Original Article

Single-cell analysis reveals the prognostic role of immune escape in the colorectal cancer microenvironment

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Background: Colorectal cancer (CRC) is a malignant disease that poses a significant threat to human health; however, early diagnostic and treatment strategies for it remain limited. Immune evasion is a critical factor contributing to treatment failure in CRC. Various cell subtypes within the tumor microenvironment (TME) play essential roles in this process. However, there is currently a lack of a systematic and novel classification of immune evasion-related cell subtypes and an analysis of their dynamic interaction networks within the CRC TME. This study aims to explore a novel classification of immune evasion-related subtypes in CRC, elucidate their underlying mechanisms, and assess their value for immunotherapy and prognosis.

Methods: This study investigates immune escape-related gene expression profiles utilizing single-cell RNA sequencing (scRNA-seq), which were subsequently validated through multiple immunohistochemistry (mIHC) techniques. Non-negative matrix factorization (NMF) clustering was employed to identify novel subtypes associated with immune escape. Additionally, CellChat and pseudotime analysis were utilized to explore intercellular interactions and differentiation pathways. Kyoto Encyclopedia of Genes and Genomes (KEGG), Single-Cell Regulatory Network Inference and Clustering (SCENIC), and immune checkpoint analyses were conducted to elucidate the functional characteristics of these novel subtypes. Furthermore, Cox proportional hazards regression analysis and Kaplan-Meier survival analysis were performed to assess the response to immunotherapy and prognosis.

Results: The expression profile of immune escape-related genes in the TME of CRC was initially plotted. This analysis identified 11 distinct types of immune escape-related cells in the TME, and confirmed that TGF-β$^+$JAK1$^+$Calretinin$^+$ could serve as a candidate cell marker for the immunosuppressive state of the TME. Furthermore, novel subtypes of cancer-associated fibroblasts (CAFs), CD8$^+$ T cells, macrophages, and B cells were identified. These subtypes exhibit unique gene expression profiles and functional characteristics associated with CRC immune escape. The cell interaction network, formed by these subtypes and other cells within the TME, facilitates CRC immune escape by reshaping the immunosuppressive microenvironment.

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Additionally, CFLAR$^+$B_cells-C3, CALR$^+$CD8$^+$T_cells-C2, and TAP1$^+$Mac-C2 may serve as potential biomarkers for predicting responses to immunotherapy in CRC patients. In contrast, HEXIM1$^+$CAF-C1 may act as an independent risk factor for poor prognosis in CRC.

Conclusions: Our findings enhance understanding of immune escape mechanisms in CRC, show how novel subtypes affect prognosis, and offer insights for new diagnostic and treatment strategies.

Keywords: Colorectal cancer (CRC); immune escape; tumor microenvironment (TME); prognosis; single-cell

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Introduction

Colorectal cancer (CRC) represents a significant global health challenge, being the third most common malignant tumor worldwide and the second leading cause of cancer-related mortality. The incidence rate of CRC continues to rise in many countries (1-4), particularly in recent years, with a notable increase among younger populations (5,6). Despite advancements in diagnosis and treatment, the prognosis for CRC remains poor; only 20% to 35% of patients survive beyond three years post-diagnosis, and fewer than 20% survive for five years (7,8). Therefore, it is crucial to explore the biological mechanisms underlying the occurrence and progression of CRC, as well as to identify novel biomarkers that may enhance the prognosis for CRC patients.

The tumor microenvironment (TME) represents a dynamic and heterogeneous ecosystem comprising immune cells, stromal cells, and extracellular matrix components. It plays a critical role in the development and progression of CRC. Various cell types within the TME interact with CRC cells by secreting chemokines, growth factors, and cytokines, which are essential for CRC proliferation, metastasis, angiogenesis, and immune evasion (9-11). Immune evasion is a hallmark of advanced CRC and contributes to treatment resistance (12). The primary mechanism of CRC immune evasion involves tumor cells suppressing T-cell activity through immune checkpoint pathways, such as programmed cell death protein-1 (PD-1) and its ligand programmed death ligand 1 (PD-L1), as well as cytotoxic T-lymphocyte antigen-4 (CTLA-4). Concurrently, tumor cells remodel the TME via cytokines like transforming growth factor beta (TGF-β) and interleukin-10 (IL-10) (13,14). Additionally, this study found that key cell types within the TME exhibit distinct functions. Tumor-associated macrophages (TAMs) inhibit T-cell activation and proliferation by binding to CTLA-4 ligands on T cells through CTLA-4 expression; they can also secrete IL-10 and TGF-β via M2 polarization, collectively promoting immune suppression (15,16). Cancer-associated fibroblasts (CAFs) remodel the extracellular matrix by secreting collagen and C-X-C motif chemokine ligand 12 (CXCL12), creating a physical barrier that impedes immune cell infiltration (17-19). Although existing studies have partially elucidated the role of these cells in immune evasion, there remains a lack of systematic

Highlight box

Key findings

- This study mapped immune escape-related gene expression in the colorectal cancer (CRC) tumor microenvironment (TME), confirming TGF-β$^+$JAK1$^+$Calretinin$^+$ as a candidate cell marker for the immunosuppressive TME state. Novel subtypes of cancer-associated fibroblasts (CAFs), CD8$^+$ T cells, macrophages, and B cells were identified. A cell interaction network involving these subtypes facilitates CRC immune escape by reshaping the immunosuppressive microenvironment. CFLAR$^+$B_cells-C3, CALR$^+$CD8$^+$T_cells-C2, and TAP1$^+$Mac-C2 were identified as potential biomarkers for predicting immunotherapy response, while HEXIM1$^+$CAF-C1 emerged as an independent risk factor for poor CRC prognosis.

What is known and what is new?

- Immune escape is a critical barrier to effective CRC treatment, with the TME playing a pivotal role through complex cellular and signaling networks.
- This study provides novel insights into the cellular and molecular mechanisms of immune escape in CRC, identifying specific cell subtypes and interaction networks that contribute to this process. It also proposes new biomarkers for predicting immunotherapy response and prognosis.

What is the implication, and what should change now?

- These findings enhance our understanding of CRC immune escape mechanisms, offering potential targets for diagnostic and therapeutic intervention.

classification of immune escape-related cell subtypes and analysis of dynamic interaction networks within the CRC TME. This gap poses a significant obstacle to further elucidating the underlying molecular mechanisms and developing more effective diagnostic and therapeutic approaches for CRC.

Single-cell RNA sequencing (scRNA-seq) enables the analysis of tissue and cell heterogeneity at the single-cell level, serving as a revolutionary tool for elucidating disease mechanisms (20-22). The scRNA-seq technology has significantly advanced our understanding of the intratumoral heterogeneity of CRC by identifying key cells and molecular markers that drive CRC progression, as well as elucidating the remodeling of the CRC microenvironment. Existing studies have demonstrated that scRNA-seq can reconstruct the lineage structure and evolutionary tree of tumor cells, revealing the dynamic relationship between CRC and T cells (23). Additionally, a specific type of polyp cell with stem cell characteristics has been identified, which promotes both early formation and late-stage invasive progression of CRC through the expression of tumorigenesis-related genes such as KRAS and APC (24). Utilizing scRNA-seq technology, cells such as $EMP1^+$, $PTPRO^+$, and $ASCL2^+$ have been identified as biomarkers for cell metastasis and stemness (25,26), while $SPP1^+$ macrophages and $LAMP3^+$ dendritic cells have been recognized as important cellular subtypes involved in tumor immunity (27). However, there remains a significant gap in the further analysis of single-cell transcriptomic data to elucidate the regulatory networks and interaction mechanisms among different cells within the CRC immune microenvironment. Furthermore, the classification of CRC immune subtypes remains relatively ambiguous. Additionally, the distribution of immune escape-related genes across different subtypes, along with their potential roles and clinical implications, remains largely unexplored. This gap in knowledge impedes the stratified diagnosis and personalized treatment of CRC patients.

To investigate the intricate relationship between immune escape mechanisms and the TME in CRC, we employed scRNA-seq to delineate the expression profiles of immune escape-related genes within the CRC TME. For the first time, we systematically elucidated the interaction patterns and potential functional specialization of novel immune escape-associated cellular subtypes in the CRC TME, while also exploring potential cellular markers indicative of immunosuppressive states within this environment. This study addresses a significant gap in existing research by providing a comprehensive lineage analysis of cellular subtypes within the CRC TME. The aim is to establish a novel theoretical foundation for the development of advanced diagnostic tools and targeted therapeutic strategies for CRC. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-2025-1466/rc).

Methods

Single cell sequencing data collection and processing

We downloaded scRNA-seq data of CRC patients from the NCBI Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov) (dataset GSE166555) (28). This dataset comprises 25 samples, including 13 CRC samples and 12 adjacent non-cancerous samples. The scRNA-seq gene expression matrix was processed using the R package "Seurat", and stringent quality control measures were implemented to eliminate low-quality cells. The screening criteria included unique molecular identifier (UMI) counts below 200, gene expression in fewer than 200 or more than 8,000 cells, and mitochondrial UMI contributions exceeding 10% of the total UMI. The R package "Harmony" was employed to correct for batch effects. Two thousand highly variable genes (HVGs) were selected, and dimensionality reduction was performed using t-distributed stochastic neighbor embedding (t-SNE) and principal component analysis (PCA). Visualization techniques, including uniform manifold approximation and projection (UMAP) and t-SNE, were utilized.

Bulk RNA-seq data collection and processing

Bulk RNA-seq data and clinical information were obtained from the GEO under dataset GSE39582 (29) and The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/ccg/research/genome-sequencing/tcga), comprising 689 and 579 CRC cases, respectively.

Non-negative matrix factorization and subtype identification of immune escape-related genes

We refer to the method described by Puram and Chen (30,31), as follows: to analyze immune escape-related gene expression within the TME, we constructed an expression matrix encompassing 182 immune escape-associated genes, focusing exclusively on cells expressing these genes to refine

the dataset. Matrix decomposition was performed using the 'non-negative matrix factorization (NMF)' package, which separated the gene expression data into basis vectors that represent underlying gene expression patterns and coefficients indicating the contribution of these patterns to individual cells. Subsequently, dimensional reduction and clustering techniques were applied to group cells with similar immune escape-related gene expression profiles, thereby identifying distinct subpopulations within the TME. Then, based on the logarithmic fold change in gene expression (logFC), NMF subgroups were classified more accurately. Clusters with $|logFC| \geq 1$ were marked as immune escape characteristic gene clusters, while clusters with $|logFC| < 1$ were classified as unclear immune escape gene clusters.

Cell-cell communication analysis

Utilizing the CellChat package (version 1.1.3) for intercellular ligand-receptor analysis, we investigate potential interactions between cells. We construct ligand-receptor interactions based on the CellChatDB human database and utilize secreted signals as the foundation for our intercellular communication analysis (28,31,32). Genes exhibiting elevated expression levels are extracted to identify the corresponding ligand-receptor pairs. We employ the functions 'netAnalysis_computerCentricity' and 'netAnalysis_cistribution' to compute the network centrality scores and assess the contribution of each ligand-receptor pair to the signaling pathway. Interactions with a P value below 0.05 and an average logarithmic fold change greater than 0.1 are deemed significant. Subsequently, these identified pairings are mapped onto protein interaction networks for weighted analysis to evaluate their functional significance.

Pseudo-temporal trajectory analysis of immune escape-related genes in TME cells

We utilized the Monocle R package (version 1.0.0) for single-cell trajectory analysis. The preprocessing steps involved filtering cells based on mean expression levels and empirical dispersion metrics, thereby ensuring the inclusion of high-quality data for downstream analysis. Dimensionality reduction was performed using the 'DDRTree' method to effectively map the developmental trajectories of distinct TME cell subtypes. Subsequently, immune escape patterns were visualized along these trajectories within the clusters defined by NMF.

Functional enrichment analysis of immune escape gene-related subtypes

We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the differentially expressed genes (DEGs) using the clusterProfiler package (version 3.12.0), with statistical significance set at $P<0.05$. The enrichment score analysis was conducted to evaluate the functional characteristics of various subtypes. Additionally, we utilized the 'immune escape' function to randomly generate 30 enrichment scores corresponding to different signaling pathways related to immune escape.

Single-Cell Regulatory Network Inference and Clustering (SCENIC) analysis for immune escape-related subtypes

We ranked scRNA-seq data utilizing gene motifs derived from the RcisTarget database (hg19-tss-centered-10kb-7species). Subsequently, we employed SCENIC to infer transcription factor (TF) activity and the genes they directly regulate across various subtypes. Our analysis centers on transcription start sites (TSS) and gene regulatory networks. The criteria for gene screening necessitate that genes be expressed in at least 1% of cells and contribute to more than 3% of total cell expression. We elucidate regulatory relationships through correlation matrices and TF target regression analyses. To identify cell type-specific regulatory factors, we calculated a regulatory factor-specific score (RSS) using a z-score threshold of 1.5, plotted TF activity maps with FeaturePlots, and generated heatmaps specific to regulatory factors. We utilized R packages such as 'ComplexHeatmap', 'ggplot2', and 'pheatmap' for data analysis and visualization.

Immunotherapeutic response of NMF immune escape-related subtype cells

To evaluate treatment response predictions, we applied Tumor Immune Dysfunction and Exclusion (TIDE) analysis to CRC transcriptomic datasets obtained from the TIDE database. This approach facilitated the assessment of immune escape-related subtypes in the context of therapeutic efficacy. Furthermore, we analyzed the IMvigor210 immunotherapy dataset to investigate the immune response characteristics of the NMF-defined immune escape-related subtypes, thereby providing valuable insights into their potential impact on immunotherapy outcomes and patient stratification.

Prognostic analysis

We use gene set variation analysis (GSVA) to calculate genetic marker scores. Log rank test and Cox proportional hazards regression analysis were used to evaluate the relationship between subtypes and overall survival (OS). Draw Kaplan-Meier survival curves using the R package "survminer" and "ggplot2".

Human patient samples

A total of three CRC resection samples were obtained from The First Affiliated Hospital of Hunan Traditional Chinese Medical College (Hunan Provincial Directly Affiliated Hospital of Traditional Chinese Medicine). Tissues were collected under an approved protocol. All CRC patients had not received any treatment prior to sample collection. This study was reviewed and approved by The First Affiliated Hospital of Hunan College of TCM (No. KY-2025042902). Written informed consent forms were obtained from all patients before tissue collection. All experiments conducted in this study adhered to the ethical guidelines of the Helsinki Declaration and its subsequent amendments.

Multiplex immunohistochemistry (mIHC)

According to the protocol of the Opal 7-Color kit (Panovue, Beijing, China), mIHC staining was performed on formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from patients with CRC. The antibodies utilized in this study include TGF-β (Abcam, Waltham, MA, USA), JAK1 (Abcam), Calretininin (Abcam), and DAPI (Abcam). For each staining process, secondary horseradish peroxidase-conjugated antibodies (Panovue) were incubated with casein-conjugated fluorescent groups. Selected mIHC image regions were captured using the VP tissue imaging system (Akoya Biosciences, Marlborough, MA, USA). Image analysis was conducted using IHalo software (Indica Labs, Albuquerque, NM, USA).

Statistical analysis

Statistical analyses were conducted using methods such as the Student's *t*-test, Wilcoxon rank-sum test, and Kruskal-Wallis test. All analyses were performed using R software (version 4.1.2). Statistical significance was defined as $P<0.05$.

Results

The landscape of immune escape-related genes in TME cells in CRC

To investigate immune escape-related genes in CRC, we analyzed single-cell data from 13 tumor and adjacent normal tissue samples, supplemented by two prognostic datasets for CRC and one dataset related to immunotherapy. A high-quality single-cell transcriptome dataset comprising 66,050 cells was generated by evaluating gene expression levels and mitochondrial content through rigorous quality control measures, followed by cell counting (Figure S1A,S1B). We selected a set of 2,000 HVGs for downstream processing to capture key transcriptional differences (Figure S1C). Cell harmonization was performed to enhance consistency, and sample distributions were visualized using UMAP (Figure S1D). After removing batch effects and conducting dimensionality reduction, 27 distinct cell clusters were identified (*Figure 1A*).

Based on the established marker genes, 11 cell types were identified, including epithelial cells, endothelial cells, goblet cells, intestinal cells, B cells, T cells, natural killer (NK) cells, macrophages, plasma cells, fibroblasts, and mast cells (*Figure 1B*). The dot plot of feature genes provides additional insights into the harmonization, distribution, and annotation of cell types, ensuring the comprehensiveness and reliability of the analysis (Figure S1E-S1G). The heatmap illustrates the expression of marker genes across different cell types (*Figure 1C*). These 11 distinct cell types exhibit varying tissue proportions between tumors and normal tissues, particularly in fibroblasts and macrophages (*Figure 1D*). A heatmap was employed to display the activity of 171 immune escape-related genes across different cell types, revealing that in the CRC microenvironment, each cell type possesses unique biological functions and participates in CRC immune escape through specific regulatory mechanisms (*Figure 1E*).

To validate the results of the bioinformatics analysis, mIHC techniques were employed to detect the expression of candidate immune escape-related gene markers in tissue samples from different CRC patients. The analysis revealed that TGF-β^+JAK1$^+$Calretinin$^+$ cells exhibited significantly enhanced expression in CRC tissues (*Figure 2*), indicating that the TME is in a highly immunosuppressive state. This finding further corroborates the reliability of the analytical results.

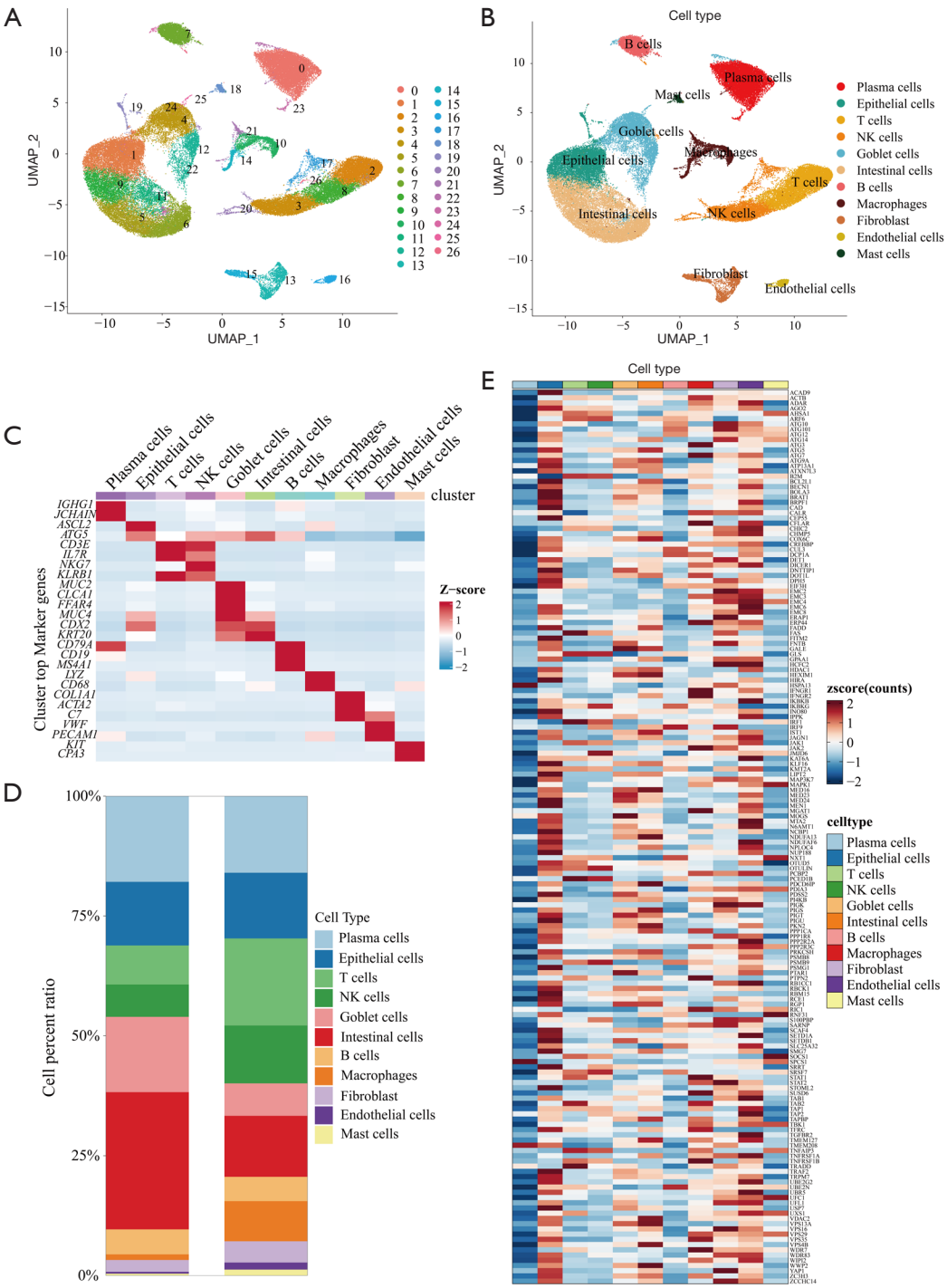

Figure 1 Overview of immune escape-associated genes in single-cell data from CRC. (A) UMAP representation displaying 27 distinct cell clusters related to the CRC microenvironment. (B) UMAP plot displaying 11 major cell types in CRC. (C) Heatmap illustrating gene expression markers across various cell types in CRC. (D) Bar chart depicting the ratio of cell types between normal and CRC tissues. (E) Heatmap showing the distribution of immune escape-related genes across 11 identified cell types. CRC, colorectal cancer; NK, natural killer; UMAP, uniform manifold approximation and projection.

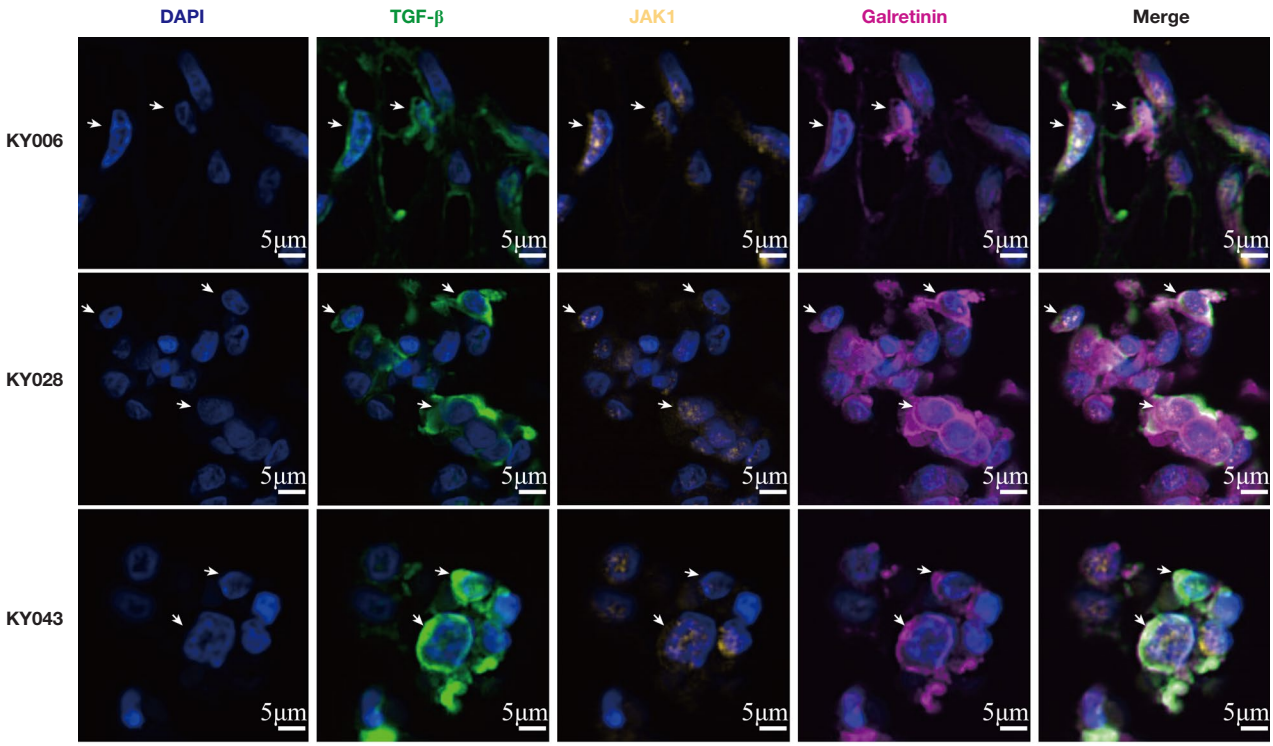

Figure 2 mIHC staining image of CRC tissues. mIHC staining of TGF-β$^+$ (green), JAK1$^+$ (yellow), and Calretininin$^+$ (pink), and DAPI (blue) in CRC tissues. Arrows indicate positive CRC cells. Images were captured at an original magnification of ×20, with a scale bar representing 5 μm. CRC, colorectal cancer; DAPI, 4',6-diamidino-2-phenylindole; mIHC, multiple immunohistochemistry; TGF-β, transforming growth factor beta.

Novel immune escape-related gene CD8$^+$ T cell subtypes and their roles in the CRC microenvironment

To investigate the role of T cells in the CRC TME, we initially categorized T cells into four groups: CD4$^+$, CD8$^+$, regulatory T cells (Treg), and unidentified T cells. Given the critical function of tumor-specific CD8$^+$T cells in anti-tumor immunity (33), we further subdivided them into seven distinct clusters using NMF clustering (*Figure 3A*). Subsequently, we classified these clusters into five novel subtypes based on the expression of immune escape genes: SOCS1$^+$CD8$^+$T_cells-C1 (CD8$^+$T-C1), CALR$^+$CD8$^+$T_cells-C2 (CD8$^+$T-C2), PCBP2$^+$CD8$^+$T_cells-C3 (CD8$^+$T-C3), JAK1$^+$CD8$^+$T_cells-C4 (CD8$^+$T-C4) and non-immune_evasion-CD8$^+$T_cells-C5 (CD8$^+$T-C5) (*Figure 3B*). The proportion of these five subtypes varies in the tissues of different CRC patients, reflecting differences in immune escape mechanisms among individuals (*Figure 3C*). The proportions of these five subtypes vary among the tissues of different CRC patients, reflecting the variations in immune escape mechanisms among individuals.

CellChat analysis revealed complex and extensive intercellular communication among the five CD8$^+$ T cell subtypes and various cell types, including epithelial cells, goblet cells, enterocytes, and endothelial cells. Notably, CD8$^+$ T-C1/C4 exhibited active expression of TGF-β signaling molecules, potentially facilitating communication with themselves or neighboring cells via the TGF-β/TGF-β RII axis, thereby activating the downstream SMAD signaling pathway. This process directly inhibits CD8$^+$ T cell proliferation and cytotoxicity, contributing to the establishment of an immune-tolerant microenvironment (34). The incoming signaling patterns of CD8$^+$ T-C1/C3 are enriched with Midkine (MDK), which may activate the PI3K-AKT signaling pathway through the MDK/PTPRZ1 axis, inducing metabolic reprogramming and T cell exhaustion (35). Additionally, the outgoing signaling patterns of CD8$^+$ T-C3 are abundant in CXCL and demonstrate the closest communication with epithelial and endothelial cells, suggesting that CXCL/CXCR 3/4 plays a role in remodeling the TME and suppressing

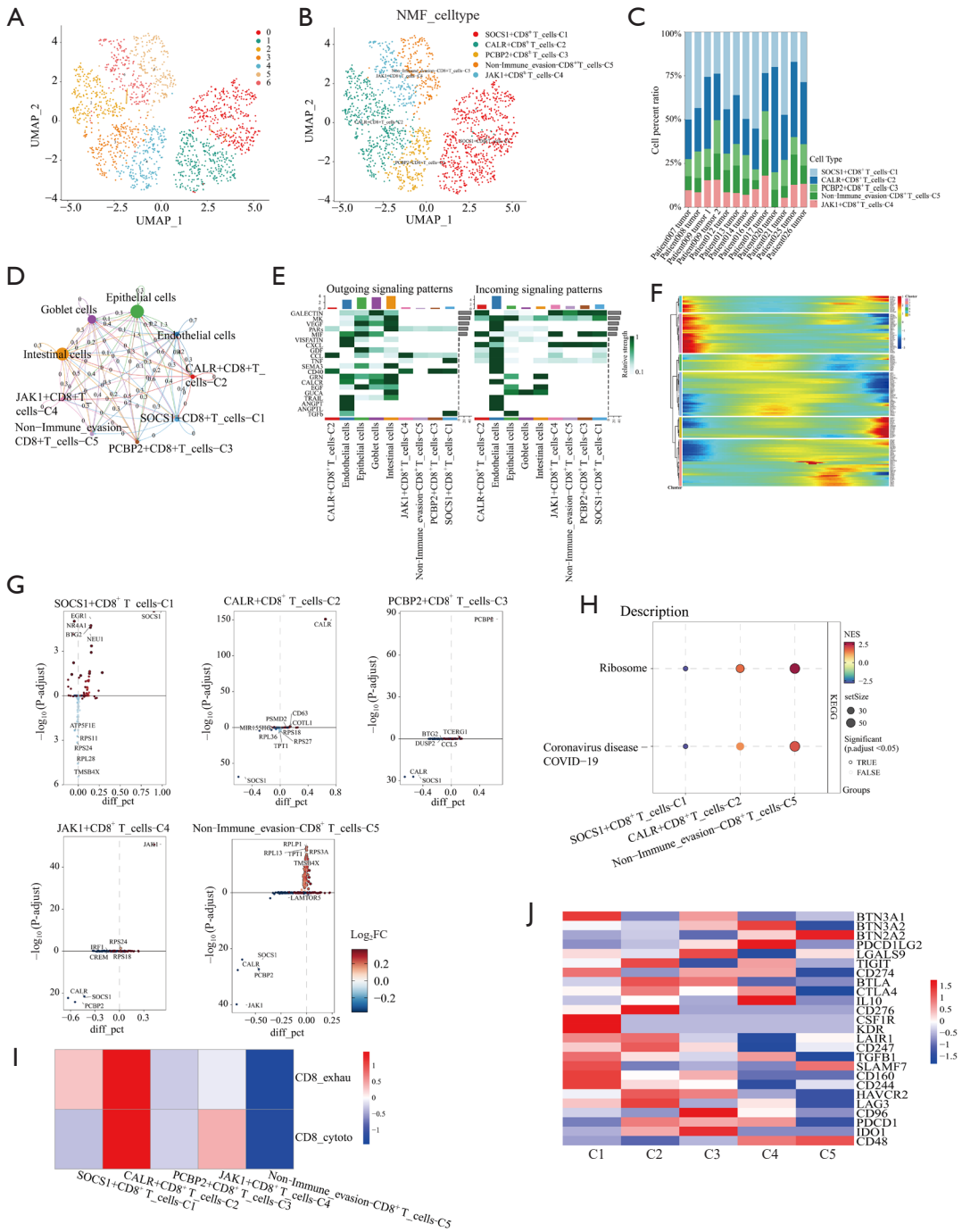

Figure 3 Novel subtypes of immune escape related CD8⁺ T cell and their functional profiles. (A,B) UMAP visualizing cell clusters associated with CD8⁺ T cell and their immune escape related subtypes. (C) The composition ratio of the five subtypes of CD8⁺ T cell across the 13 CRC samples. (D,E) Visualizing the intercellular communication network and the centrality score of CD8⁺ T cell subtypes. (F) Heatmap displaying the pseudotime expression of immune escape-related genes across CD8⁺T cell clusters. (G) Differential expression gene set of CD8⁺ T cell subtypes. (H) KEGG pathway enrichment analysis bubble plot for CD8⁺ T cell subtypes. (I) Heatmap of cellular marker expression patterns for CD8⁺ T cell subtypes. (J) Thermogram of transcription factor specific expression patterns of CD8⁺ T cell subtypes. CRC, colorectal cancer; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; UMAP, uniform manifold approximation and projection.

T cell infiltration (36-38). Furthermore, the outgoing PARs/CCL signals from CD8$^+$ T-C5 may interact with B cells and macrophages, indirectly suppressing T cell function (*Figure 3D,3E*). Additionally, Pseudotime analysis highlights the significance of immune escape-related genes in modulating the developmental trajectory of CD8$^+$ T cell subtypes, emphasizing their role in reshaping the TME and regulating dynamic anti-tumor immunity. This suggests that CD8$^+$ T cell subtypes engage in a complex and active regulatory network of interactions with other cells within the TME (*Figure 3F*).

DEG revealed that five CD8$^+$ T cell subtypes exhibit unique sets of differentially expressed genes (*Figure 3G*). KEGG pathway analysis revealed that the Ribosome/coronavirus disease-COVID-19 pathway was activated in CD8$^+$T-C2/5 but suppressed in CD8$^+$T-C1. This suggests that these cell subtypes may participate in TME immune escape through metabolic reprogramming (*Figure 3H*). Immune checkpoint analysis identified a unique gene expression pattern in CD8$^+$T-C5, which showed a negative correlation with most immune checkpoints, potentially serving as novel anti-tumor targets. Furthermore, genes associated with CD8$^+$ T cell exhaustion were primarily expressed in CD8$^+$T-C1/2, while cytotoxic genes were mainly enriched in CD8$^+$T-C2/4. This further highlights the functional heterogeneity among different CD8$^+$ T cell subtypes (*Figure 3I*).

The SCENIC results revealed a high expression of TFs associated with immune checkpoint signaling pathways, including BTN3A1, CSF1R, KDR, CD244, and SMAD4, in the CD8$^+$T-C1. In contrast, exhaustion-related TFs such as CD276, TIGIT, BTLA, and LAG3 were highly expressed in the CD8$^+$T-C2. This suggests that the two subtypes may play opposing roles in tumor immune evasion: CD8$^+$T-C1 likely resists immune escape by enhancing anti-tumor activity, whereas CD8$^+$T-C2 may facilitate immune escape by inducing T cell exhaustion. These findings provide a theoretical basis for stratified immunotherapy in tumors (*Figure 3J*).

Novel immune escape-related gene CAF subtypes and their roles in the CRC microenvironment

Previous analyses have shown that the expression ratio of fibroblasts is elevated in CRC tissue. To further investigate the role of CAFs in the CRC microenvironment, we categorized CAFs into six distinct clusters using NMF clustering (*Figure 4A*). Subsequently, we classified these clusters into four novel subtypes based on the expression of immune escape genes: HEXIM1$^+$CAF-C1 (CAF-C1), TGFBR2$^+$CAF-C2 (CAF-C2), unclear-CAF-C3 (CAF-C3), and none-immune-evasion-CAF-C4 (CAF-C4) (*Figure 4B*). The proportions of these four subtypes vary among the tissues of different CRC patients (*Figure 4C*).

Cell chat analysis revealed that CAF-C3 exhibited the strongest associations with both incoming and outgoing signals, serving as a signaling hub within the TME. The semaphorin 3 (SEMA3) signals emitted by CAF-C3 may induce T cell dysfunction by influencing neuropilin-1 (NRP1) on T cell surfaces, while simultaneously promoting the M2 polarization of macrophages. Meanwhile, TGF-β signals from CAF-C1, CAF-C2, and CAF-C3 could facilitate the formation of an immune-tolerant microenvironment through TGF-β type II receptors (TGF-β RII) on T cells (34), and may also collaborate with CAFs to construct physical barriers that impede T cell infiltration (39,40). There is a significant interaction between CAF-C4 and CAF-C3, both of which exhibit the most prominent associations with the GAS signaling pathway network. It is hypothesized that they may bind to TAM receptors on the surface of T cells through the growth arrest-specific protein 6 (GAS6) signaling molecule, thereby activating downstream pathways such as PI3K-AKT and MAPK. This process induces PD-L1 overexpression in CRC cells and immunosuppression of CD8$^+$ T cells, contributing to the remodeling of the immunosuppressive microenvironment (41,42). Moreover, the CXCL10/CXCR3 signaling axis on CAF-C3 and CAF-C4 is highly active and may participate in recruiting myeloid-derived suppressor cells (MDSCs), promoting M2-like polarization of macrophages, thereby indirectly suppressing T cell function while also facilitating CAF proliferation and matrix deposition (43). Additionally, CAF-C1 demonstrates strong associations with goblet cells and endothelial cells, indicating complex cellular communication between mucosal cells and fibroblasts, suggesting its potential role in regulating tumor angiogenesis and immune cell infiltration (*Figure 4D-4F*). Pseudotime analysis revealed that distinct clusters of CAFs can differentiate into two developmental stages along their trajectories, underscoring the plasticity of CAFs within the CRC microenvironment (*Figure 4G*). The heat map further underscores the pivotal role of immune escape-related genes in facilitating the differentiation and development of CAFs, forming the foundation for the cellular heterogeneity of CAFs in the CRC microenvironment (*Figure 4H*).

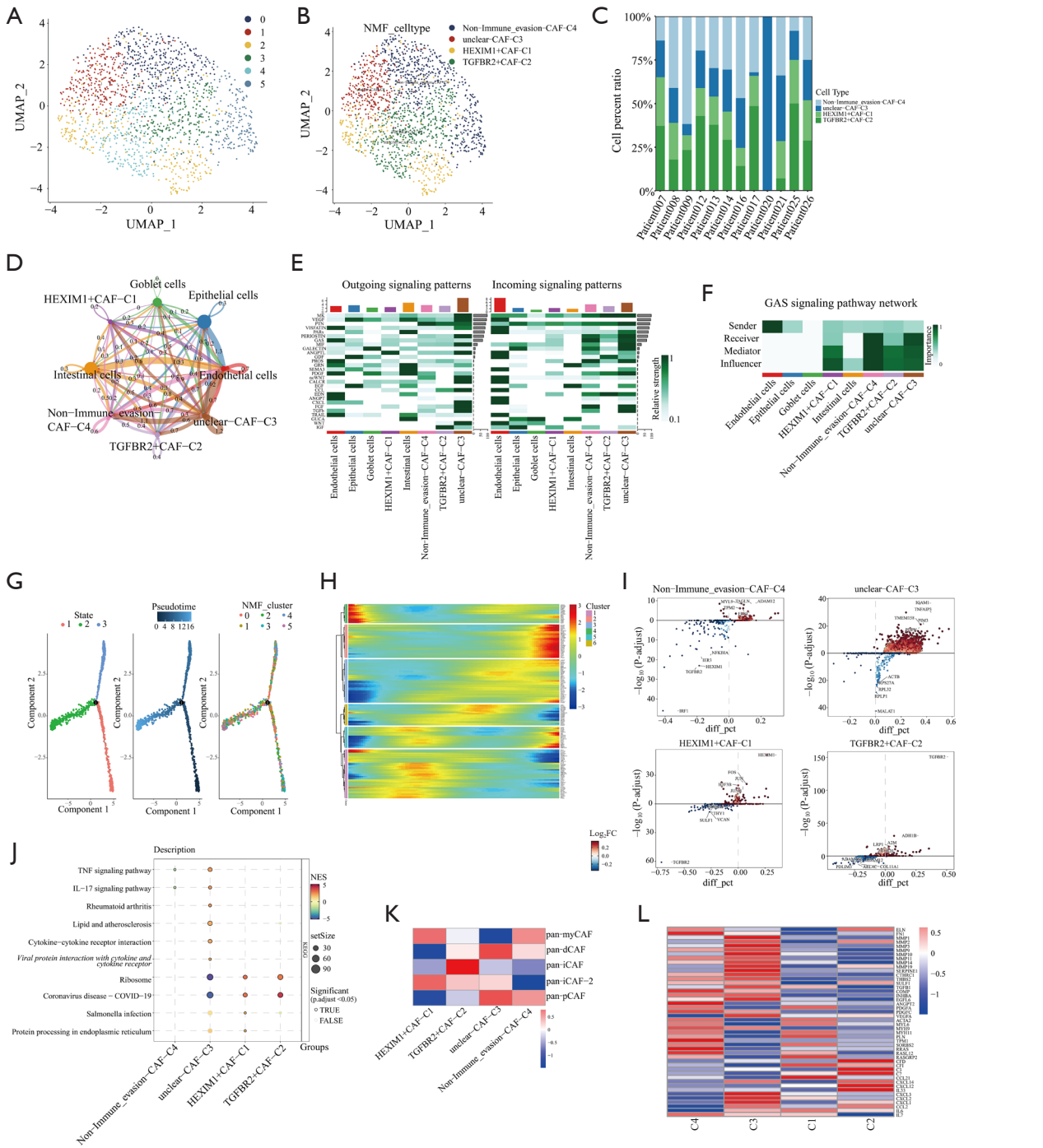

Figure 4 Novel subtypes of immune escape related CAF and their functional profiles. (A,B) UMAP visualizing cell clusters associated with CAF and their immune escape related subtypes. (C) The composition ratio of the four subtypes of CAF across the 12 CRC samples. (D-F) Visualizing the intercellular communication network and the centrality score of CAF subtypes. (G) Visualizing the differentiation trajectory of CAF clusters. (H) Heatmap displaying the pseudotime expression of immune escape-related genes across different CAF clusters. (I) Differential expression gene set of CAF subtypes. (J) KEGG pathway enrichment analysis bubble plot for CAF subtypes. (K) Heatmap of cellular marker expression patterns for CAF subtypes. (L) Thermogram of transcription factor specific expression patterns of CAF subtypes. CAF, cancer-associated fibroblast; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; NMF, non-negative matrix factorization; UMAP, uniform manifold approximation and projection.

Differential expression analysis (DEG) of the four subtypes of CAFs revealed that each subtype possesses a distinct set of differentially expressed genes. Notably, the gene set contains a significant number of genes involved in immune regulation, particularly in the CAF-C3 subtype (*Figure 4I*). The KEGG pathway enrichment analysis revealed distinct functional characteristics among various CAF subtypes. Notably, genes associated with the Ribosome/coronavirus disease-COVID-19 pathways are significantly upregulated in CAF-C1 and CAF-C2, whereas this trend is reversed in CAF-C3. Additionally, genes involved in protein processing in endoplasmic reticulum signaling pathway exhibited significant upregulation in CAF-C1. The abnormal activity in protein synthesis, processing, and secretion observed in CAF-C1 and CAF-C2 suggests their potential involvement in immune regulation through the endoplasmic reticulum stress response or by simulating pathogen-associated molecular patterns (PAMPs). The genes associated with TNF signaling, IL-17 signaling, and the cytokine-cytokine receptor interaction pathway are significantly upregulated in CAF-C3. This suggests that CAF-C3 serves as a central hub for regulating immune and inflammatory responses within the CRC microenvironment, thereby reshaping the immunosuppressive milieu through cytokines. In contrast, CAF-C4 exhibits downregulation of genes related to TNF and IL-17 signaling pathways, which aligns with the expression pattern of immune escape genes. This indicates that CAF-C4 may sustain immune tolerance by inhibiting pro-inflammatory signals (*Figure 4J*).

Through marker expression analysis, we differentiate the cellular status and functional characteristics of these subtypes. The results indicate that pan-myCAF and pan-iCAF-2 are highly expressed in CAF-C1, while pan-iCAF-2 is predominantly expressed in CAF-C2. Conversely, pan-dCAF and pan-pCAF are concentrated in CAF-C3. This suggests that CAF-C1 and CAF-C2 may reshape the extracellular matrix by secreting specific proteins. Additionally, CAF-C3 may regulate the proliferation and differentiation of CAFs by activating particular metabolic states, thereby participating in immune regulation and immune escape within the TME. Furthermore, the specific high expression of pan-iCAF-2 in CAF-C1 indicates that CAF-C1 may possess unique biological functions. The expression patterns of cellular markers underscore the functional heterogeneity among different CAF subtypes (*Figure 4K*).

The specific expression patterns and potential functions of key TFs in different subtypes of CAFs were analyzed using the SCENIC methodology. The results indicated that the key TFs identified in CAF-C1 included RASGRP2, CFI, and CCL21, suggesting a role for CAF-C1 in the chemotaxis, recruitment, and activation of immune cells (44-49). In contrast, the key TFs identified in CAF-C2 encompassed CFD, C3, C7, CXCL12, and IL33, implying that CAF-C2 may be linked to immune cell infiltration, dysfunction of the complement system, cell invasion, angiogenesis, and the polarization of TAMs (50-56). Furthermore, the key TFs identified in CAF-C3 included members of the matrix metalloproteinases (MMPs) family, such as MMP1, MMP3, MMP10, and CXCL3, indicating that CAF-C3 may play a role in the degradation of the extracellular matrix and the chemotaxis of immune cells. These findings provide crucial molecular insights into the roles of various CAF subtypes in immune regulation, extracellular matrix remodeling, and the remodeling of the TME (*Figure 4L*).

Novel immune escape-related gene macrophage subtypes and their roles in the CRC microenvironment

To investigate the role of macrophages in the CRC microenvironment, we utilized NMF clustering to categorize them into eight distinct cell clusters (*Figure 5A*). Subsequently, we classified these clusters into four novel subtypes based on the expression of immune escape genes: IRF1^{+}Mac-C1 (Mac-C1), TAP1^{+}Mac-C2 (Mac-C2), unclear-Mac-C3 (Mac-C3), and non-immune-evasion-Mac-C4 (Mac-C4) (*Figure 5B*). The proportions of these subtypes varied across tissues from different CRC patients, with Mac-C4 being the most predominant subtype, exhibiting the highest proportion in CRC tissues (*Figure 5C*).

Cell chat analysis results revealed robust intercellular communication among the four macrophage subtypes and various cell types, including epithelial cells, goblet cells, enterocytes, and endothelial cells, with particularly strong interactions noted between Mac-C2/C4 and endothelial cells. Furthermore, the active VEGF/VEGFR axis in the outgoing signaling patterns of Mac-C2/C4 may facilitate angiogenesis and inhibit immune cell infiltration by interacting with endothelial cells, thereby establishing an immune escape 'physical barrier' (57). Additionally, the outgoing signaling patterns of Mac-C2 exhibit strong CXCL signals, indicating its potential to recruit immunosuppressive cells, such as MDSCs and Tregs, to the TME via the CXCL/CXCR axis (58,59). Concurrently, the CXCL/CXCR axis may also play a role in activating

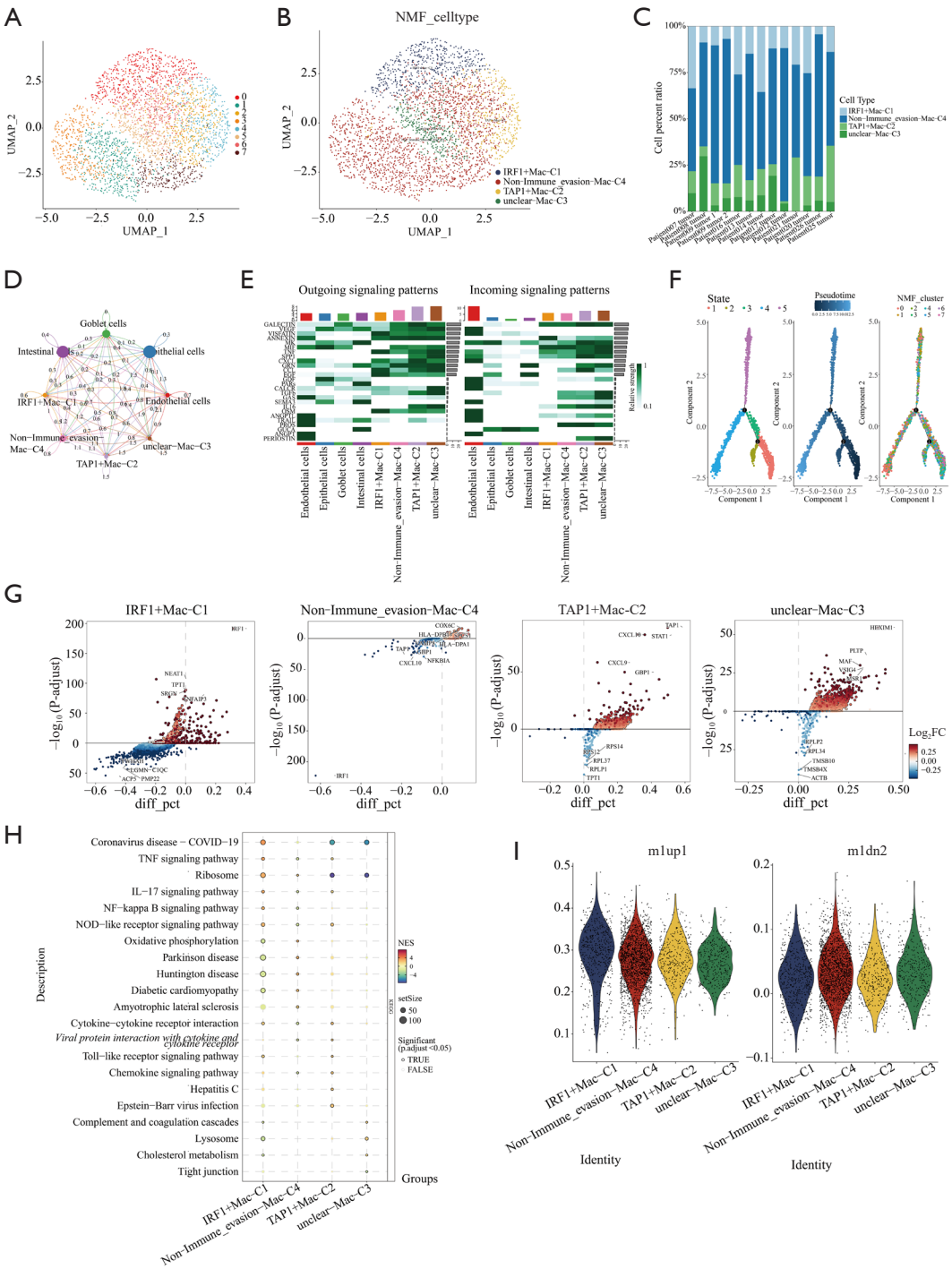

Figure 5 Novel subtypes and functional profiles of immune escape related macrophage subtypes. (A,B) UMAP visualizing cell clusters associated with macrophage and their immune escape related subtypes. (C) The composition ratio of the four subtypes of macrophage across the 13 CRC samples. (D,E) Visualizing the intercellular communication network and the centrality score of macrophage subtypes. (F) Visualizing the differentiation trajectory of macrophage clusters. (G) Differential expression gene set of macrophage subtypes. (H) KEGG pathway enrichment analysis bubble plot for macrophage subtypes. (I) Gene co-expression patterns of macrophage subtypes and M1 macrophages. CRC, colorectal cancer; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; NMF, non-negative matrix factorization; UMAP, uniform manifold approximation and projection.

cancer-associated fibroblasts (CAFs) and promoting stromal deposition, consequently impeding T cell migration (43). Conversely, the anti-tumor potential of Mac-C1 may be partially counterbalanced by the pro-tumor signals from MIF/TNF/SPP1. Strong communication was also observed between Mac-C2 and Mac-C3, suggesting that they may function as 'dual hubs' within the TME through signal integration. Furthermore, we identified that the TGF-β/TGF-β RII axis, GALECTIN/glycoprotein receptor axis, CXCL/CXCR axis, and MIF/CD74 axis were highly active among these four macrophage subtypes. This indicates that different cell subtypes can collectively remodel the TME through multiple mechanisms and circuits, thereby promoting CRC progression and contributing to therapy resistance (*Figure 5D,5E*). Pseudotime analysis revealed the dynamic regulatory role of immune escape-related genes in macrophage cluster development (*Figure 5F*).

DEG further delineated distinct sets of differentially expressed genes among the four macrophage subtypes (*Figure 5G*). KEGG pathway analysis revealed that the Coronavirus disease-COVID-19/TNF/Ribosome/IL-17/NF-κB/NOD-like receptor signaling pathway was activated in Mac-C1. Combined with the previous CellChat analysis results, it can be inferred that Mac-C1 may bind to the tumor necrosis factor receptor (TNFR) through tumor necrosis factor (TNF), thereby activating the TNF/NF-κB signaling pathway. This not only maintains its own highly pro-inflammatory state but also promotes CRC proliferation and migration (60,61). The Coronavirus disease-COVID-19/Ribosome signaling pathway was significantly downregulated in Mac-C2/C3, suggesting that they may promote CRC immune escape by upregulating immunosuppressive molecules. In contrast, Mac-C4 played a crucial role in maintaining metabolic homeostasis (*Figure 5H*).

We further analyzed the correlation between immune escape genes in the newly identified subtypes of macrophages and M1 macrophages. Our findings indicate that the immune escape genes identified in the novel subtypes predominantly align with those present in M1 macrophages, suggesting that the novel subtypes and M1 macrophages may share similar functional characteristics in pro-inflammatory responses and immune regulation (*Figure 5I*).

Novel immune escape-related gene B cell subtypes and their role in the CRC microenvironment

We also investigated the role of B cells in the CRC microenvironment. B cells were categorized into ten distinct clusters using NMF clustering (*Figure 6A*) and further classified into five novel subtypes based on the expression of immune escape genes: JAK1$^+$B_cells-C1 (B-C1), TAPBP$^+$B_cells-C2 (B-C2), CFLAR$^+$B_cells-C3 (B-C3), IRF1$^+$B_cells-C4 (B-C4), and unclear-B-cells C5 (B-C5) (*Figure 6B*). The proportions of these five subtypes vary among the tissues of different CRC patients. Notably, the combined proportions of B-C2, B-C3, and B-C5 exceed 80% of the total subgroup, suggesting that they may represent the core subgroups involved in immune suppression within the CRC microenvironment (*Figure 6C*).

Cell chat analysis revealed active intercellular communication between B cell subtypes, particularly B-C1/C2, and endothelial cells or enterocytes within the CRC microenvironment. However, communication among different B cell subtypes was relatively inactive, potentially mediated through intermediary cells or signaling molecules. Among all B cell subtypes, B-C4 received the highest number of incoming signals, suggesting its potential role as a "signaling hub". B-C2 could enhance antigen presentation via TAP-associated glycoprotein (TAPBP) expression while simultaneously suppressing T cell function and promoting angiogenesis through active EGF and CXCL signaling, thereby forming a dual "pro-tumor and immunosuppressive" phenotype. The GDF signaling in the incoming signaling patterns of B-C2/C4/C5 is highly active, potentially inhibiting T cell differentiation and cytotoxicity by activating the BMP/SMAD pathway, while promoting CRC cell proliferation and the formation of an immunosuppressive microenvironment (62). The EGF/EGFR axis, GALECTIN/glycoprotein receptor axis, CXCL/CXCR axis, and MIF/CD74 axis all exhibit robust activity across various B cell subtypes, indicating that different cell subtypes can remodel the CRC TME and suppress T cell function through similar signaling pathways, collectively contributing to the formation of an immunosuppressive milieu (*Figure 6D,6E*).

Pseudotime analysis revealed that B cells can differentiate into five distinct developmental stages along their trajectory, underscoring the plasticity of B cells within the CRC microenvironment. The heatmap further illustrates the dynamic changes in immune escape-related genes during B cell development, which serve as the molecular basis for the heterogeneity of B cell subtypes and the remodeling of the CRC microenvironment (*Figure 6F,6G*).

We analyzed the intercellular interactions of novel subtypes within the TME and found that CRC dynamically regulates immunosuppression through a multidimensional intercellular communication network (*Figure 6H*). Among

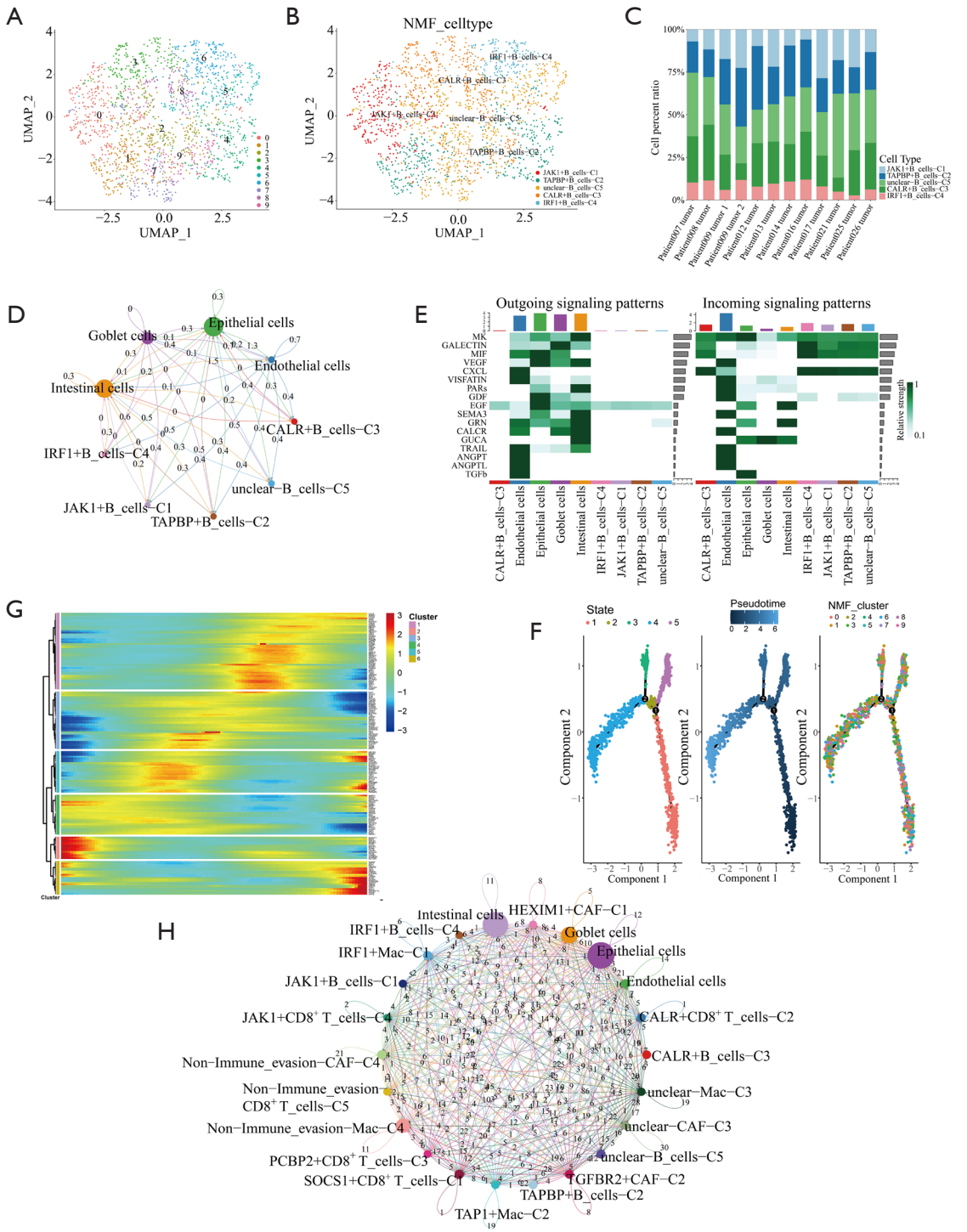

Figure 6 Novel subtypes of immune escape related B cell and their functional profiles. (A,B) UMAP visualizing cell clusters associated with B cell and their immune escape related subtypes. (C) The composition ratio of the five subtypes of B cell across the 12 CRC samples. (D,E) Visualizing the intercellular communication network and the centrality score of B cell subtypes. (F) Visualizing the differentiation trajectory of B cell clusters. (G) Heatmap displaying the pseudotime expression of immune escape-related genes across B cell clusters. (H) Visualizing the intercellular communication network of novel immune escape related cell subtypes. CRC, colorectal cancer; NMF, non-negative matrix factorization; UMAP, uniform manifold approximation and projection.

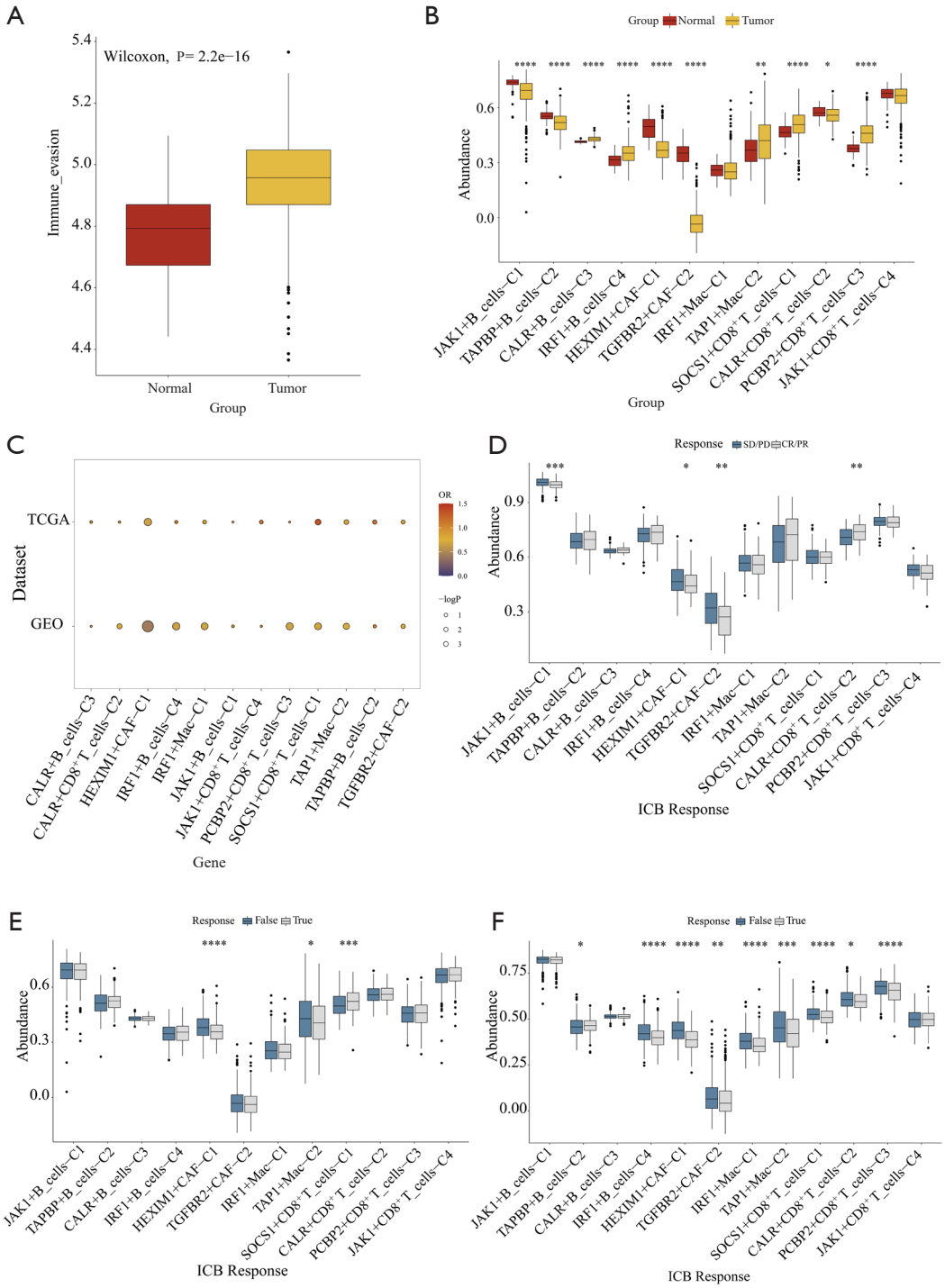

Figure 7 Immune response analysis of novel immune escape related cell subtypes in CRC. (A) Differential expression analysis between CRC tumors and normal tissues. (B) Differential expression analysis of immune escape related cell subtypes between CRC tumors and normal tissues. (C) Displaying the relationship between different cell subtypes and prognosis in TCGA and CRC patient cohorts. (D-F) Box plot showing the differences in cell subtype abundance among different immunotherapy response groups for TCGA (D), GEO (E), and IMergy (F) cohorts. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. CR, complete response; CRC, colorectal cancer; GEO, Gene Expression Omnibus; ICB, immune checkpoint blockade; PD, progressive disease; PR, partial response; SD, standard deviation; TCGA, The Cancer Genome Atlas.

the various CD8$^+$ T cell subtypes, the active TGF-β/TGFβRII, GAS6/TAM, and CXCL/CXCR axes may activate the SMAD, PI3K-AKT/MAPK, and CXCL/CXCR signaling pathways, respectively, directly inhibiting CD8$^+$ T cell proliferation and cytotoxicity while inducing T cell exhaustion phenotypes. Among CAFs subtypes, particularly CAF-C3 and CAF-C4, immune escape may be promoted through two mechanisms: first, by inducing T cell dysfunction via NRP1 receptor-mediated signaling of active molecules such as SEMA3 and VEGF; second, by facilitating angiogenesis through the VEGF/VEGFR axis in endothelial cells, thereby establishing an immune-physical barrier, while synergizing with Mac-C2/C4's VEGF signaling to suppress immune cell infiltration. Meanwhile, Mac-C2 may potentially reshape the TME and promote immunosuppression through a positive feedback loop involving the CXCL/CXCR axis. On one hand, it recruits immunosuppressive cells, such as MDSCs and Tregs; on the other hand, it hinders T cell migration by enhancing the stromal remodeling functions of CAFs. Among B cell subtypes, particularly B-C2/C4/C5, the BMP/SMAD pathway in T cells may be activated through GDF signaling molecules, suppressing T cell differentiation and cytotoxicity. Additionally, these B cell subtypes interact with epithelial cells and enterocytes to remodel the TME via the CXCL/CXCR axis, ultimately leading to the formation of an immune-tolerant microenvironment. The classification and functional analysis of these subtypes provide a molecular basis for immune evasion and treatment resistance in CRC.

The correlation between novel subtypes and immune therapy response and prognosis in CRC

DEG between CRC tumors and normal tissues revealed a significant increase in the expression of immune escape genes in tumor tissues, further confirming the activation of immune escape mechanisms in CRC (*Figure 7A*). Subtypes B-C3, B-C4, Mac-C2, CD8$^+$T-C1, and CD8$^+$T-C3 exhibited higher expression levels in tumor tissues ($P<0.05$) and may be in an activated state. Conversely, the expression of B-C1, B-C2, CAF-C1, CAF-C2, and CD8$^+$T-C2 in tumor tissues was significantly reduced ($P<0.05$), indicating a potentially inhibited state (*Figure 7B*).

Cox analysis was employed to assess the impact of the novel subtype on prognosis (*Figure 7C*). Additionally, the response of this novel subtype to immunotherapy was evaluated by comparing the differences in cell subtype abundance across various immunotherapy response groups. The results indicated that the abundance of CAF-C1 cells exhibited a negative correlation with immunotherapy across all cohorts. Furthermore, CAF-C2 and CD8$^+$T-C1 cells showed a negative correlation with immunotherapy in the TCGA and IMergy cohorts, while Mac-C2 cells were negatively correlated with immunotherapy in the GEO and IMergy cohorts (*Figure 7D-7F*).

The correlation between novel subtypes and prognosis in CRC

The results of the Kaplan-Meier survival analysis indicated that in the TCGA, GEO, and IMergy cohorts, B-C3, CD8$^+$T-C2, and Mac-C2 were positively correlated with the prognosis of CRC and may serve as biomarkers for predicting the response of CRC patients to immunotherapy. Conversely, HEX1M1$^+$CAF-C1 exhibited a negative correlation with CRC prognosis, suggesting its potential as an independent risk factor for poor outcomes in CRC (*Figure 8A,8B*). These findings provide valuable insights for evaluating the immune status and drug resistance in CRC patients, thereby guiding the selection and optimization of immunotherapy, combination therapy, or adjuvant therapy strategies.

Discussion

CRC is a malignant disease that poses a significant threat to health, with the limitations of current diagnostic and treatment strategies contributing to its high mortality rate. Immune escape is a critical factor that promotes CRC progression and drug resistance, closely associated with the complex TME. Existing research predominantly focuses on a single dataset, gene, or cell type, which limits the understanding of the roles and heterogeneity of various cells within tumors. Furthermore, such studies are often disconnected from the TME, failing to capture the dynamic interactions between cells, thereby restricting our comprehension of immune escape mechanisms. Our research utilizes scRNA-seq data to investigate the role of immune escape within the CRC TME. By analyzing a comprehensive dataset comprising 66,050 high-quality single cells from tumor and adjacent normal tissues, we identified the expression patterns of immune escape-related genes across different cell types and elucidated their contributions to the immune escape mechanisms in CRC. Through the integration of NMF clustering and analysis of

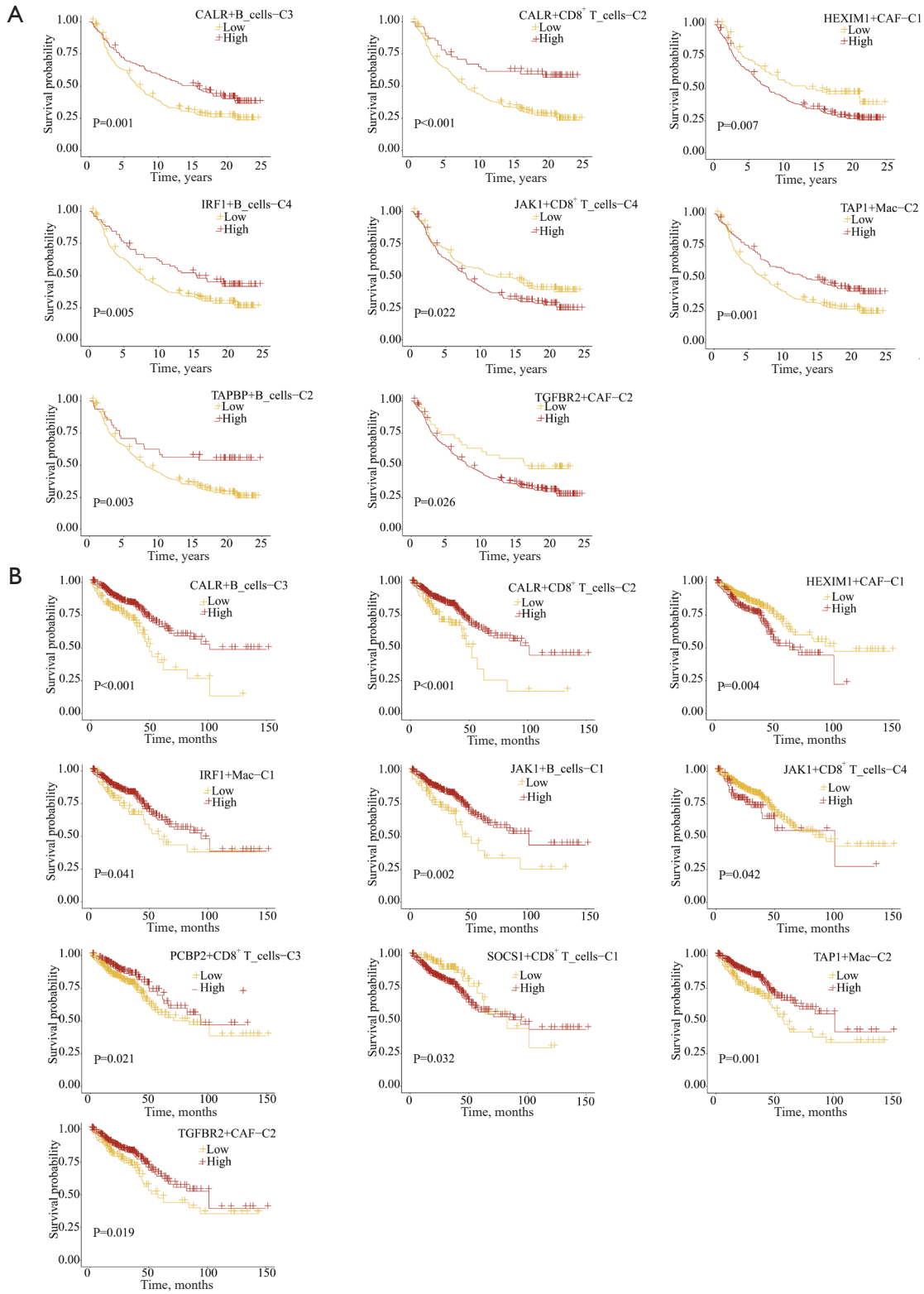

Figure 8 Prognostic analysis of novel immune escape related cell subtypes in CRC. Kaplan Meier survival analysis of the correlation between novel immune escape related cell subtypes and CRC prognosis in the TCGA (A) and GEO (B) cohort. CRC, colorectal cancer; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas.

immune escape gene expression profiles, we have subdivided CAFs, TAMs, CD8$^+$T cells, and B cells in the CRC microenvironment into distinct subtypes for the first time. We also analyzed their biological functions and dynamic intercellular interactions. Our findings underscore the significance of cell heterogeneity in CRC immune escape and prognosis, providing a foundation for the selection of targeted diagnostic and therapeutic strategies.

The TME of CRC facilitates immune escape via a multidimensional immune regulatory network. Its core mechanisms encompass the functional remodeling of immune cells, metabolic reprogramming, and interactions among signaling pathways. The primary immune cell subpopulations within the TME include TAMs, T cells, B cells, and tumor-associated neutrophils (TANs). Each component collaboratively constructs an immunosuppressive network through their differentiated functions (63,64).

The anti-tumor immune function of CD8$^+$ T cells in the CRC microenvironment is consistently inhibited. The activation of TGF-β and IFN diminishes the cytotoxic activity of CD8$^+$ T cells (65,66). Abnormal metabolism of amino acids and sugars leads to impaired proliferation and cytotoxic function of CD8$^+$ T cells (67,68), while sustained expression of immune checkpoint molecules such as PD-1 results in the depletion of CD8$^+$ T cells (69,70). Blocking the PD-1 pathway can restore their anti-tumor effects (71,72). Functional analysis reveals a bidirectional regulatory mechanism among CD8$^+$ T cell subtypes. Specifically, CD8$^+$T-C1 and CD8$^+$T-C2 exhibit functional antagonism in immune evasion. The robust communication between CD8$^+$T-C3 and epithelial/endothelial cells plays a pivotal role in regulating tumor dynamics and TME remodeling. Although CD8$^+$T-C5 shows potential as a therapeutic target, caution is warranted regarding its interaction with CAF-C4, which may compromise its anti-tumor efficacy. Consequently, coordinated interventions targeting specific multi-subpopulations could optimize immunotherapy strategies.

CAFs play a crucial role in reshaping the extracellular matrix, secreting specific proteins, and undergoing metabolic reprogramming, thereby influencing the TME (73-75). This modulation significantly affects the efficacy of anti-PD-1/PD-L1 therapy (76,77). Functional subtype analysis revealed that CAF-C3 directly suppresses CD8$^+$ T cell function through multiple signaling molecules while simultaneously activating the IL-17 and TNF signaling pathways. This activation promotes the formation of an inflammatory immunosuppressive microenvironment, effectively serving as a signaling hub. Key TFs, particularly members of the MMP family such as MMP1/3/10, may also contribute to extracellular matrix degradation, thereby exacerbating immunosuppression. In contrast, CAF-C1 and CAF-C2 are associated with angiogenesis and stromal remodeling, potentially facilitating immune cell infiltration. Meanwhile, CAF-C4 may play a role in maintaining immune tolerance by suppressing pro-inflammatory signals.

TAMs promote self-infiltration by secreting chemokines such as CXCL10 and CXCL11, creating a vicious cycle that enhances tumor proliferation, metastasis, and immune evasion (78). The functional phenotype of TAM exhibits dynamic plasticity: the M1 subtype possesses pro-inflammatory properties, while the M2 subtype is involved in immune suppression. Regulating TAM polarization may help to reconstruct anti-tumor immunity, and its combination with immune checkpoint inhibitors shows promising potential (79-81). The Mac-C1 identified in this study exhibits pro-inflammatory properties and demonstrates dynamic associations with immune evasion genes. In contrast, Mac-C2 is involved in TAP1$^+$ antigen presentation and may enhance the stromal barrier through the activation of endothelial cells or CAFs. Furthermore, Mac-C3 may restrict T cell function by regulating cytoskeletal dynamics or matrix deposition. Additionally, Mac-C4 indirectly supports the immunosuppressive microenvironment by maintaining metabolic homeostasis and the integrity of the stromal barrier. The functional similarities between Mac-C4 and classical M1-type macrophages also warrant further investigation. These findings provide a molecular foundation for understanding the functional heterogeneity of TAMs.

B cells play a crucial role in immune surveillance and immune evasion by secreting IgA antibodies, as well as the cytokines IL-10 and IL-6, which are linked to the response to chemotherapy (82-84). Additionally, they interact closely with immune cells such as T cells (85-87). Functional analysis revealed that B-C2 promotes immunosuppression by coordinating antigen presentation, while B-C5 constitutes the core of this immunosuppressive process. Additionally, B-C4 serves as a critical signaling hub, orchestrating interactions among multiple cellular subtypes and amplifying immunosuppressive effects. Collectively, these components form a hierarchical communication network that operates through a division of labor and dynamic regulation of immunosuppression.

Through this research, we found that the intercellular interactions of the new subtype in the TME are complex and dynamic, involving interactions among tumor cells,

immune cells, fibroblasts, and other microenvironmental components. This intercellular communication influences TME remodeling and immune evasion through multiple signaling pathways and molecular mechanisms. These findings are consistent with previous studies on glioblastoma and hepatocellular carcinoma (88-91), which validate the reliability of our analytical results and demonstrate the universality of this interactive network.

The analysis of the response of novel subtypes to immunotherapy and its relationship with CRC prognosis reveals new potential biomarkers and therapeutic targets for evaluating the immune status of CRC patients and selecting appropriate immunotherapy strategies. Notably, the responses of B-C3, $CD8^+$T-C2, and Mac-C2 subtypes to immunotherapy offer significant insights for individualized treatment. Furthermore, CAF-C1, identified as an independent risk factor for poor CRC prognosis, serves as a valuable reference for the stratified diagnosis of CRC patients in clinical practice. Furthermore, the strategy of combined blockade of TGF-β, galectin, CXCL/CXCR, and immune checkpoints, such as PD-1, shows promise in reversing immunosuppression in CRC. Targeted drug development and therapies aimed at hub subtypes, such as B-C4 and CAF-C3, may enhance anti-tumor immune responses. This approach provides robust theoretical support for the precise and personalized treatment of CRC patients.

While this study has made significant progress in elucidating cellular immune evasion within the CRC microenvironment, there remains a need for more comprehensive clinical data and tissue samples to validate our findings. Furthermore, additional functional studies utilizing *in vitro* and *in vivo* cellular and animal models are essential to explore more deeply the interactions and molecular mechanisms between the newly identified cell subtypes, CRC cells, and the TME. Moreover, integrating multi-omics data, including proteomics, metabolomics, spatial transcriptomics, and glycomics, will facilitate a systematic analysis of how cellular heterogeneity in the CRC microenvironment influences immune evasion. This integrative approach will provide a robust foundation for the development of new clinical diagnostic and therapeutic strategies.

In conclusion, this study utilized single-cell sequencing analysis to characterize the molecular features of immune escape-related subtypes within the CRC TME. It elucidated how cell-cell interactions can suppress anti-tumor immune responses by reshaping the TME. These findings highlight the clinical significance of identifying immune escape-related subtypes, their influence on patient prognosis, and their potential to inform the development of more effective treatment strategies. Ultimately, this research lays the groundwork for advancing treatment approaches and improving outcomes for CRC patients.

Conclusions

In this study, we used scRNA-seq to reveal gene expression profiles linked to immune evasion in the TME, found 11 immune escape-related cell subtypes and confirmed TGF-β^+JAK1$^+$Calretinin$^+$ as a candidate TME immunosuppressive cell marker. Novel subtypes of CAF, $CD8^+$ T cell, macrophage and B cell with unique CRC features related to immune escape were identified, and their interaction network aids CRC immune escape by altering the immunosuppressive microenvironment. CFLAR$^+$B_cells-C3, CALR$^+$CD8$^+$T_cells-C2, and TAP1$^+$Mac-C2 may serve as potential biomarkers for predicting responses to immunotherapy, and HHEXIM1$^+$CAF-C1 may act as an independent risk factor for poor prognosis in CRC patients. This deepens the understanding of CRC immune evasion and provides guidance for new diagnosis and treatment.

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None.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-2025-1466/rc

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Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-2025-1466/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. The study was approved by the Ethics Committee of The First Affiliated Hospital of Hunan College of Traditional Chinese Medicine (also recognized as Hunan Province Directly Affiliated TCM Hospital) (approval No. KY-2025042902) and informed consent was taken from all the patients.

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