Research Paper

Single-cell immune escape correlation analysis: unraveling the prognostic influence of intercellular communication in the tumor microenvironment of colorectal cancer

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Keywords: immune escape, single-cell, tumor microenvironment, colorectal cancer, prognosis
Received: September 26, 2023 **Accepted:** June 3, 2024 **Published:** June 27, 2024

ABSTRACT

Background: The role of immune escape-related genes in the tumor microenvironment (TME) in CRC remains unclear but is known to be crucial for CRC development.
Methods: We analyzed single-cell RNA-seq data from 13 CRC tumor samples, comprising 66,050 cells, using NMF to identify immune escape-related genes. We predicted the prognosis and immune response of novel TME cell clusters using a public CRC cohort and immunotherapy cohort.
Results: CRC single-cell analysis revealed distinct cell types, including plasma cells, epithelial cells, T cells, NK cells, goblet cells, intestinal cells, B cells, macrophages, fibroblasts, endothelial cells, and mast cells. These cell types were further grouped into new clusters based on immune escape-related gene annotations. Immunohistochemistry (IHC) confirmed the high expression of TGF-β+, JAK1+, and Calretinin+ in CRC tissues, validating key bioinformatics findings on their potential relevance in CRC pathology. Pseudo-temporal trajectory analysis showed the differentiation trajectory of different immune escape-related subtype cell clusters. Cellular communication analysis revealed extensive interactions between endothelial cells and immune escape-related metabolizing TME cell-related subtypes. SCENIC analysis identified transcription factors upstream of TME cell clusters with varying immune responses. Moreover, TME cell clusters associated with immune escape processes exhibited enrichment in the expression of CAF subtypes, CD8-depleted, M1, and M2 macrophages. Bulk-seq analysis demonstrated the significant prognostic importance of immune escape-related TME cell clusters in CRC. Remarkably, the immunotherapy cohort showed a significant immune response, especially in patients treated with immune ICB, involving CAFs, T cells, and macrophages.
Conclusion: Our study is the first to reveal the role of immune escape in mediating intercellular communication within the CRC microenvironment, elucidating the anti-tumor mechanisms and immune prognostic responses of distinct cell cluster subtypes.

INTRODUCTION

Colorectal cancer (CRC) ranks as the third most prevalent cancer worldwide and the second leading cause of cancer-related deaths. The incidence of CRC in younger individuals has been increasing globally [1]. The development of CRC is influenced by both hereditary factors and modifiable risk factors such as

smoking, processed meat consumption, alcohol intake, red meat consumption, low intake of vegetables and fruits, and high body fat levels [2]. Unfortunately, the survival rates for those diagnosed with colorectal cancer are discouraging, with only around 20%–35% of patients surviving beyond 3 years and less than 20% surviving beyond 5 years after diagnosis [3, 4]. Hence, a deeper understanding of CRC's molecular mechanisms may lead to novel prevention and treatment strategies.

CRC immunoediting involves three processes: elimination, equilibrium, and escape [5]. Successful immune escape leads to the mutation of tumor cells, rendering them insensitive to immunological monitoring and causing uncontrolled growth, resulting in clinically diagnosed cancer [6, 7]. Immune escape is also believed to play a crucial role in CRC metastasis, occurrence, and resistance to immunotherapy [8, 9]. Although the reciprocal mechanisms between CRC and the immune system have been well-documented, the molecular characterization of immune escape in CRC remains insufficiently studied.

The tumor microenvironment (TME) is a complex integrated system consisting of tumor cells, immune cells (T-cells, B-cells, dendritic cells, MDSCs, TAMs), inflammatory cells, cytokines, tumor-associated fibroblasts, and the extracellular matrix [10, 11]. TME can be modified by tumors to promote tumor angiogenesis, establish a metabolic symbiosis with stromal cells, and induce peripheral immune tolerance. Likewise, immune cells in the microenvironment can influence the proliferation and evolution of cancer cells [12, 13]. Recent studies indicate that poor responses to immunotherapy might be related to immune escape in the TME, where various cytokines and tumor-derived exosomes can promote tumor immune escape [14]. Therefore, there is a need to investigate changes in the cellular immune system and the activation of different immune subpopulations.

To explore the connection between immune escape and the TME in CRC, we conducted a study based on single-cell sequencing data from 13 CRC patients, encompassing 689 single cell samples. We utilized non-negative matrix factorization (NMF) to identify distinct subpopulations of TME cell types in CRC. We then investigated the relevance of these new immune escape subtypes to different immune characteristics, metabolic pathways, cellular interactions, and prognosis in CRC. This comprehensive single-cell analysis of immune escape may shed light on the interactions between the TME and tumor cells, ultimately aiding in the development of new staging and prognostic tools for CRC.

RESULTS

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

In order to investigate immune escape-related genes in the landscape map of colorectal cancer (CRC), we utilized single-cell data from 13 tumor tissues and their adjacent tissues, in addition to two datasets on CRC prognosis and one on immunotherapy. We conducted a single-cell quality control process, which included analyzing gene, mitochondrial, and cell counts after the acquisition (Supplementary Figure 1A), and selecting 2000 highly variable genes for subsequent analysis (Supplementary Figure 1B). We visualize the distribution of samples using UMAP after harmonizing cells to enhance data accuracy and reliability (Supplementary Figure 1C). Then we employed marker genes (Supplementary Figure 1D–1F) from the original literature to annotate the following cell types: epithelial cells, T cells, goblet cells, NK cells, plasma cells, intestinal cells, macrophages, B cells, fibroblasts, mast cells, and endothelial cells (Figure 1A–1C). Various cell types exhibited differential variation in ratios between tumor and normal tissues, such as fibroblasts and macrophages (Figure 1D). Subsequently, a thermal visualization was created to illustrate the activity of 171 immune escape-associated genes within various types of immune cells. (Figure 1E).

In this investigation, immunohistochemistry (IHC) was employed to explore the expression of select genes previously identified as positive in bioinformatics analyses of colorectal cancer (CRC) patient samples. Among these, TGF-β+, JAK1+, and Calretinin+ were notably found to be highly expressed in tumor tissues, as evidenced through IHC staining. This observation highlights the presence of these genes as part of the subset of positive findings from the initial bioinformatics screening, emphasizing their potential relevance in CRC pathology. The figure associated with these findings showcases a representative example of three CRC tumors (Figure 2A–2C), demonstrating the enhanced expression of TGF-β+, JAK1+, and Calretinin+ via multi-colored IHC staining. This visualization serves to validate the elevated expression levels of these markers in the tumor samples, aligning with a fraction of the bioinformatics analysis's positive results.

Novel immune escape gene-associated CAF subtype cells for TME of CRC

By applying MNF clustering to cancer-associated fibroblast (CAF) cells, we categorized them into five distinct clusters (Figure 3A). Subsequently, the clusters were then categorized into specific subtypes, which

were determined by the gene expression profiles associated with immune escape: none-immune_evasion-CAF-C4, unclear-CAF-C3, HEX1M1+CAF-C1, and TGFBR2+CAF-C2 (Figure 3B, 3C). Our cell chat analysis revealed specific and robust interactions within the tumor microenvironment, illustrating the complex interplay at play. Notably, non-immune evasion CAF-C4 demonstrated significant interaction with an unclear CAF subtype (CAF-C3), suggesting a potential novel communication pathway within the CAF population.

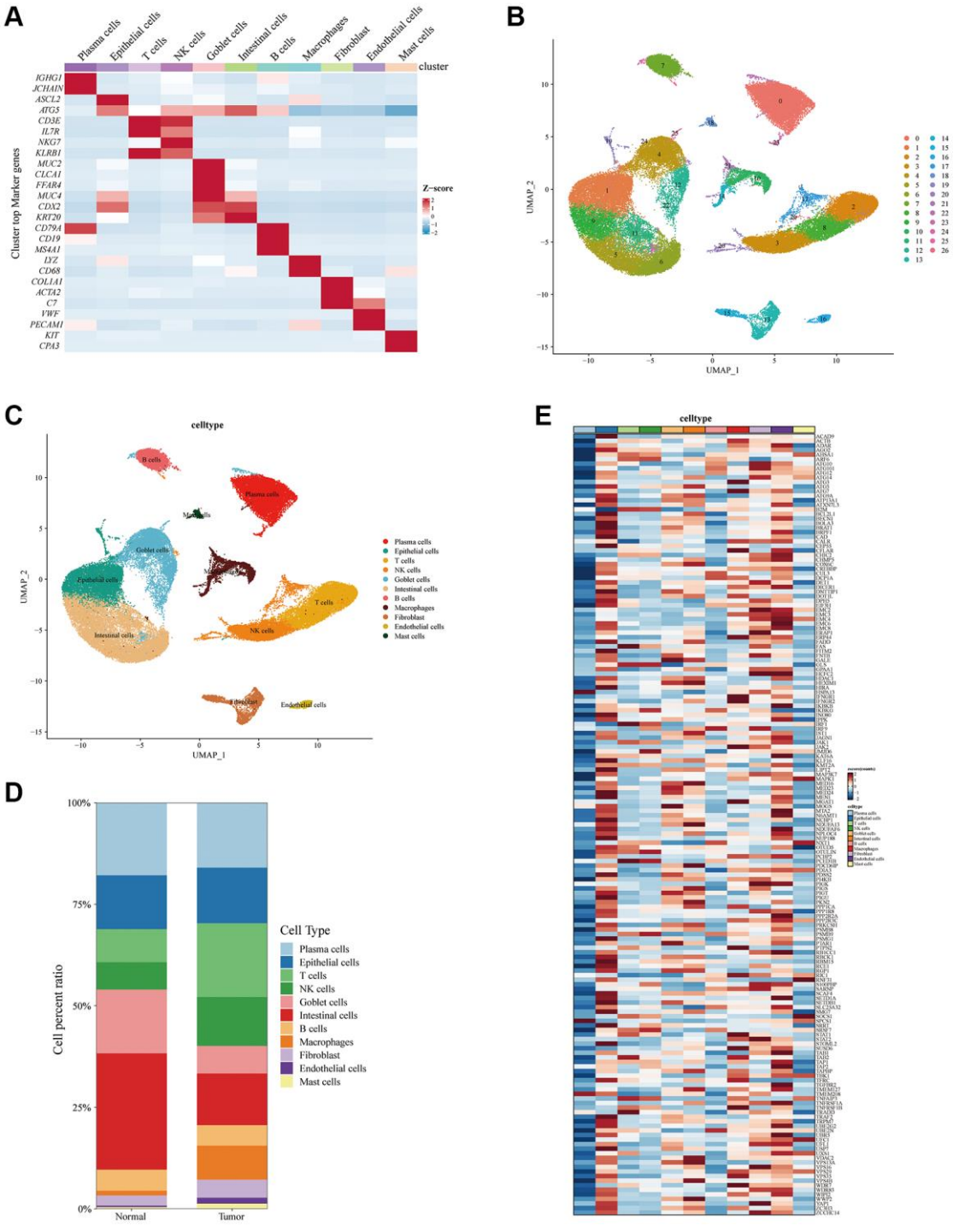

Figure 1. Overview of immune escape-related genes in colorectal cancer single-cell data. (**A**) Heat map showing maker genes annotating subpopulations of colorectal cancer cells. (**B**) The UMAP visualizes 26 cell clusters of colorectal cancer cells. (**C**) The UMAP visualizes 11 cell subpopulations of colorectal cancer cells. (**D**) Cell percentage diagram showing the proportion of cell subpopulations in normal and colorectal cancer tissues. (**E**) Distribution of immune escape-related genes in the heat map of epithelial cells, T cells, goblet cells, NK cells, plasma cells, intestinal cells, macrophages, B cells, fibroblasts, mast cells, and endothelial cells.

Furthermore, goblet cells were found to be closely connected with HEXIMI-positive CAF-C1, hinting at an intricate dialogue between mucosal cells and fibroblasts that may influence tumor microenvironment dynamics. Additionally, a pronounced interaction was observed between HEXIMI-positive CAF-C1 and endothelial cells, indicating a crucial cross-talk that could impact angiogenesis and tumor vasculature (Figure 3D). Notably, unclear-CAF-C3 cell clusters exhibited a significant association with outgoing signaling patterns, incoming signaling patterns, and the GAS signaling pathway network (Figure 3E, 3F). Our investigation identified three distinct branches encompassing different states or clusters (Figure 3G). This trichotomy suggests diverse developmental trajectories or functional phenotypes within the cell population, indicative of cellular heterogeneity and plasticity. Each branch likely signifies unique gene expression patterns and biological activities, highlighting the intricate dynamics and potential transitions among different cellular subpopulations. Furthermore, pseudo-temporal analysis unveiled the pivotal role of immune escape-related genes in the developmental trajectory of various cells within the tumor microenvironment (TME), including epithelial cells, T cells, goblet cells, NK cells, plasma cells, intestinal cells, macrophages, B cells, fibroblasts, mast cells, and endothelial cells (Figure 3H).

Differential gene expression analysis (DEGs) unveiled sets of genes exhibiting positive and negative correlations with the four subtypes of CAF cells (Figure 4A). In our study, we observed a distinct expression of immune-related genes within novel CAF clusters, particularly noting a significant presence in the enigmatic CAF-C3 cluster. SCENIC analysis further identified key transcription factors, such as RASGRP2, CFI, and CCL21 in HEXIM1+CAF-C1, alongside CFD, C3, C7, CXCL12, and IL33 in TGFBR2+CAF-C2, pointing to their roles in immune modulation and tumor microenvironment dynamics (Figure 4D). Furthermore, we found that the unclear-CAF-C3 cell clusters were enriched in signaling pathways such as TNF signaling, IL-17 signaling, rheumatoid arthritis, lipid metabolism, and atherosclerosis (Figure 4B). The HEX1M1+CAF-C1 and TGFBR2+CAF-C2 cell clusters were found to be enriched in signaling pathways related to ribosome function and Coronavirus disease (COVID-19) (Figure 4B). The pan-myCAF typing-related gene marker was predominantly expressed in the HEX1M1+CAF-C1 cell cluster, while the pan-dCAF and pan-pCAF typing-related gene markers were concentrated in the unclear-CAF-C3 cell cluster. The pan-iCAF typing-related gene marker showed high expression in the TGFBR2+CAF-C2 cell cluster, and the pan-iCAF-2 typing-related gene marker was expressed in the HEX1M1+CAF-C1 cluster (Figure 4C).

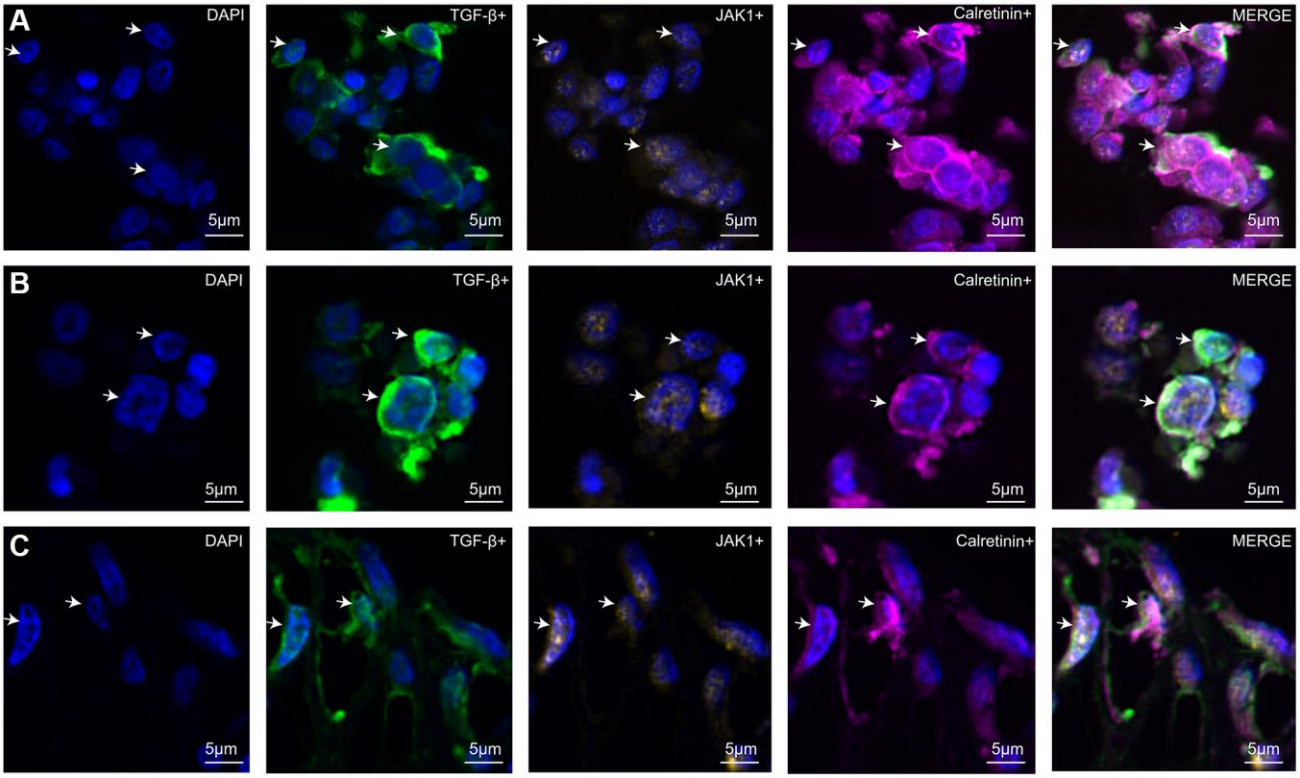

Figure 2. Representative example of three CRC tumors stained by multi-colored IHC. The panel indicates the expression of TGF-β+, JAK1+, and Calretinin+ in three different patients: Patient A (**A**), Patient B (**B**), and Patient C (**C**). Original magnification, x20; scale bar, 5 μm.

Novel immune escape gene-associated CD8+ T subtype cells for TME of CRC

The NK T cells were further classified into different subpopulations, including CD4+ T cells, CD8+ T cells, Treg cells, and unidentified cells. The CD8+ T cells were subsequently divided into seven cell clusters and five distinct cell clusters using NMF clustering, annotated based on immune escaTMEpe genes: SOCS1+CD8+T_cells-C1, CALR+CD8+T_cells-C2, PCBP2+CD8+T_cells-C3, Non-immune_evasion-CD8+T_cells-C5, and JAK1+CD8+T_cells-C4 (Figure 5A, 5B). The proportions of CD8+ T cell clusters associated with immune escape genes exhibited differential expression patterns in 13 CRC patients (Figure 5C). Pseudo-temporal analysis revealed the significant role of immune escape-related genes in shaping the trajectory of CD8+ T cell subtypes (Figure 5F). Additionally, our cell chat analysis has uncovered a pronounced correlation between epithelial and endothelial cells with a novel immune escape gene-associated cluster, specifically the PCBP2+CD8+ T cell cluster C3. This correlation highlights a highly intricate network of cellular interactions, underscoring the crucial communication pathways that may facilitate immune escape mechanisms within the tumor microenvironment. The close interactions observed between these cell types and the PCBP2+CD8+ T cell cluster C3 suggest a complex interplay that potentially impacts tumor immunity and the effectiveness of immune surveillance (Figure 5D). Furthermore, cell-chat signaling analysis indicated that the incoming signaling patterns of endothelial cell clusters were predominantly associated with MK, VEGF, PARs,

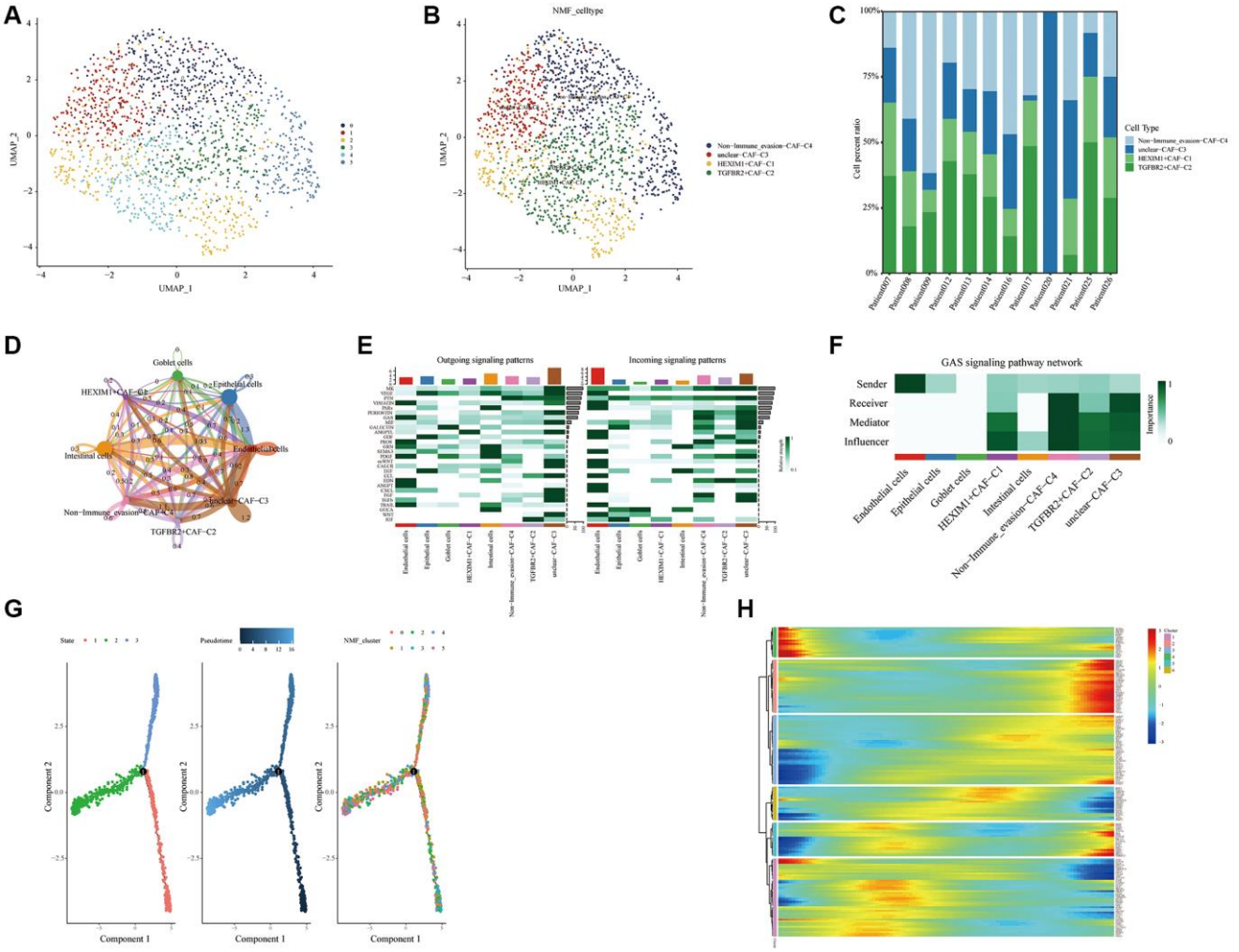

Figure 3. Novel fibroblast clusters under immune escape related gene modifications. (**A**, **B**) UMAP showed fibroblast clusters after immune escape related gene annotation. (**C**) Cell percentages showed expression of novel fibroblast clusters in 12 CRC patients. (**D**) Cell chat revealed the interaction of novel fibroblast clusters with kinds of cells. (**E**, **F**) The signaling pathways of the novel fibroblast cluster inputs and outputs and the GAS signaling pathway network were shown by heat map. (**G**) Pseudo time showed the differentiation of fibroblast clusters after NMF classification. (**H**) Trajectory analysis reveals the role of immune escape related genes in fibroblast clustering after NMF classification.

VISFATIN, and GDF signaling pathways. The MK, PARs, SEMA3, GRN, and CALCR signaling pathways were primarily involved in the outgoing signaling patterns of the novel immune escape gene-associated CD8+ T cell subtype (Figure 5E). DEGs identified groups of genes that exhibited positive and negative correlations with the five subtypes of CD8+ T cells (Figure 6A). Further analysis of key immune checkpoint genes in the novel CD8+ T cell sub-populations revealed that the Non-immune_evasion-CD8+T_cells-C5 cell cluster exhibited a negative association with most immune checkpoint-related genes (Figure 6C). Our SCENIC analysis revealed a unique transcriptional profile in the SOCS1+CD8+T_cells-C1 cluster, with high expression levels of BTN3A1, CSF1R, KDR, and CD244. This suggests a role in modulating immune responses, particularly through regulating immune checkpoints, which balance immune activity and prevent autoimmunity but can also facilitate tumor immune evasion. Similarly, the CALR+CD8+T_cells-C2 cluster showed increased expression of CD276, TIGIT, BTLA, and LAG3, hinting at its potential involvement in promoting immunosuppression within tumors (Figure 6D). Most of the cell clusters showed enrichment in Ribosome and Coronavirus disease signaling pathways, particularly the Non-immune_evasion-CD8+T_cells-C5 cluster (Figure 6B). CD8+ T cell depletion-related genes were predominantly expressed in the SOCS1+CD8+T_cells-C1 and CALR+CD8+T_cells-C2 cell clusters. Additionally, cytotoxic-related genes of CD8+ T cells were primarily expressed in the CALR+CD8+T_cells-C2 and JAK1+CD8+T_cells-C4 cell clusters (Figure 6C).

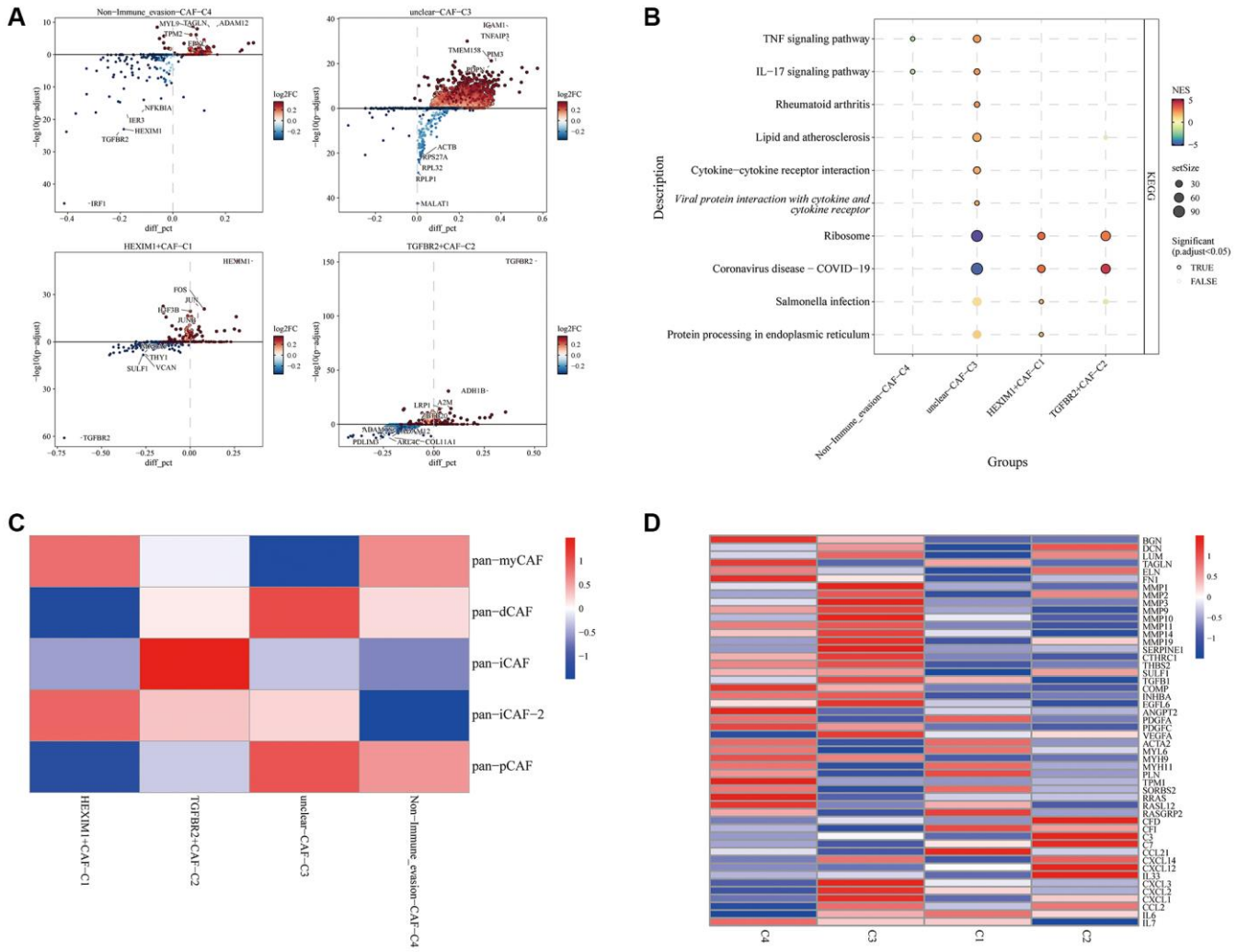

Figure 4. Novel fibroblast clusters under immune escape related gene modifications. (**A**) Differential gene expression analysis (DEGs) unveiled sets of genes exhibiting positive and negative correlations with the four subtypes of CAF cells. (**B**) Enrichment of immune escape related signaling pathways in fibroblast clusters was shown by bubble diagram. (**C**) Heat map demonstrating immune escape related fibroblast clusters enriched in a characteristic subpopulation of previous fibroblasts. (**D**) Transcription factor activities of immune escape related fibroblast clusters are shown by heat maps.

Novel immune escape gene-associated macrophage subtype cells for TME of CRC

After applying MNF clustering, macrophage cells were divided into seven distinct cell clusters and annotated based on immune escape genes, resulting in the identification of four cell clusters: IRF1+Mac-C1, TAP1+Mac-C2, unclear-Mac-C3, and Non-immune_evasion-Mac-C4 (Figure 7A, 7B). Notably, the Non-immune_evasion-Mac-C4 cell cluster constituted the majority of macrophage cells (Figure 7C). Pseudo-temporal analysis revealed the pivotal role of immune escape-related genes in shaping the developmental trajectory of macrophage subtypes, with the pseudo-time analysis of cell clusters resulting in the identification of three branches (Figure 7F). This branching pattern suggests diverse functional states or transitional phases within the macrophage population, shedding light on the complexity of immune responses within the tumor microenvironment. Cell chat analysis depicted interactions between the cell clusters of novel immune escape gene-associated macrophage subpopulations (Figure 7D). Outgoing signaling patterns involving GALECTIN, ANNEXIN, CXCL, and GRN were predominantly associated with the TAP1+Mac-C2 cell clusters. On the other hand, incoming signaling patterns involving GALECTIN, ANNEXIN, MIF, TNF, and SPP1 were primarily associated with the IRF1+Mac-C1 cell clusters (Figure 7E). DEGs revealed sets of genes exhibiting positive and negative correlations with the four subtypes of macrophage cells (Figure 8A). The IRF1+Mac-C1, TAP1+Mac-C2, and Non-immune_evasion-Mac-C4 clusters were enriched in most immune escape-related signaling pathways (Figure 8B). Additionally, we compared the novel immune escape gene-associated macrophage subpopulations with the M1 and M2 macrophage classifications. As depicted in Figure 8C, the novel immune escape gene-associated macrophage cell clusters were predominantly associated with the M1-type cell subpopulation.

Novel immune escape gene-associated B subtype cells for TME of CRC

B cells were categorized into nine distinct cell clusters through MNF clustering (Figure 9A). Utilizing immune escape-related genes, B cells were further divided into JAK1+B_cells-C1, TAPBP+B_cells-C2, unclear-B_cells-C5, CFLAR+B_cells-C3, and IRF1+B_cells-C4

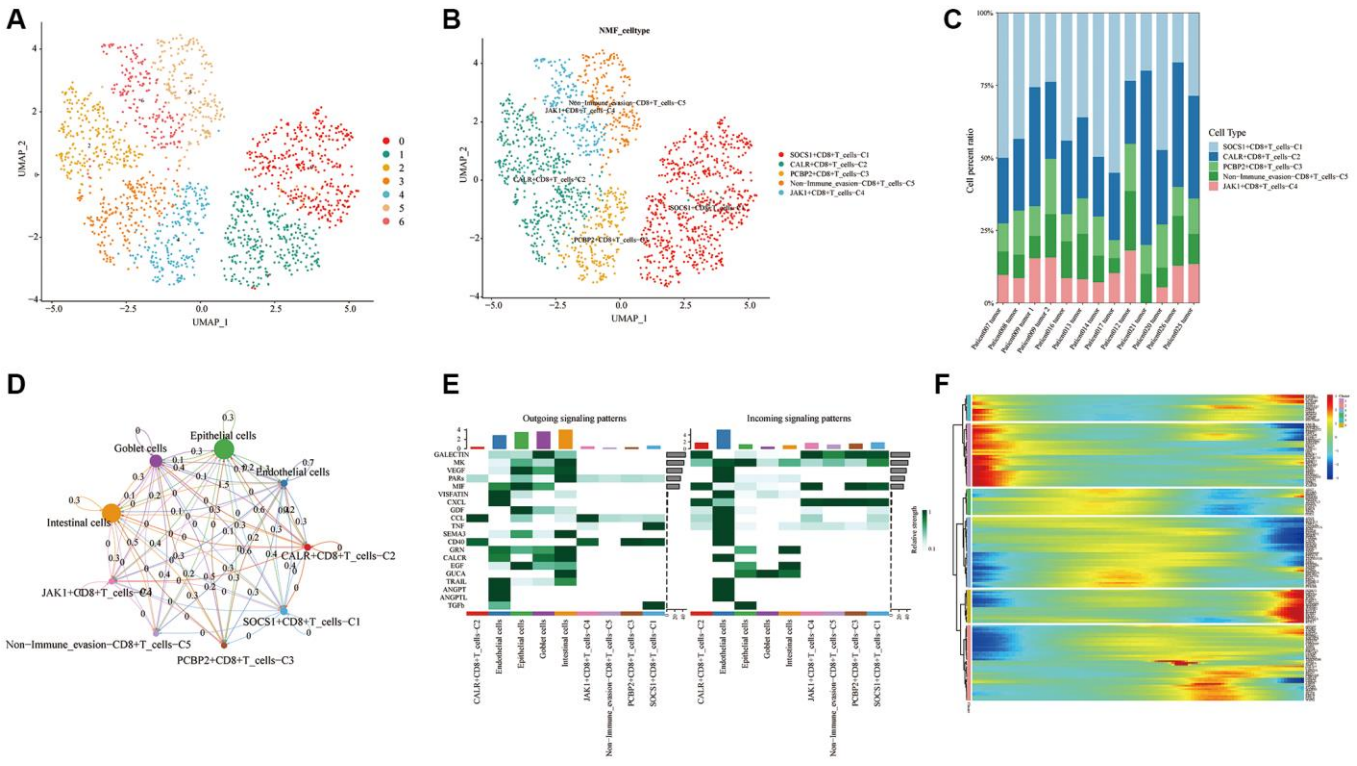

Figure 5. Novel CD8+ T cell clusters under immune escape related gene modifications. (**A**, **B**) UMAP showed CD8+ T cell clusters after immune escape related gene annotation. (**C**) Cell percentages showed expression of novel fibroblast clusters in 13 CRC patients. (**D**) Cell chat revealed the interaction of novel CD8+ T cell clusters with kinds of cells. (**E**) The signaling pathways of the novel CD8+ T cell cluster inputs and outputs were shown by heat map. (**F**) Trajectory analysis reveals the role of immune escape related genes in CD8+ T cell clustering after NMF classification.

(Figure 9B). The cell clusters of TAPBP+B_cells-C2, unclear-B_cells-C5, and CFLAR+B_cells-C3 accounted for approximately 80% of the B cell population (Figure 9C). Cell-chat analysis revealed interactions between the novel B cell subtypes and endothelial cells and intestinal cells, primarily involving JAK1+B_cells-C1 and TAPBP+B_cells-C2 (Figure 9D). Pseudo-temporal analysis unveiled the significant role of immune escape-related genes in shaping the developmental trajectory of B cell subtypes, with the pseudo-time analysis of cell clusters resulting in the identification of four branches (Figure 9F, 9G). These branches likely represent different functional states or transitional stages within the B cell population, indicating the diverse roles and responses of B cells in the tumor microenvironment. Outgoing signaling patterns involving CXCL, VISFATIN, TRAIL, ANGPT, ANGPTL, and TGFβ were primarily associated with endothelial cell clusters, while incoming signaling patterns involving MK, GALECTIN, MIF, and CXCL were predominantly associated with IRF1+B_cells-C4 cell clusters (Figure 9E).

Prognostic and immunological effects of novel immune escape gene-associated cell clusters on CRC

Additionally, we investigated the prognostic implications of subtype cells based on transcriptional and prognostic information in CRC. Cell chat analysis demonstrated intercellular interactions among all immune escape gene-associated subtypes of cells in CRC (Figure 10A). The box plot revealed higher expression levels of immune escape genes in tumor tissues compared to normal tissues in the CRC

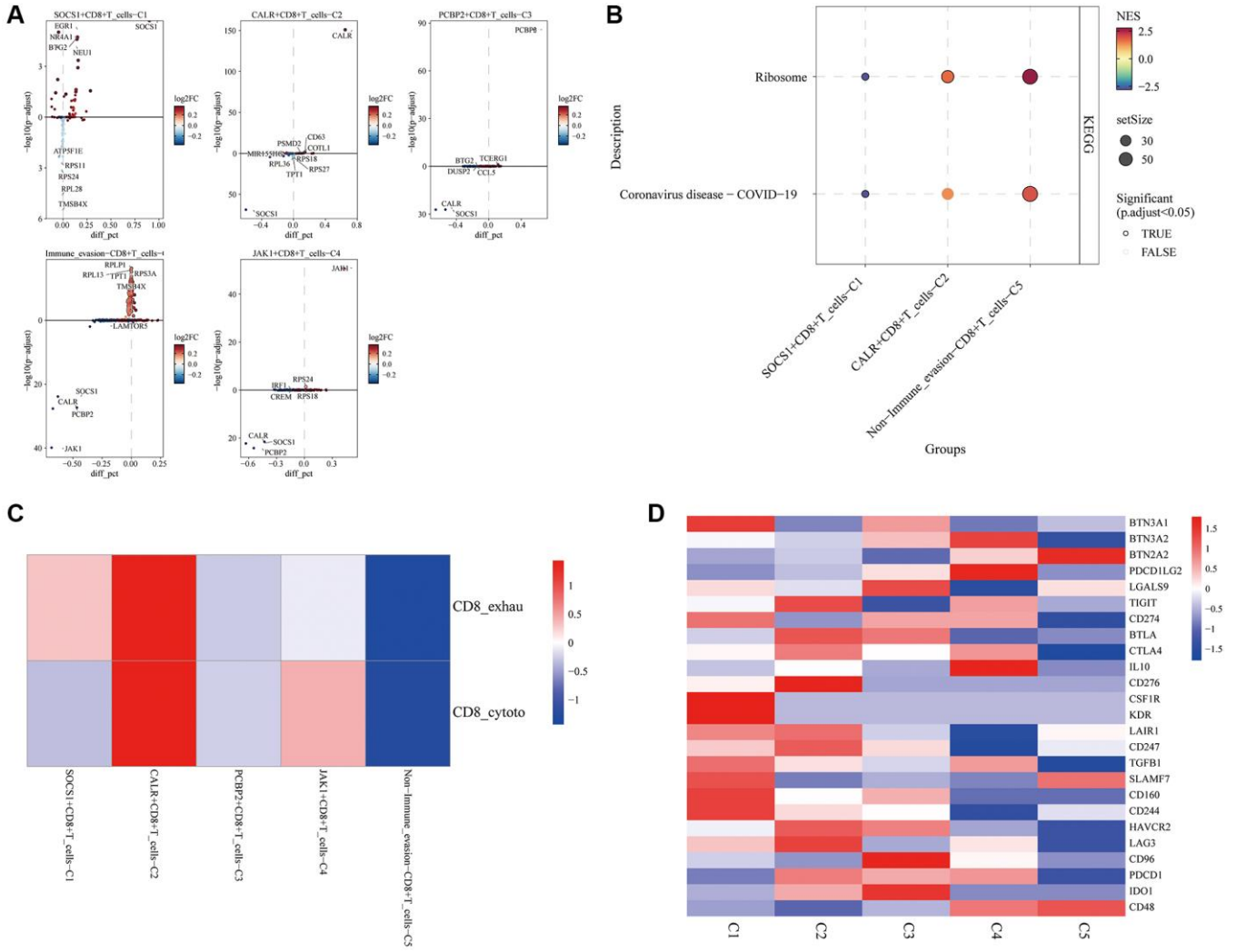

Figure 6. Novel CD8+ T cell clusters under immune escape related gene modifications. (**A**) Differential gene expression analysis (DEGs) unveiled sets of genes exhibiting positive and negative correlations with the five subtypes of CD8+ T cells. (**B**) Enrichment of immune escape related signaling pathways in CD8+ T cell clusters was shown by bubble diagram. (**C**) Heat map demonstrating immune escape related CD8+ T cell clusters enriched in a characteristic subpopulation of previous CD8+ T cells. (**D**) Transcription factor activities of immune escape related CD8+ T cell clusters are shown by heat maps.

population (Figure 10B). Moreover, we identified specific cell types that exhibited higher expression in tumor tissues compared to normal tissues, including CFLAR+B_cells-C3, IRF1+B_cells-C4, TAP1+Mac-C2, SOCS1+CD8+T_cells-C1, CALR+CD8+T_cells-C2, and PCBP2+CD8+T_cells-C3 ($p < 0.05$). Conversely, JAK1+B_cells-C1, TAPBP+B_cells-C2, HEX1M1+CAF-C1, TGFBR2+CAF-C2, and CALR+CD8+T_cells-C3 were found to exhibit lower expression in tumor tissues compared to normal tissues ($p < 0.05$) (Figure 10D). Furthermore, univariate Cox analysis demonstrated consistent prognostic significance for different novel immune escape gene-associated cell subtypes across different datasets (Figure 10C). The HEX1M1+CAF-C1 and SOCS1+CD8+T_cells-C1 cell clusters may serve as risk factors for CRC patients. In the CRC population cohorts, distinct immunotherapeutic responses were observed among multiple immune escape gene-associated cell subtype clusters ($p < 0.05$) (Figure 10F, 10G). Similarly, in the IMvigor210 cohort, we identified differences in JAK1+B_cells-C1, HEX1M1+CAF-C1, TGFBR2+CAF-C2, and CALR+CD8+T_cells-C2 cell clusters among patients with varying immunotherapeutic responses ($p < 0.05$) (Figure 10E). Finally, we conducted K-M prognostic analysis in the IMvigor210 and CRC population cohorts, highlighting the importance of several novel immune escape gene-associated cell clusters in predicting overall survival in the CRC population (Figures 11–13). The results demonstrate that the high expression of TGFBR2+CAF-C2, CALR+CD8+T_cells-C2, PCBP2+CD8+T_cells-C3, IRF1+Mac-C1, TAP1+Mac-C2, JAK1+B_cells-C1, TAPBP+B_cells-C2, CFLAR+B_cells-C3, IRF1+B_cells-C4 cell subtypes is associated with a favorable prognosis in colorectal cancer patients, with statistically significant differences. Conversely, the elevated expression of HEX1M1+CAF-C1, TGFBR2+CAF-C2, JAK1+CD8+T_cells-C4, SOCS1+CD8+T_cells-C1 cell subtypes is correlated with a poor prognosis in colorectal cancer patients, also with statistically significant differences.

DISCUSSION

Until now, several studies have elucidated the connection between immune escape and the pathogenesis, progression, or immunotherapy resistance in CRC [6, 15–18]. Nevertheless, the majority of these studies have mainly focused on investigating the action mechanisms of immune escape-related genes in

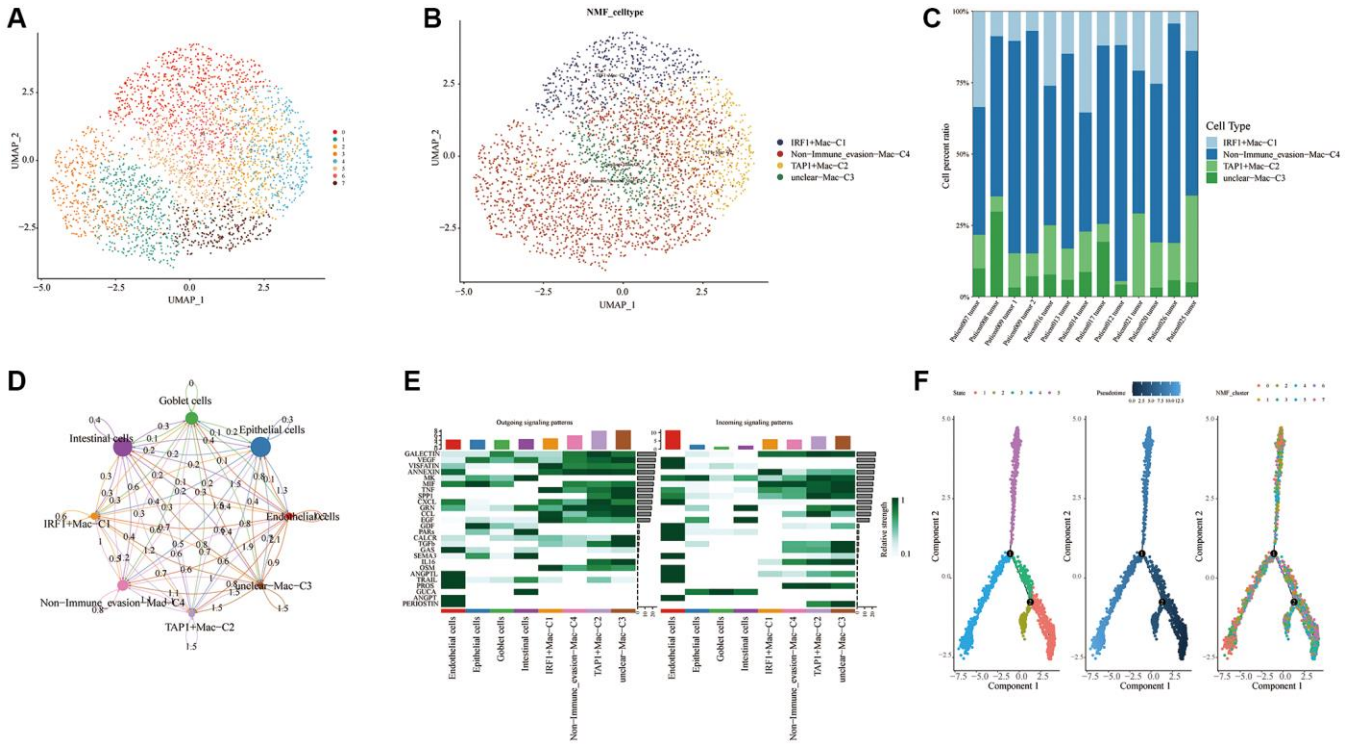

Figure 7. Novel macrophage cell clusters under immune escape related gene modifications. (**A**, **B**) UMAP showed macrophage cell clusters after immune escape related gene annotation. (**C**) Cell percentages showed expression of novel macrophage cell clusters in 13 CRC patients. (**D**) Cell chat revealed the interaction of novel macrophage cell clusters with kinds of cells. (**E**) The signaling pathways of the novel macrophage cell cluster inputs and outputs were shown by heat map. (**F**) Pseudo time showed the differentiation of macrophage cell clusters after NMF classification.

individual tumor microenvironment cells of CRC. In this research, we introduce the first comprehensive TME mapping study of immune escape-related genes in CRC, along with the identification of interaction pathways among multiple immune cell subtypes. We observed that various clusters of immune subtypes are associated with the prognosis of CRC. This novel perspective offers a fresh approach to exploring the mechanisms of immune escape in CRC.

Cancer-associated fibroblasts (CAFs) represent a crucial element within the tumor microenvironment, significantly contributing to tumor progression. CAFs suppress immune cell function and promote tumor development by secreting various cytokines to exchange information with other stromal cells and tumor. Fibroblasts associated with tumors, known as CAFs, form a key part of the tumor microenvironment and are instrumental in the progression of tumors. By secreting various cytokines and interacting with stromal cells and tumor cells, CAFs suppress immune cell function and promote tumor growth [19, 20]. Intricate control systems, which include established signaling cascades like transforming growth factor-β (TGF-β) and Hedgehog (Hh), along with a range of transcription factors, govern the development, activation, diversity, metabolic traits, and tumorigenic properties of tumor-associated fibroblasts (CAFs) [21]. Pei's study demonstrates the influence of CAFs on the efficacy of anti-PD-1/PD-L1 immunotherapy [22]. The classical analyses widely used in several tumors include pan-myCAFs, pan-dCAFs, pan-iCAFs, pan-nCAFs, and pan-pCAFs [20]. In our study, enrichment analysis revealed that the HEX1M1+CAF-C1 cell cluster participated in multiple pathways related to Ribosome, Coronavirus disease, Salmonella infection, and Protein processing in the endoplasmic reticulum. Additionally, various transcription factor activities (RASGRP2, CCL21, CF1, etc.) were highly expressed in this cell cluster. Highly expressed PPP1CB+CAF-C4 cell clusters in CRC patients were associated with a poor prognosis, suggesting potential immunosuppressive

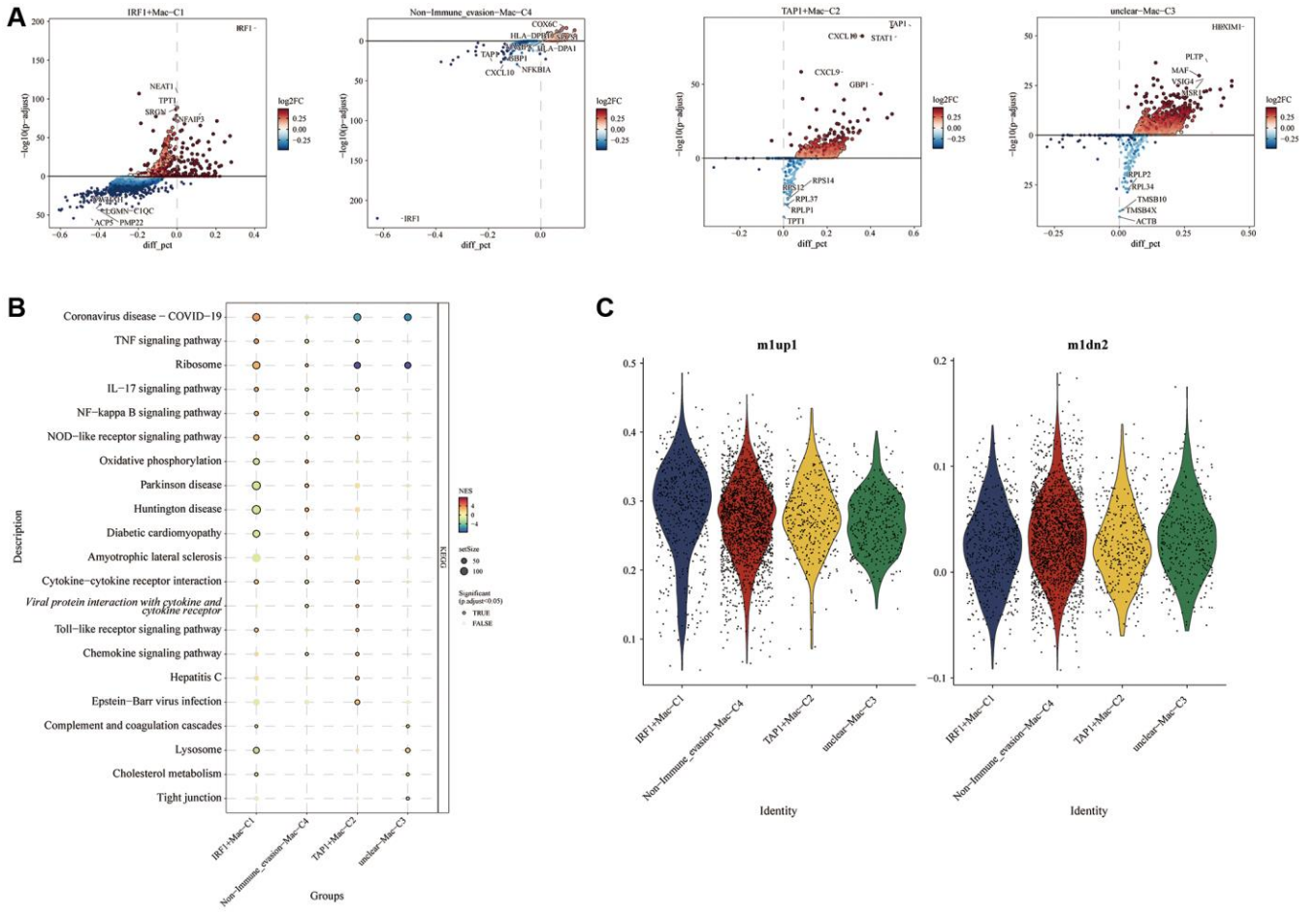

Figure 8. Novel macrophage cell clusters under immune escape related gene modifications. (**A**) Differential gene expression analysis (DEGs) unveiled sets of genes exhibiting positive and negative correlations with the four subtypes of macrophage cells. (**B**) Enrichment of immune escape related signaling pathways in Macrophage cell clusters was shown by bubble diagram. (**C**) Violin diagram showing the expression of novel immune escape related macrophage clusters in M1 and M2 phenotypes.

interactions between immune escape-related CAFs and tumor cells that promote tumor progression.

The microenvironment of colorectal tumors encompasses diverse cellular subsets of the innate immune system, including macrophages, neutrophils, natural killer (NK) cells, and T and B lymphocytes, which are essential components of adaptive immunity [23]. In CRC patients, immune cells not only contribute to anti-tumor responses but also play a role in cancer transformation, particularly CD8+ T cells [24]. Studies suggest that CRC patients with a lack of CD8+ T cells in the tumor microenvironment may not benefit from immunotherapy [25, 26]. Our study identified SOCS1+CD8+ T cells-C1 cell clusters as the dominant population among CD8+ T cells, and these cells were highly expressed in CD8+ T cell exhaustion. This finding suggests that SOCS1 may inhibit tumor immunity through immune escape, leading to a poor prognosis for CRC with high expression of SOCS1+CD8+ T cells-C1 cell clusters. Moreover, these cells are highly expressed in most immune-related signaling pathways, indicating that immune escape may be a prognostic factor in colorectal cancer patients.

To provide a detailed atlas, we present the immune escape-related B-cell and macrophage cell clusters in the TME of CRC. Accumulation of tumor-associated macrophages is common in CRC patients and is associated with CRC progression and poor prognosis [27–29]. Macrophages can differentiate into classical (M1) and alternative (M2) activated polar cells

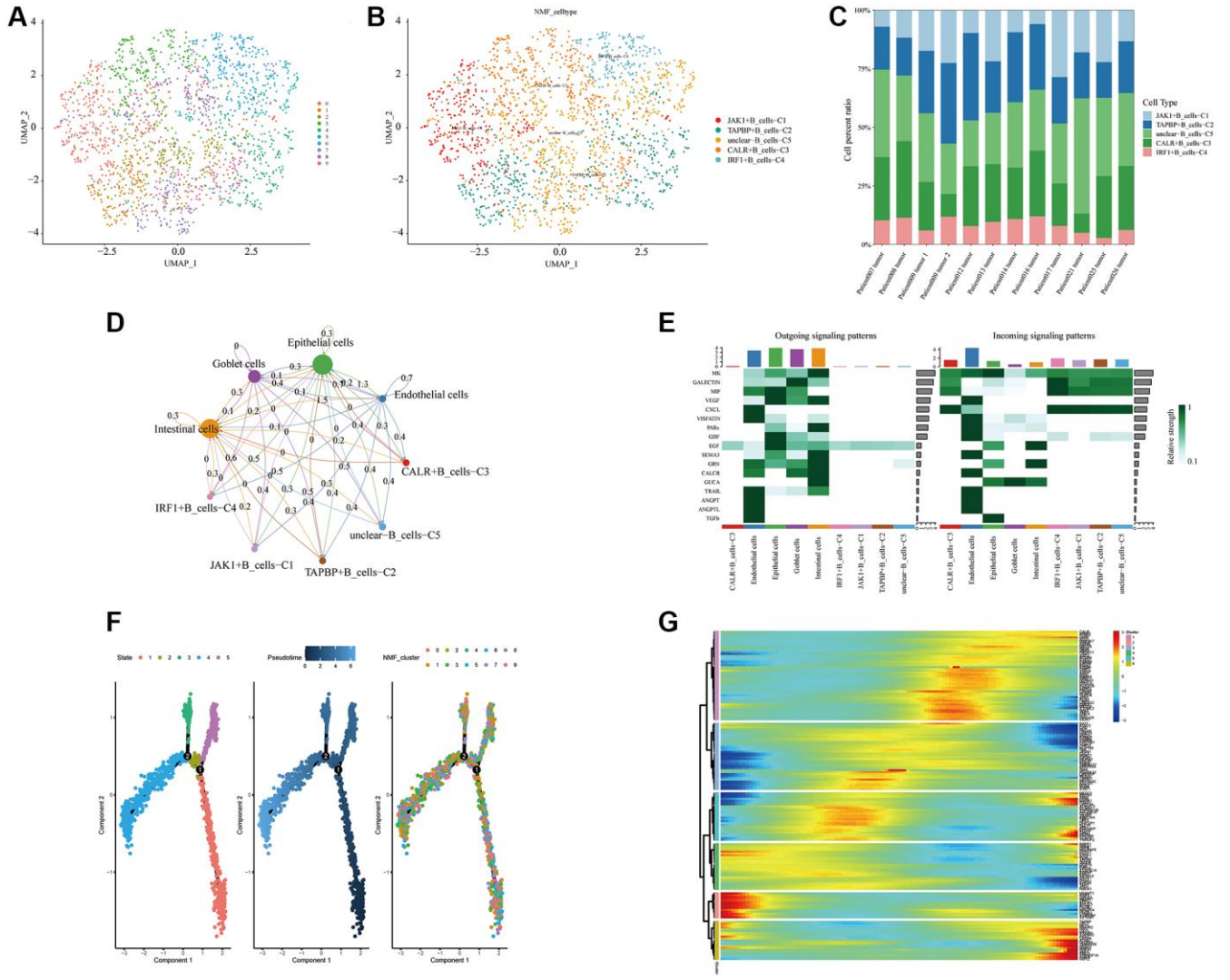

Figure 9. Novel B cell clusters under immune escape related gene modifications. (**A**, **B**) UMAP showed B cell clusters after immune escape related gene annotation. (**C**) Cell percentages showed expression of novel B cell clusters in 12 CRC patients. (**D**) Cell chat revealed the interaction of novel B cell clusters with kinds of cells. (**E**) The signaling pathways of the novel B cell cluster inputs and outputs were shown by heat map. (**F**) Pseudo time showed the differentiation of B cell clusters after NMF classification. (**G**) Trajectory analysis reveals the role of immune escape related genes in B cell clustering after NMF classification.

depending on environmental conditions. M1 macrophages generally exert an inflammatory response through the expression of nitric oxide synthase (iNOS). In contrast, M2 macrophages, by producing anti-inflammatory cytokines like IL-10, facilitate the advancement and spread of tumors. Our study revealed that the majority of immune escape-related macrophage clusters in CRC were highly expressed in the M1 type. Prognostic analysis demonstrated that IGF1+ Mac-C1 cell clusters were highly expressed in CRC patients with a good prognosis, suggesting the role of immune escape-related subtype cells in inhibiting tumor metabolism, progression, and metastasis.

To analyze the complex heterogeneity of immune escape-related genes in the TME of CRC, we comprehensively summarized the relationship between the scores of these subclusters with prognosis and immune response from a large publicly available RNA-seq cohort. Our findings underscored the dramatic variation in prognosis for CRC patients with TME cell clusters of immune escape-related subtypes, particularly

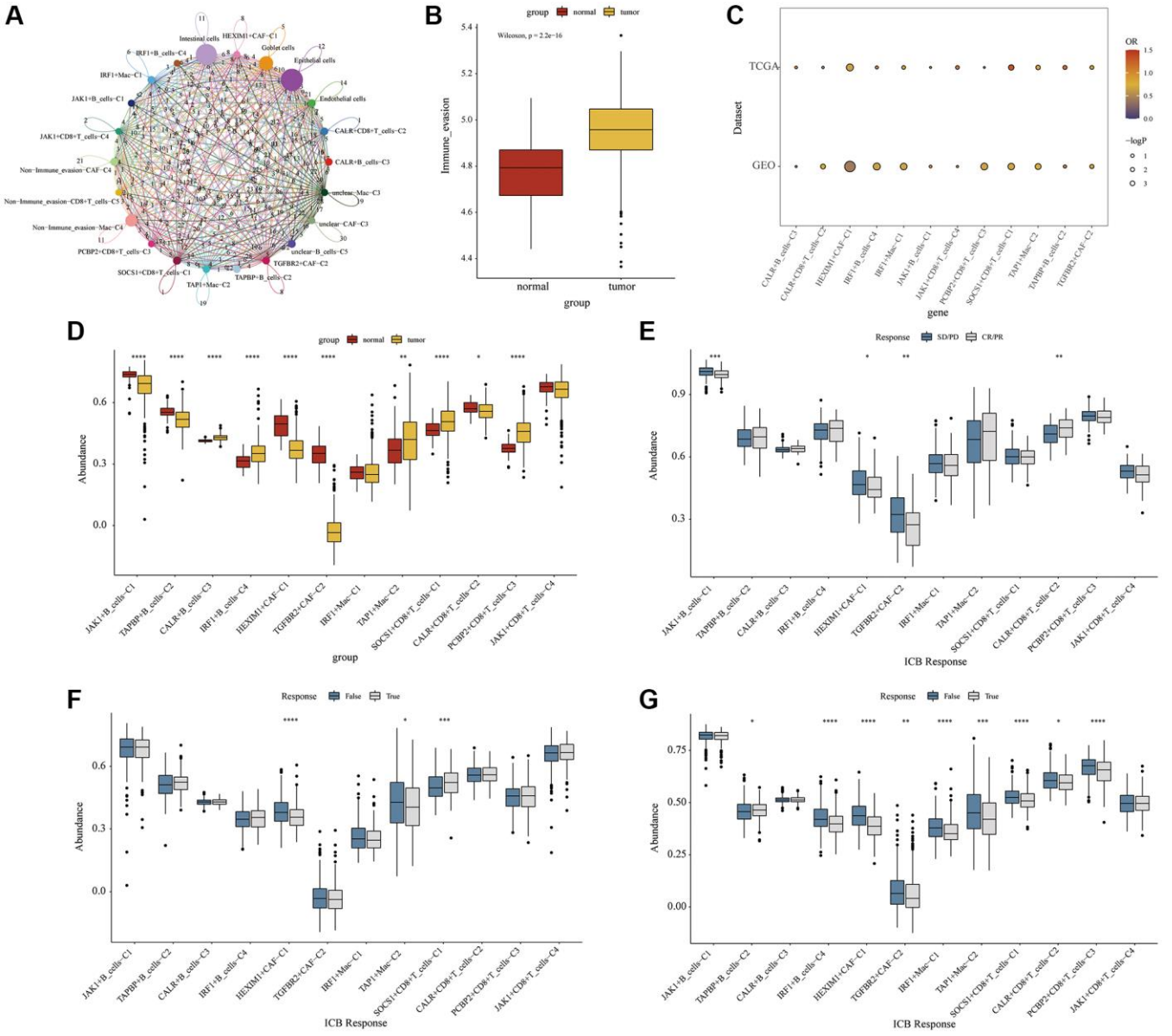

Figure 10. Novel immune escape related cell clusters prognosis and immune response in patients with colorectal cancer. (**A**) Cell chat demonstrates interactions between all novel immune escape related cell clusters. (**B**) Box plot showing the difference in immune escape related gene expression in CRC tumor tissue and normal tissue. (**C**) Bubble plot demonstrating one-way Cox analysis of novel immune escape related cell clusters in the prognosis of TCGA and CRC patient cohorts. (**D**) Box plot demonstrating the difference in expression of immune escape related cell clusters in CRC tumor tissues and normal tissues. (**E**–**G**) Box plot demonstrating novel immune escape related cell clusters in immune response in TCGA, CRC, and IMvigor patient cohorts. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

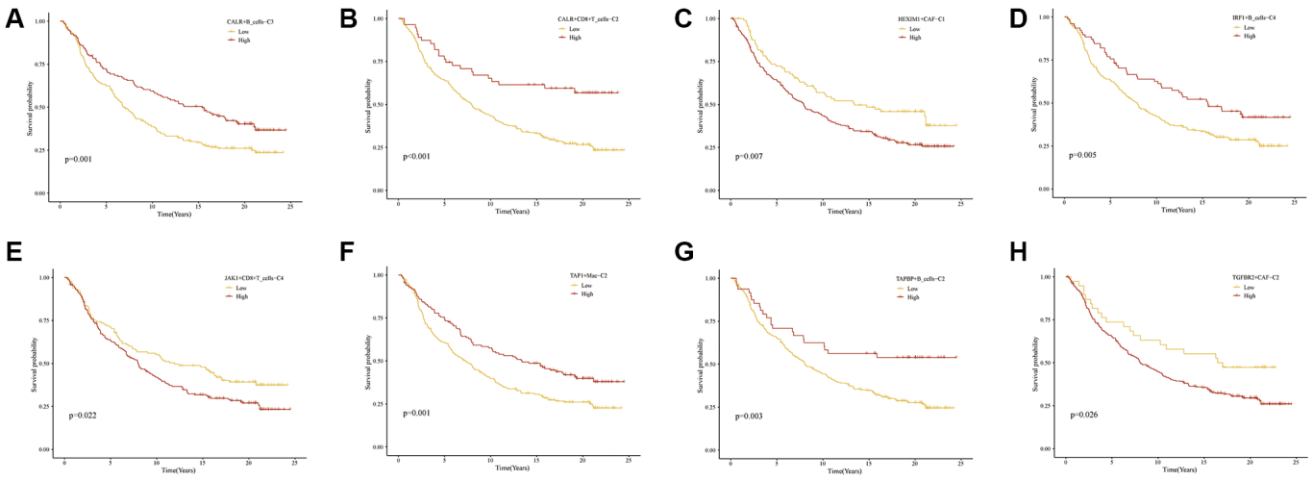

Figure 11. Prognosis of novel immune escape related cell clusters in the TCGA cohort. The panels indicate the following cell clusters: CALR+B_cells-C3 (**A**), CALR+CD8+T_cells-C2 (**B**), HEX1M1+CAF-C1 (**C**), IRF1+B_cells-C4 (**D**), JAK1+CD8+T_cells-C4 (**E**), TAP1+Mac-C2 (**F**), TAPBP+B_cells-C2 (**G**), and TGFBR2+CAF-C2 (**H**).

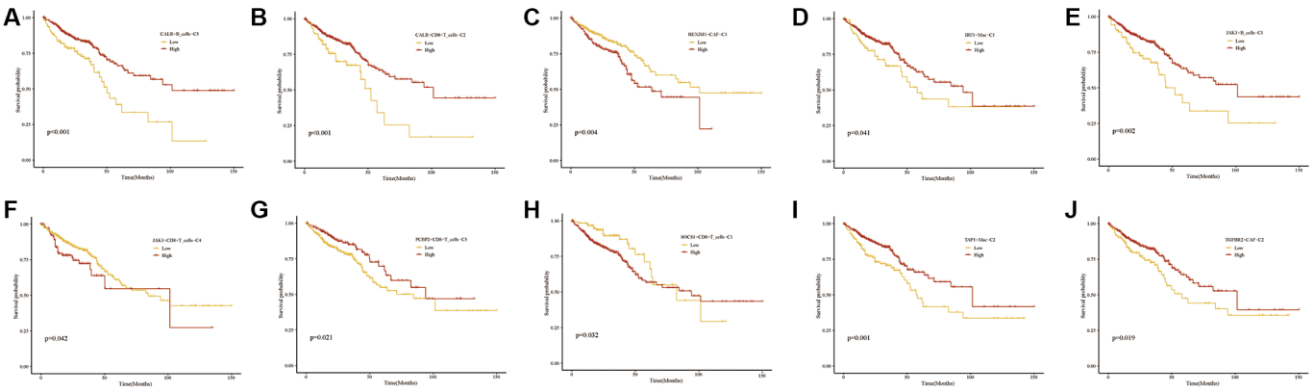

Figure 12. Prognosis of novel immune escape related cell clusters in the CRC cohort. The panels indicate the following cell clusters: CALR+B_cells-C3 (**A**), CALR+CD8+T_cells-C2 (**B**), HEX1M1+CAF-C1 (**C**), IRF1+Mac-C1 (**D**), JAK1+B_cells-C1 (**E**), JAK1+CD8+T_cells-C4 (**F**), PCBP2+CD8+T_cells-C3 (**G**), SOCS1+CD8+T_cells-C1 (**H**), TAP1+Mac-C2 (**I**), and TGFBR2+CAF-C2 (**J**).

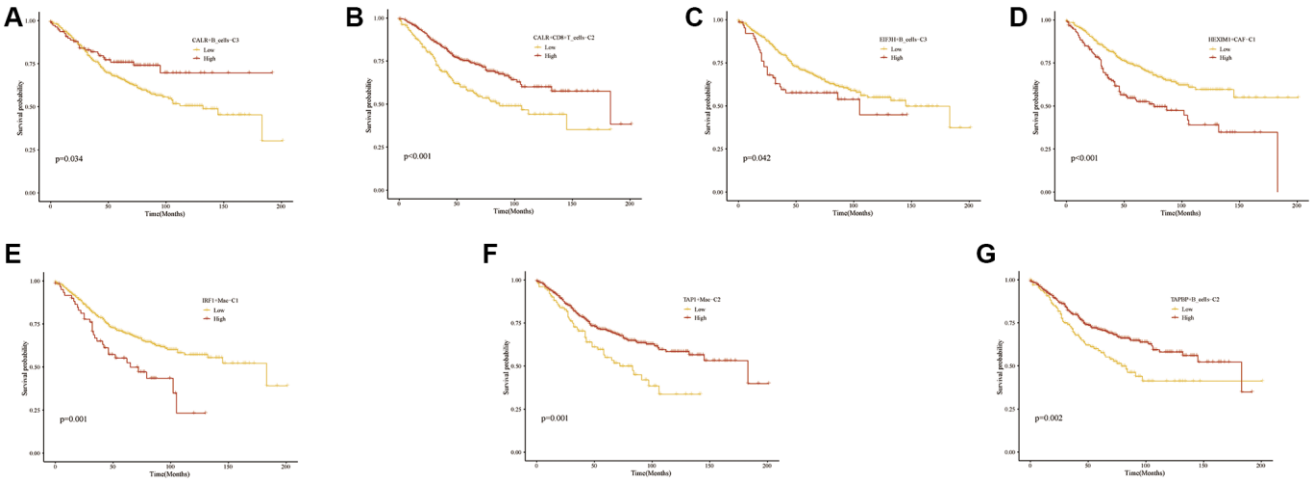

Figure 13. Prognosis of novel immune escape related cell clusters in the IMvigor cohort. The panels indicate the following cell clusters: CALR+B_cells-C3 (**A**), CALR+CD8+T_cells-C2 (**B**), EIF3H+B_cells-C3 (**C**), HEXIM1+Mac-C1 (**D**), IRF1+Mac-C1 (**E**), TAP1+Mac-C2 (**F**), and TAPBP+B_cells-C2 (**G**).

CAFs and macrophages, highlighting the crucial role of TME immune escape in CRC patients. As a preliminary study, our investigation of immune escape-related mapping in TME cells of CRC revealed the need for more samples to validate our analysis. The scRNA-seq approach provided a new perspective on the characteristics of immune escape-related genes in various TME monocytes, shedding light on the critical clinical step forward in reducing CRC tumor heterogeneity.

In conclusion, based on single-cell sequencing analysis, presents the molecular profiles of immune-associated TME cell subtypes in CRC, highlighting the role of immune escape-mediated intercellular communication in tumor growth regulation and anti-tumor immune modulation. The identification of immune escape-related subtypes and their impact on CRC patient outcomes provides valuable insights for future investigations aiming to enhance therapeutic responses and patient prognosis in CRC.

In providing a detailed atlas, we present the immune escape-related B-cell and macrophage cell clusters within the tumor microenvironment (TME) of colorectal cancer (CRC). It's well-documented that the accumulation of tumor-associated macrophages (TAMs) is common in CRC patients and is linked to CRC progression and poor prognosis [27–29]. Macrophages can differentiate into classical (M1) and alternative (M2) activated polar cells, depending on environmental conditions. M1 macrophages are known for their pro-inflammatory activity, characterized by the production of nitric oxide synthase (iNOS). On the other hand, M2 macrophages secrete cytokines with anti-inflammatory effects, such as IL-10, which have been implicated in aiding the growth and spread of tumors. Our research revealed that the majority of immune escape-related macrophage clusters in CRC were predominantly of the M1 type. Additionally, prognostic analysis showed that IGF1+ Mac-C1 cell clusters were highly expressed in CRC patients with favorable prognosis, suggesting the role of these immune escape-related subtype cells in inhibiting tumor metabolism, progression, and metastasis.

MATERIALS AND METHODS

Data collection

We conducted single-cell transcriptome sequencing on four relevant tissue types, including non-tumor colorectum and primary tumor tissue, using 13 CRC patients from the GSE166555 dataset. Primary tumors and non-tumor colorectum tissues were selected for further analysis. Transcriptomic data and clinical prognosis-related information were obtained from The Cancer Genome Atlas (TCGA) dataset, which included 689 CRC cases. Additionally, transcriptomic data from 579 CRC cases from the Gene Expression Omnibus (GEO) colorectal cancer cohort were used as a validation cohort (GSE39582). The immune escape gene set comes from the previously published literature [30].

Visualization of TME cell types in CRC

The scRNA-seq gene expression matrix of CRC was used to create Seurat objects using the R package "Seurat". Low-quality cells were filtered out based on criteria such as unique molecular identifiers (UMI) count (<200 UMI), number of cells expressing a gene (<200 or >8,000 cells), and mitochondrial genome UMI count (>10% UMI). Consequently, a collection of 66,050 high-quality transcriptomes at the single-cell level was established from all the samples. Dimensionality reduction was performed using a scale matrix of 2,000 highly variable genes (Supplementary Figure 1B), and tissue and patient samples were merged using the R package "harmony" for data scaling and integration. Data visualization was achieved through UMAP/tSNE techniques. Cellular subpopulations were annotated using markers obtained from the literature [31].

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

To investigate the single-cell trajectory analysis with immune escape in CRC TME, we employed the Monocle R package to perform developmental sorting of all cell subtypes in CRC. The version of the Monocle R package used was 1.0.0. Data preprocessing steps included filtering cells based on mean expression and empirical dispersion criteria. Downscaling of data was performed using the 'DDRTree' method. The distribution of immune escape in the NMF cluster developmental trajectory of different TME cells was visualized.

Non-negative matrix factorization of immune escape related genes in TME cells

First, we constructed an expression matrix specifically for the 182 immune escape-related genes. This matrix represents the gene expression profiles of individual cells within the TME. Cells that did not express any of these immune escape-related genes were excluded from the analysis to focus solely on relevant cellular populations. Next, we employed the "NMF" package, a widely used tool in the field of computational biology, to perform matrix decomposition. This process involves decomposing the original gene expression matrix into

two lower-dimensional matrices: a matrix of basis vectors (representing the features or patterns) and a matrix of coefficients (indicating the contribution of each basis vector to each cell). By iteratively optimizing these matrices, NMF identifies latent factors that capture the underlying structure of the data. Following matrix decomposition, we conducted dimensional reduction clustering to group cells with similar gene expression profiles into clusters. This step helps identify distinct cellular subpopulations within the TME based on their immune escape-related gene expression patterns. It's worth noting that our approach is consistent with methodologies described in previous studies by Sidharth and Chen [32, 33], ensuring the robustness and reproducibility of our analytical pipeline.

Identification of cellular subtype marker genes associated with immune escape related genes in TME cells

To further delineate NMF cellular subsets, we conducted reannotation using characteristic genes from each cluster. The reannotation criteria comprised a log-fold change (logFC) greater than 1 for immune escape-related signature genes, logFC greater than 1 for non-immune escape-related genes, and logFC less than 1 for immune escape-related genes with ambiguous annotation. This meticulous process ensured the precise identification of distinct cellular subtypes associated with immune escape mechanisms.

Functional enrichment analysis of NMF glycogen immune escape genes-related subtypes

We conducted enrichment score analysis to delve into the immune escape-related subtypes identified through NMF typing. The "immune escape" function was utilized to randomly generate 30 scores for immune escape-related signaling pathways. This approach enabled comprehensive exploration of the immune escape mechanisms operating within different cellular subtypes.

Cell communication analysis for NMF immune escape genes-related subtypes of cells

To probe the cellular interactions among NMF immune escape gene-related cell types, we conducted intercellular ligand-receptor analysis using the CellChat function. This process entailed extracting MNF-related cell subtypes and immune cells, identifying overexpressed genes, and detecting ligand-receptor pairs. Subsequently, ligands and receptors were mapped onto the protein-protein interaction network for weight analysis. Outgoing and incoming signaling pathways of different NMFs were then visualized, providing insights into the dynamic communication networks operating within the tumor microenvironment.

SCENIC analysis for immune escape-related subtypes

Transcription factors are a group of proteins that directly interact with the genome, binding to specific DNA sequences known as transcription factor binding sites (TFBS/motifs), and regulating the process of DNA transcription. In our study, we performed cell-cell communication analysis specifically for NMF immune escape-related subtypes. We examined transcription start sites (TSS) and gene regulatory networks in CRC single-cell RNA sequencing (scRNA-seq) data using gene motif ranking from the RcisTarget database, specifically the hg19-tss-centered-10kb-7species dataset. Initially, we applied default parameters for gene filtering, where the filtering criteria required that the sum of gene expression be greater than 3% of the total cell count and expressed in at least 1% of cells. Subsequently, we calculated the correlation matrix and performed TF-Targets correlation regression analysis. Due to the large number of cell types, we utilized the Regulon Specificity Score (RSS) to identify cell type-specific regulons. We selected a z-score threshold of 1.5 to further map the transcription factor FeaturePlot. Additionally, we generated a heatmap of regulon scores. For further analysis and visualization, we employed the "ComplexHeatmap", "ggplot2", and "pheatmap" packages. To enhance the visualization of our findings, we utilized the "ComplexHeatmap", "ggplot2", and "pheatmap" packages for further analysis and visualization.

Prognostic analysis of immune escape-related genes for CRC

Prognostic assessment at the bulk level of immune escape related cell subtypes in CRC was conducted by calculating genetic marker scores using the "GSVA" function on public CRC datasets. The immune escape-related NMF characteristics and overall patient survival (OS) were explored using log-rank tests and Cox proportional hazard regression. Kaplan-Meier curves were plotted for different cell subtypes using the "survminer" R package. Prognosis of the different immune escape related NMF cell subtypes was visualized using the "ggplot" function.

Immunotherapeutic response of MNF immune escape-related subtype cells

Tumor Immune Dysfunction and Exclusion (TIDE) analysis, which predicts patient response to treatment, was performed using the CRC transcriptomic datasets from the TIDE database. The IMvigor210

immunotherapy dataset was used to explore the immune response of NMF immune escape-related subtypes.

Statistical analysis

Statistical tests such as Student's *t*-test, Wilcoxon rank-sum test, and Kruskal-Wallis test were utilized for subgroup comparisons. Correlations between subtype cells and the immune microenvironment were examined using TME-related genes and CAF subtypes. All statistical analyses were conducted using R 4.1.2 software, and *p*-values below 0.05 were considered statistically significant.

Abbreviations

CRC: colorectal cancer; TME: tumor microenvironment; NMF: non-negative matrix factorization; IHC: immunohistochemistry; CAF: cancer-associated fibroblast; ICB: immune checkpoint blockade; DEGs: differential gene expression analysis; TCGA: The Cancer Genome Atlas; ICGC: International Cancer Genome Consortium; UMI: unique molecular identifiers; TSS: transcription start sites; RSS: Regulon Specificity Score; OS: overall survival; TIDE: Tumor Immune Dysfunction and Exclusion.

AUTHOR CONTRIBUTIONS

Yuejun Li and Qixin Gan participated in the manuscript of the article, the conception of the article and the immunohistochemistry experiment by designing the experimental approach, conducting the experiments, analyzing the data, and interpreting the results; Qixin Gan, Haifen Liu and Hong Wang edited the picture and data analysis; Fangting Tang and Ruoxuan Wang participated in the review of the article. Yuejun Li modified the article.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL STATEMENT

Our study strictly adhered to the publishing guidelines provided by TCGA and ICGC, and all data were directly obtained from publicly accessible databases.

FUNDING

This study was supported by the National Natural Science Foundation of China (No. 81703916 to Yuejun Li), the Natural Science Foundation of Hunan Province (No. 2018JJ6042 to Yuejun Li), and the Health Commission Research Program Project of Hunan Province (No. D202309037763 to Qixin Gan).

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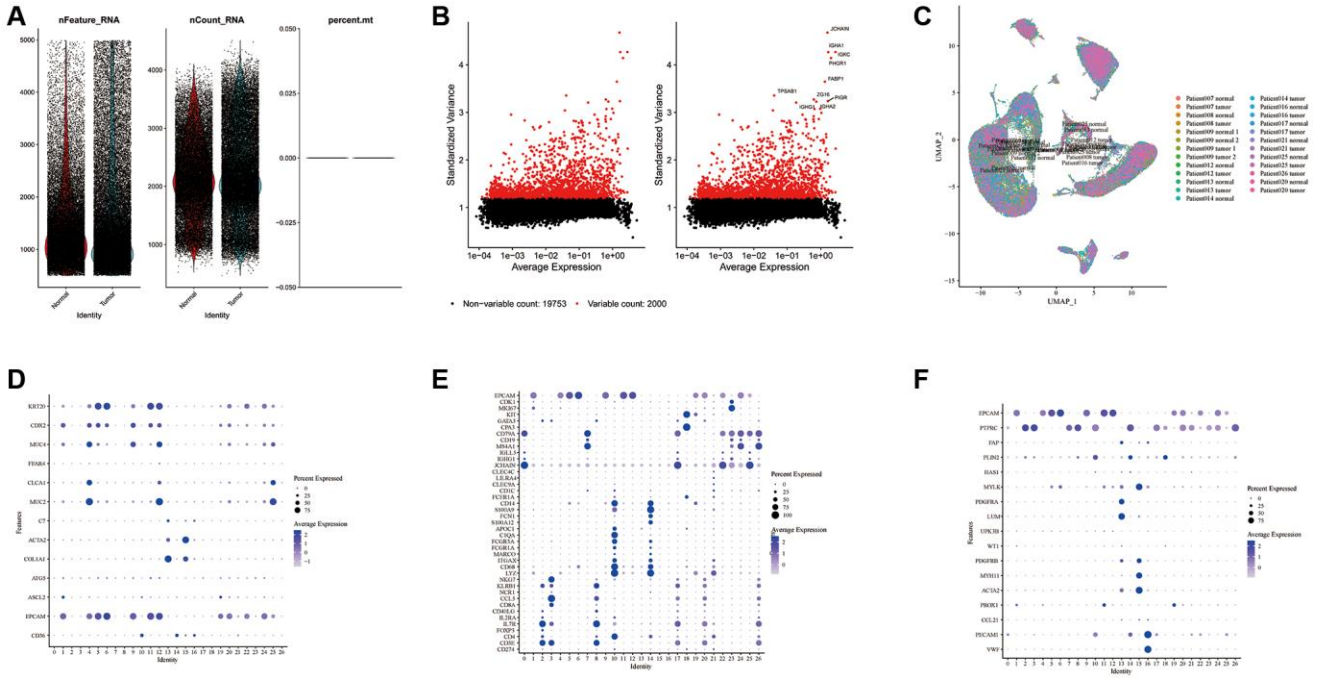
SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Single-cell quality control process. (**A**) Gene, mitochondrial, and cell count after the accusation. (**B**) Selection of 2000 highly variable genes for subsequent analysis. (**C**) Visualization of sample distribution using UMAP after harmonizing cells. (**D**–**F**) Identification of marker genes for cell annotation.