte proliferation, represents the pathological hallmark of RA. Given the specific expression of *APOE* and *CCR2* in macrophages, we examined the correlation between these two hub genes and the GSVA enrichment score of the immune mechanisms-related gene sets. As a result, *APOE* was negatively correlated with sprouting angiogenesis and inflammatory response, potentially through its involvement in negative regulation by reducing endothelial cell proliferation and inhibiting respiratory burst (Figure 7A and Supplementary Table S3). On the contrary, there was a trend for a positive correlation between *CCR2* and inflammatory response, likely mediated by its role in promoting the activation and migration of leukocytes and the production of inflammatory mediators (Figure 7B and Supplementary Table S4).

Changes in Macrophage Cluster Upon in Vitro Stimulation with LPS/IFN-γ or IL-4

To explore the changes in macrophage subpopulations upon pro- and anti-inflammatory polarization of RAW 264.7 cells in vitro, we utilized the immunofluorescence staining method to detect the protein levels of genes enriched in different macrophage clusters. Here, one representative high-variable gene was selected as a molecular marker for this cell cluster (macrophage clusters annotated to osteoclasts were not included for analysis). Cell clusters 0, 2, 3, and 4 were defined as $APOE^+$ macrophage, $PDPN^+$ macrophage, $CD74^+$ macrophage, and $PLAC8^+$ macrophage (Figure 8A). Although the markers in Clusters 0 and 1 appeared to be similar, they differed in gene expression levels. Immunofluorescence staining revealed weak APOE staining in RAW 264.7 cells stimulated with LPS/IFN-γ ($P < 0.001$), whereas IL-4-stimulated cells exhibited intense immunofluorescent staining of APOE and CD74 ($P < 0.001$) (Figure 8B). Even though both inductions resulted in enhanced PDPN ($P < 0.05$ or $P < 0.001$) and PLAC8 ($P < 0.001$) staining, the staining intensity was significantly higher after LPS/IFN-γ induction when compared with IL-4 ($P < 0.001$).

The Level of APOE and CCR2 in LPS/IFN-γ or IL-4-Stimulated RAW 264.7 Cells

The hub genes *APOE* and *CCR2* were not only essential for biologically relevant pathways and functions, but also specifically expressed in macrophages. We assessed their mRNA and protein expression levels in RAW 264.7 cells stimulated with LPS/IFN-γ or IL-4 using RT-qPCR and ELISA. As expected, the APOE level in RAW 264.7 cells decreased within 24 h of LPS/IFN-γ stimulation ($P < 0.01$), but a gradual increase was observed after IL-4 exposure ($P < 0.001$) (Figure 9A and B). Meanwhile, both LPS/IFN-γ and IL-4 stimulation for 24 hours resulted in increased CCR2 protein expression ($P < 0.001$ or 0.01), with LPS/IFN-γ inducing a stronger and more rapid upregulation compared to IL-4 ($P < 0.001$).

Discussion

There is increasing evidence to support the irreplaceable role of synovial macrophages in the pathogenesis of RA. Here, we identified two hub genes, *APOE* and *CCR2*, specifically expressed in synovial macrophages and found them to be

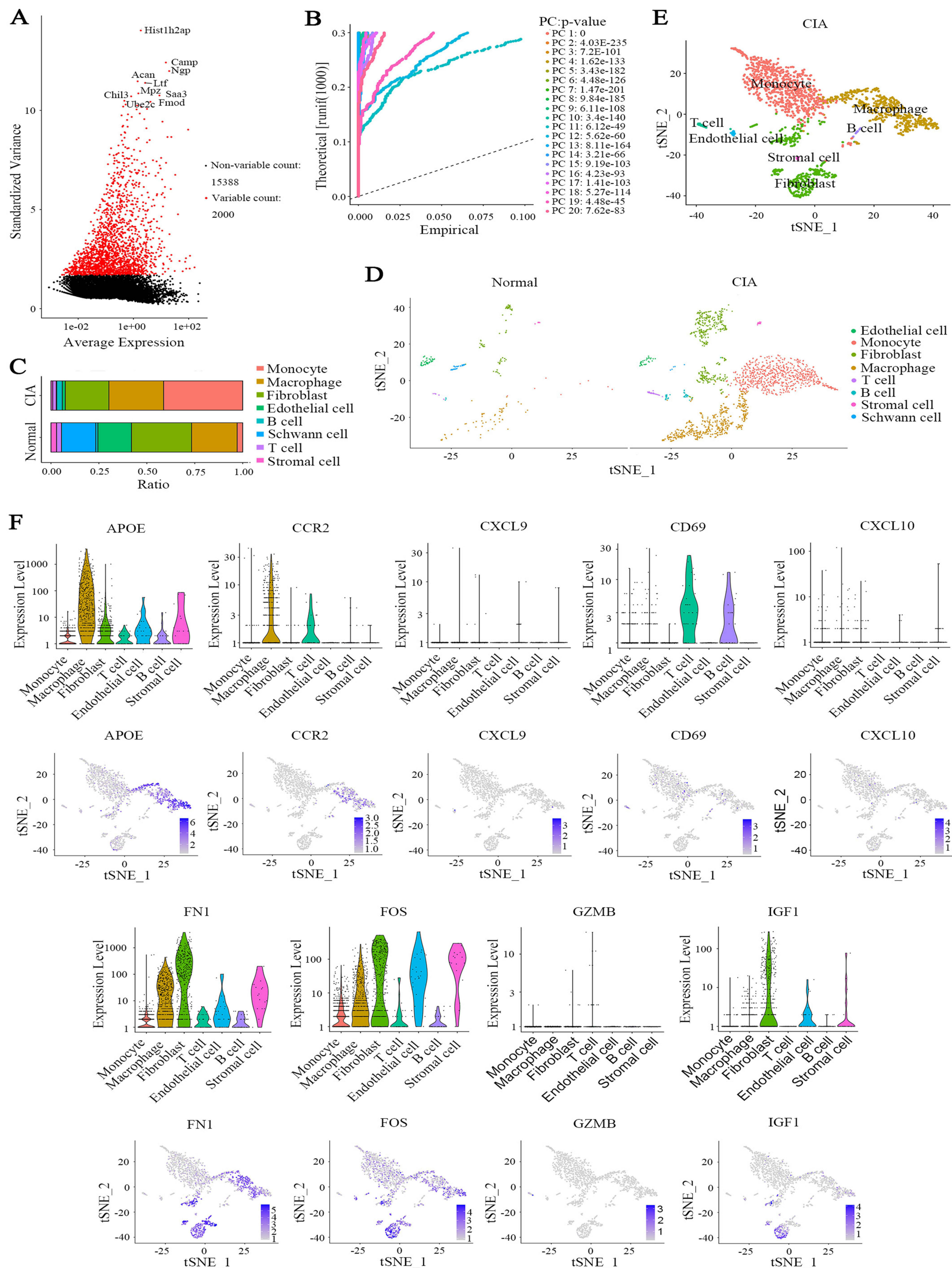

Figure 5 Preprocessing of single-cell RNA sequencing (scRNA-seq) dataset and identification of synovial cell in GSE192504 database. (**A** and **B**) Highly variable genes filtering and PCA clustering of scRNA-seq data on the synovial cells isolated from the collagen-induced arthritis (CIA) mice group and the healthy control mice (normal) group. (**C** and **D**) Annotations and the proportions of different cell types between the CIA and normal group. Monocytes, macrophages, and fibroblasts accounted for significant proportions of synovial cells in the CIA group compared to the normal group. (**E**) t-SNE projections and cell annotation of scRNA-seq data extracted from the CIA group. Synovial cells mainly included monocytes, macrophages, and fibroblast-like synoviocytes in the CIA group. Cell types were annotated based on canonical marker gene expression (see Methods). (**F**) The expression levels and distribution of 10 hub genes in different synovial cells of the CIA group. Violin plots and t-SNE plots both showed specifical high expression of *APOE* and *CCR2* in macrophages.

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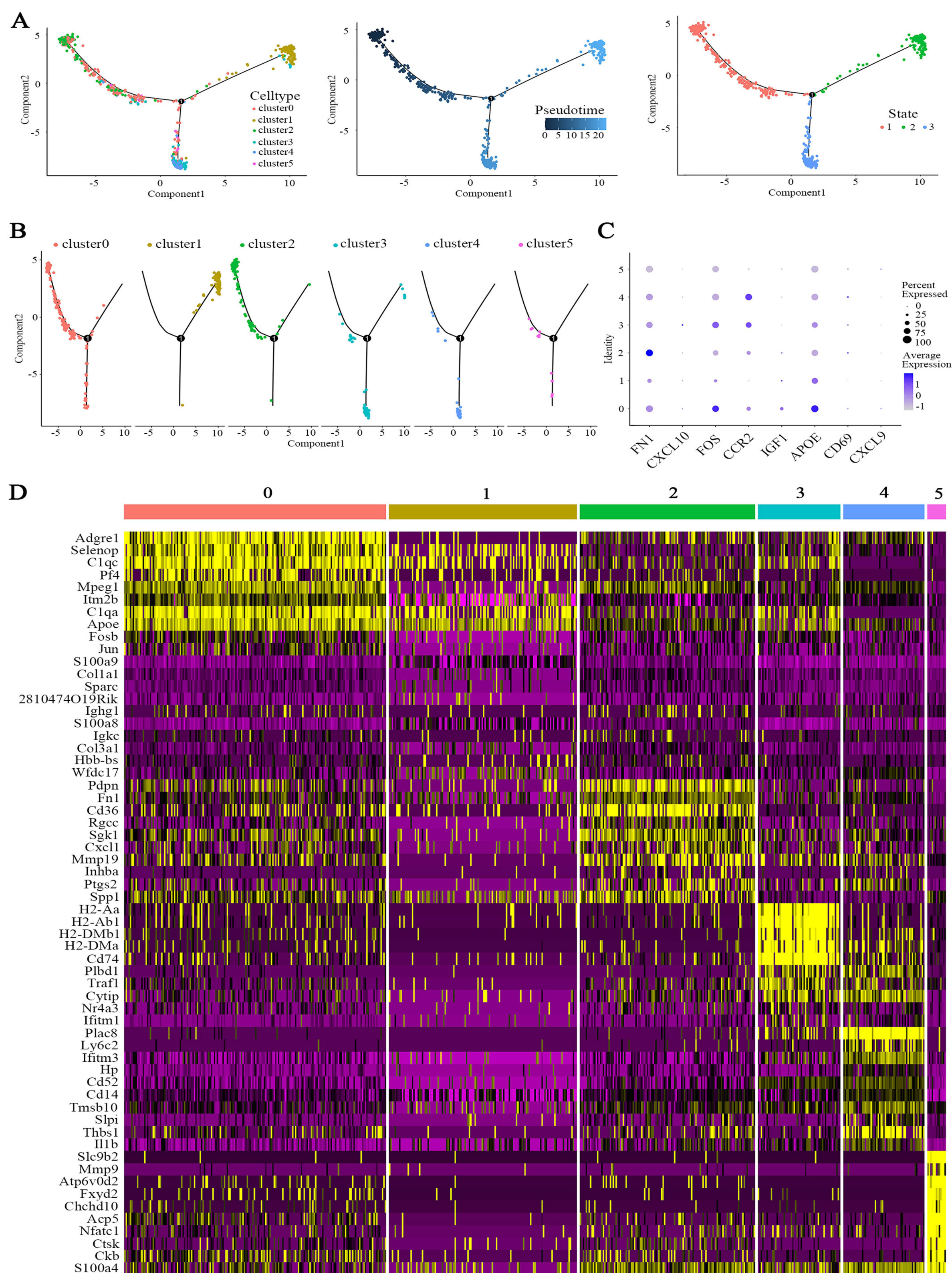

Figure 6 Clustering and cell trajectory analysis of macrophages. (**A**) Single-cell trajectory analysis of the clusters. The macrophages in the CIA group were divided into 6 cell clusters, which were in three states. (**B**) Cell trajectory analysis for each subpopulation. Clusters 0 and 2 were mainly located in the root, while other cell subtypes were distributed along branches, of which cluster 1 was located alone in one of the branches. (**C**) The expression levels of hub genes in each subpopulation. The lighter and darker color represented lower and higher expression in the dot plot, respectively, and dot size represented the average percentage of cells. (**D**) The heatmap of highly variable genes in each cluster.

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Figure 7 Correlation analysis between two hub gene expressions and GSVA immune-enrichment scores. (**A** and **B**) The heatmaps showed *APOE* and *CCR2* expression and functional enrichment scores of RA patients in the GSE48780 database. The Spearman R and P values were respectively visualized by using the right bar graphs and line graphs.

closely associated with the regulation of inflammatory and angiogenic responses, which are major pathological processes in RA. In addition, six macrophage subclusters were annotated, among which clusters 0 and 1 both had the protective gene *APOE* as a marker gene. Finally, we experimentally validated these two hub gene expression changes in macrophage cells under different stimulations in vitro.

Our study demonstrated that RA synovial macrophages were closely associated with cytokine-receptor interaction and migration of inflammatory mediators, thereby playing a positive regulatory role in inflammatory response. However, previous studies have established that macrophages not only drive joint inflammation but also promote tissue repair, due mainly to the great phenotypic and functional diversity of synovial macrophages in the immune response.[9,10] Moreover, macrophages undergo functional and phenotypic changes in response to microenvironmental alterations. Specifically, LPS/IFN-γ and IL-4 exposure respectively polarize macrophages toward pro-inflammatory (M1) phenotype characterized by high expression of CD86 and iNOS, and anti-inflammatory (M2) phenotype marked by elevated CD206 and CD163.[11,12] Therefore, we analyzed the distribution of synovial macrophage phenotypes in RA and observed a higher proportion of M1 macrophages and a lower proportion of M2 macrophages in RA patients compared to healthy subjects. These findings are largely consistent with earlier reports of M1/M2 imbalance in RA synovium.[13] A predominant infiltration of pro-inflammatory macrophages into the affected synovium represents an early hallmark of RA disease activity.[14] M1-like macrophages initially trigger an inflammatory response by releasing inflammatory mediators, including IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α), recruit neutrophils and monocytes through the release of chemokines CXCL8 and CXCL2, and stimulate synovial angiogenesis by the production of vascular endothelial growth factor (VEGF), culminating in continued joint inflammation and progressive bone erosions.[15,16] In contrast, M2-like macrophages correlate with reduced inflammation and disease activity,[17] which can be further divided into M2a, M2b, M2c, and M2d subcategories with highly functional diversity. M2a, M2c, and M2d subtypes contribute to wound resolution and tissue repair, whereas the M2b subtype has pro-inflammatory properties similar to the M1 subtype, such as secretion of IL-1β, TNF-α, and IL-6.[18] It is important to note that M1 and M2 subtypes just represent two

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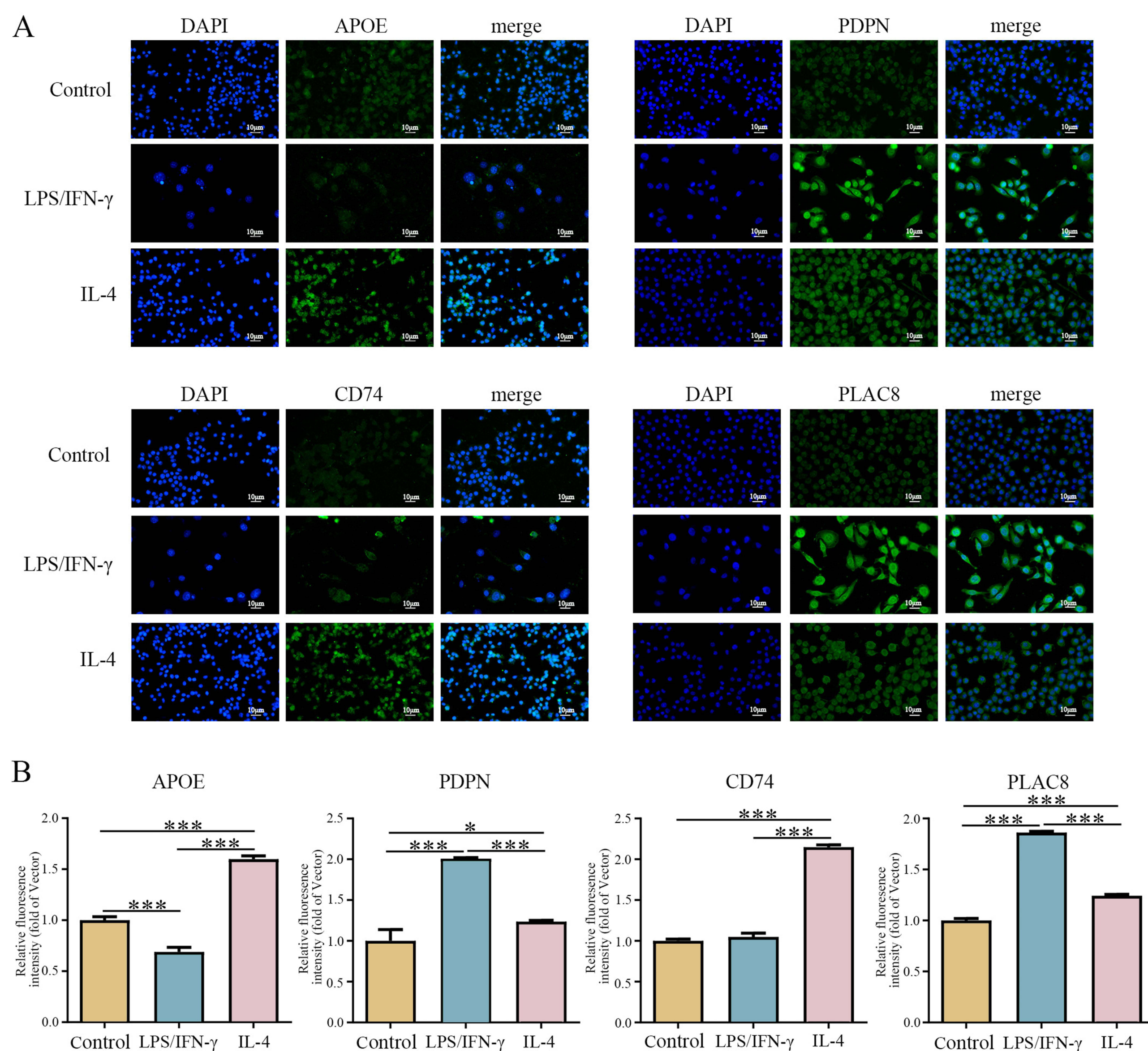

Figure 8 Changes in each macrophage cluster upon stimulation with LPS/IFN-γ or IL-4. (**A**) Representative images of immunofluorescence of each macrophage cluster. Blue represented the genetic material stained with DAPI and green showed fluorescence of APOE, PDPN, CD74, and PLAC8, which are regarded as specific protein markers of macrophage clusters. (**B**) Fluorescence intensity of APOE, PDPN, CD74, and PLAC8. Data are shown as mean ± SD from three independent biological experiments. *$P < 0.05$; ***$P < 0.001$.

extremes of a functional spectrum, and further research is required to explore macrophage phenotype switching in response to dynamic microenvironmental cues.

While considerable efforts have recently been directed toward discovering RA biomarkers for early diagnosis, outcome prediction, and disease monitoring, the understanding of macrophage-related biomarkers is still quite limited. In our study, we identified 10 hub genes (*FN1*, *CXCL10*, *FOS*, *CCR2*, *GZMB*, *CD69*, *CXCL9*, *FCGR1A*, *APOE*, and *IGF1*) through bioinformatic analysis, among which only *APOE* and *CCR2* were most abundantly and specifically expressed in synovial macrophages. However, the clinical relevance of these genes—specifically their relationship to disease activity or treatment response—could not be determined due to insufficient clinical metadata in the public datasets. Furthermore, the distribution of macrophage subclusters across different RA stages, such as early, active, or remission phases, could not be analyzed for the same reason. Future studies using well-annotated clinical cohorts are needed to validate their potential as clinically applicable biomarkers. Furthermore, while these two opposing hub genes

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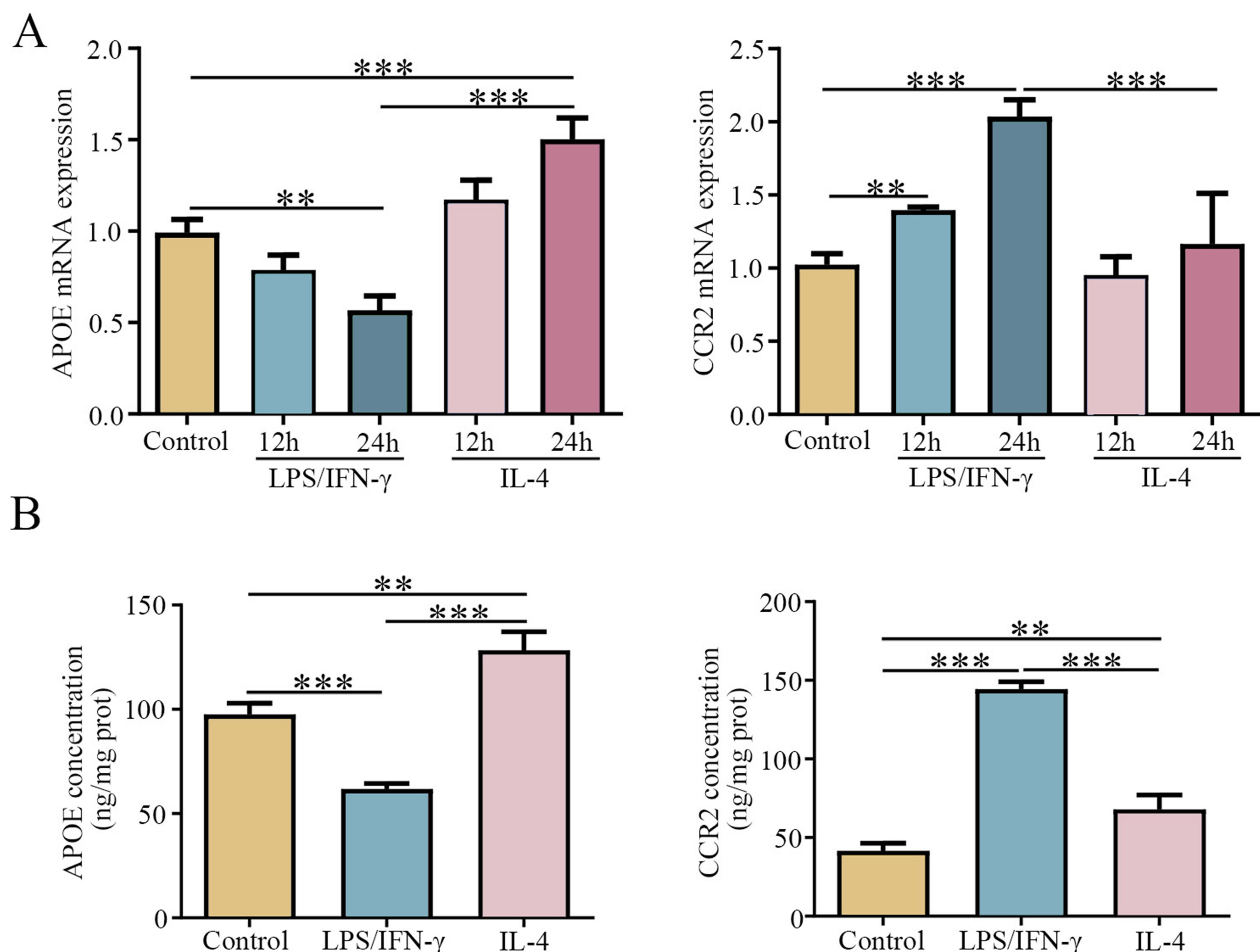

Figure 9 Gene and protein expression levels of APOE and CCR2 in LPS/IFN-γ or IL-4-stimulated RAW 264.7 cells. (**A**) Relative gene expression levels of *APOE* and *CCR2* at 12h and 24 h after the addition of LPS/IFN-γ or IL-4. (**B**) APOE and CCR2 protein levels analyzed by ELISA at 24h upon stimulation with LPS/IFN-γ or IL-4. Data are shown as mean ± SD from three independent biological experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

hold promise as effective biomarkers for RA, their potential requires further validation through functional studies—such as siRNA-mediated knockdown or overexpression—to fully elucidate their mechanistic roles in the disease.

APOE is a lipid transport protein that mediates low-density lipoprotein receptor-dependent lipoprotein clearance as the primary physiological function.[19] Current research has concentrated on the contribution of macrophage-derived APOE in protecting against lipid metabolism disorders such as hyperlipidemia and atherosclerosis.[20–22] Indeed, APOE can assume anti-inflammatory properties by mediating intracellular cholesterol efflux, thereby reducing lipid-driven monocytosis.[23] Our findings align with previous studies indicating that APOE is inversely correlated with acute inflammatory responses, potentially through its inhibition of respiratory burst. Besides serving the function of lipid-lowering and anti-inflammatory, APOE also modulates immune responses. It enhances apoptotic neuron phagocytosis by microglia, the resident macrophages of the central nervous system,[24] suppresses $CD4^+$ T cell activation and proliferation via macrophage extracellular vesicles,[25] and promotes macrophage polarization towards M2 phenotype along with production of anti-inflammatory cytokines.[26] Additionally, APOE expression in macrophages is regulated by environmental signals: TNF-α and IFN-γ inhibit APOE production, whereas TGF-β enhances its secretion.[27] In agreement, our in vitro experiments also established that diverse exogenous stimuli induce significant changes in APOE expression in macrophage cell lines. IL-4 polarization of RAW 264.7 cells toward the M2 phenotype significantly increased both mRNA and protein expression of APOE, whereas LPS/IFN-γ stimulation led to a decrease in APOE expression. These

data suggest that modulating APOE expression in macrophages to suppress inflammatory responses may represent a promising therapeutic strategy for RA.

C-C motif chemokine ligand 2 (CCL2) /CCR2 axis plays a major role in cell migration and invasion. CCR2-expressing macrophages, which are maintained through blood monocyte recruitment and proliferation, exhibit phenotypic and functional similarities to M1 macrophages and serve as key mediators of multiple tissue damage, including myocardial inflammation, tissue fibrosis, glomerular lesions, and spinal cord injury.[28–31] After tissue injury, $CCR2^+$ macrophages are recruited from peripheral blood to affected sites, where they participate in the initiation of inflammation.[32] In the context of RA, synovial fibroblasts and activated macrophages themselves can produce CCL2, creating a chemotactic gradient that guides CCR2-expressing monocytes into the joint, where they differentiate into inflammatory macrophages and perpetuate synovitis.[32,33] Beyond recruitment, CCR2 signaling may directly influence macrophage polarization and function. Evidence suggests that $CCR2^+$ monocytes are predisposed to differentiate into pro-inflammatory (M1-like) macrophages upon tissue entry, characterized by heightened production of cytokines such as TNF-α and IL-6, which are central to RA pathogenesis.[34,35] Furthermore, CCR2 has been implicated in other RA-relevant pathological processes, including the promotion of synovial angiogenesis and the enhancement of osteoclastogenesis, thereby potentially linking macrophage infiltration to both pannus formation and bone erosion.[36,37] Our in vitro data confirmed significant upregulation of CCR2 under pro-inflammatory (LPS/IFN-γ) stimulation, with a modest yet clear increase also observed under IL-4.This observation challenges a strict binary classification of CCR2 as an exclusive M1 marker and suggests a potential complex role beyond pure inflammation, possibly involved in the recruitment of macrophages necessary for the resolution phase or tissue remodeling, as indicated in some tissue repair models.[38,39] Furthermore, CCR2 is likely a marker of monocyte recruitment regardless of polarization, rather than a purely M1 marker. Therefore, CCR2 likely serves as a critical regulator at the intersection of immune cell recruitment, macrophage functional polarization, and tissue damage in RA.

As dynamically influenced by the environment, synovial macrophages exhibit remarkable functional diversity and undergo sequential phenotypic changes, which are essential to trigger the initiation and maintenance of RA. In this study, we identified six clusters of synovial macrophages from CIA mice synovial cells, using representative marker genes to infer their functional characteristics. Clusters 0 and 1, highly expressing complement-related genes (*C1qa*, *C1qc*) and hub gene APOE, were inferred to possess anti-inflammatory properties, albeit at different stages of cellular differentiation. Unlike other complement components such as C3a and C5a, C1q has the capability of promoting cell phagocytosis of targets and limiting inflammasome activation.[40] It also shifts the polarization of macrophages towards the M2 phenotype with increased secretion of anti-inflammatory cytokines.[40] Cluster 2 predominantly expressed *PDPN*, *CD36*, *SPP1*, and *MMP19*—genes linked to inflammatory infiltration and angiogenesis—suggesting its potential involvement in synovial inflammation and pannus formation in RA. Cluster 3 appeared to be an antigen-presenting cell that is neither M1 nor M2 phenotype, with abundant histocompatibility complex (MHC) class II genes (*H2-Aa*, *H2-Ab*, *CD74*). Such MHC class II-rich macrophages, which resemble dendritic cells, have been reported to be primarily responsible for antigen processing and presentation, as well as $CD4^+$ T cell activation and amplification of their effector functions.[41] Cluster 4 expressed inflammatory-related genes (*PLAC8*, *LY6C2*, *IL-β*) as did Cluster 2, but they were at the branch and root of the trajectory of synovial macrophage differentiation, respectively, implying that they were at different stages of differentiation. Cluster 5 was considered likely to be osteoclasts or pre-osteoclasts; this could be attributed to its significant expression of *ACP5*, *CTSK*, and *SLC9B2*, which are phenotypic markers of osteoclasts. We subsequently performed in vitro experiments to verify the speculation. Stimulation of RAW 264.7 cells with LPS/IFN-γ reduced the proportion of $APOE^+$ macrophages, while increasing the prevalence of $PDPN^+$ and $PLAC8^+$ macrophages. In contrast, the expression of *APOE* and *CD74* was increased following IL-4 stimulation of a macrophage cell line. Given significant difference the distribution of $APOE^+$ macrophages exposed to diverse stimuli, it holds particular promise as a promising therapeutic target.

While this study provides novel insights into macrophage-associated hub genes in rheumatoid arthritis, several limitations should be acknowledged. First, we identified candidate hub genes from human RA synovial macrophages (GSE97779) but performed functional validation in murine systems (the CIA model and RAW 264.7 cell line). We acknowledge that interspecies differences in immunology and disease pathophysiology could affect the direct translatability of findings. The CIA model, while a well-established surrogate for human RA synovitis and bone erosion, does not fully replicate the complex autoimmune etiology of the human disease. Similarly, the RAW 264.7 cell line, although a standard in vitro model for macrophage polarization, is a leukemic, virus-transformed line and may not fully replicate the complex behavior of primary

human synovial macrophages. Moreover, the LPS/IFN-γ stimulus used herein models acute septic inflammation via PAMP recognition, which differs from the sterile inflammation characteristic of RA; nevertheless, as a widely adopted protocol, it robustly induces a conserved pro-inflammatory transcriptional program, allowing assessment of gene responses within a defined inflammatory context. The primary goal of employing these systems was to provide in vivo and in vitro proof-of-concept that these genes are dynamically regulated within an inflammatory arthritic context. Future studies must verify their expression patterns and therapeutic relevance directly in human RA synovial tissue and primary human macrophages. Second, the absence of detailed clinical metadata in publicly available datasets limited our ability to correlate *APOE* or *CCR2* expression with disease severity, prognosis, or treatment response. Prospectively collected, well-annotated human cohorts are needed to establish the clinical relevance of these candidate biomarkers. Third, the distribution of these subsets across different disease stages—such as early, active, or remission phases—as well as their correlation with treatment response, could not be assessed due to data availability. Future single-cell analyses should include samples from different disease phases. Finally, functional experiments such as genetic manipulation are required to establish causal roles for *APOE* and *CCR2* in RA pathogenesis. Despite these limitations, our findings provide a foundation for further investigation into macrophage-specific biomarkers in RA.

Conclusion

Taken together, this bioinformatics-driven study identified *APOE* and *CCR2* as key hub genes associated with synovial macrophages in RA, showing strong links to inflammatory responses. We further characterized macrophage heterogeneity by annotating six distinct clusters. Our findings, derived from and validated in murine models, nominate *APOE* and *CCR2* as high-priority candidate biomarkers. Their clinical translation requires definitive validation in human RA synovial tissues.

Data Sharing Statement

All data are in the manuscript and/or Supporting Information Files. Further inquiries can be addressed directly to the corresponding author.

Ethics Statement

This study utilized publicly available datasets from the Gene Expression Omnibus (GEO) database. No new human or animal samples were collected for this study. According to the National Health Commission of China and the Ethical Review Measures for Biomedical Research Involving Human Subjects, retrospective analyses of publicly available genomic data do not require explicit ethical approval from an institutional review board (IRB) or ethics committee. All experimental procedures involving RAW264.7 cells were conducted in accordance with relevant guidelines and regulations.

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Author Contributions

Dongyi Wang: Conceptualization, Writing – original draft, Funding acquisition; Le Lu: Data curation, Writing – original draft, Formal analysis; Yuping Zhang: Writing – original draft, Software, Visualization; Wei Shang: Supervision, Methodology, Writing – review and editing. All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare there are no competing interests in this work.

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