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Single-Cell Analysis Reveals Immune Profile of T Cell Exhaustion-Associated Microenvironment in Colorectal Cancer

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Original Article

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Abstract

Background: Distinguishing the unique immune microenvironment of left and right colorectal cancer (CRC) is essential for better the development of tailored therapeutic strategies and improving patient outcomes. However, few data was available on single-cell RNA-seq (scRNA-seq)-based analysis of the immune profile in these regions. Therefore, we aimed to depict and compare the immune landscape among left and right colorectal cancer and the paired normal tissue. Targeted genes and pathways was analyzed for constructing transgenic Tumor Infiltrating Lymphocyte (TIL) to improve its efficacy in solid tumors.

Methods: Bioinformatics' analysis was performed on GSE200997 obtained from GEO (Gene Expression Omnibus) database. By analyzing the scRNA-seq data, immune cell clusters were identified and their proportions and differentially expressed genes (DEGs) on the meaningful immune cell clusters were compared among left and right colorectal cancer tissues and the paired normal tissue. Monocle3 was used to analyze the developmental sequence of cell clusters and genes involved in the progression of CRC. CellChat package was conducted to characterize the underlying mechanism of immune cell recruiting.

Results: In this study, we found that more CD8 T cells were in exhausted state in right tumor tissue (RT) than in normal tissue (N) and left tumor tissue (LT) microenvironments, where CXCL13 and MTRNR2L8 were not only mainly involved in the differentiation of CD8 from naive T cells to exhausted T cells but also a factor in the poor prognosis of CRC patients. CD8 T cell exhaustion was also associated with the presence of BATF, FOXP3, TIGIT, and TNFRSF4 on Treg that inhibit CD8-mediated immune responses. In addition to that, we found that in tumor tissue, especially RT and MSI-H state the proportion of epithelial cells was higher, wherein invasive cancer cells developed from epithelial cells and shared the same input and output signal patterns as epithelial cells. Further crosstalk analysis of epithelial cells on cytotoxic T lymphocytes (CTLs) revealed that comparing to epithelial cells, the invasive cancer cells expressed TFG-β and CXCL signaling axis in LT, and TGF-β was highly expressed by epithelial and endothelial cells with CTL cells in RT, which may be a key mechanism to promote tumor invasion, progression, and treatment resistance.

Conclusions: Most CD8 in RT are effector memory T cells and are more likely to differentiate into exhausted T cells with high expression of CXCL13 and MTRNR2L8 and less expression of chemokines. Cells responsible for this phenomenon are proliferative cancer cell, Treg, invasive cancer cell, and epithelial cell in LT, and proliferative cancer cell, endothelial cell, Treg, and cancer stem cell in RT. The pathway involved in the interactions between above cells and cytotoxic T cell is mainly TGFB-TGFBR.

Keywords: Colorectal cancer; Tumor microenvironment; Single-cell RNA sequencing; Cell crosstalk

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Introduction

Colorectal Cancer (CRC) is one of the most common malignancies worldwide, ranking as the third most frequently diagnosed cancer and the second leading cause of cancer-related deaths globally [1]. Despite advancements in screening and early detection, a significant number of CRC cases are diagnosed at an advanced stage, where treatment options are limited and less effective [2]. The growing burden of CRC underscores the urgent need for more precise research to better understand its profile, identify novel biomarkers for early detection, and develop targeted therapies [3]. As a highly heterogeneous disease, differences of CRC are not only between patients but also within different regions of the same tumor [4,5]. Emerging evidence suggests that the right and left halves of the colon exhibit distinct biological characteristics, including variations in their genetic, epigenetic, and immune landscapes [6]. Previous literature on single-cell sequencing analysis of Colorectal Cancer (CRC) has significantly advanced our understanding of the cellular diversity and molecular characteristics of these tumors [7]. However, many studies have predominantly focused on the overall tumor microenvironment without adequately distinguishing between the right and left sides of the colon. This oversight neglects the well-documented biological differences between right-sided and left-sided CRC.

To fully understand these complex biological differences, bioinformatics analysis plays a crucial role by enabling the integration and interpretation of large-scale single-cell data, thereby identifying novel biomarkers and potential therapeutic targets specific to the right and left-sided CRC. Conducting such analyses is essential for the development of more effective, tailored therapeutic strategies and improving outcomes for CRC patients.

Methods and Materials

Data information

The series GSE200997 was obtained from GEO (Gene Expression Omnibus) database [7]. Droplet-based scRNA-seq (10X Genomics Single Cell 5' Platform) were performed on 49,589 cells from 23 samples of 16 racially diverse, resectable treatment naive CRC patients, including 8 tumor tissue from the left side colon cancer (Patient 2, 4, 6, 9, 10, 14, 15, 16) and another 8 (Patient 1, 3, 5, 7, 8, 11, 12, 13) from the right colon cancer, with 7 paired para-carcinoma tissue (Left side colon: Patient 4, 6, 10, 14, 15. Right side colon: Patient 7, 11).

Bioinformatics' Analysis

R package Seurat was used to analyze cell profile of CRC microenvironment in both tumor site and the paired normal tissue. The first cell clustering was used to define major cell groups, including T cells, B cells, Hepatocytes, Goblet cells, Monocyte, progenitor cells, endothelial cells and mast cells. The second cell clustering was used to define subgroups in T cells. Cell clusters were determined using the CellMarker website (http://xteam.xbio.top/CellMarker/search.jsp). Differential genes expression analysis was conducted use Volcano plot R package. Changes in differential gene expression were > 2-fold (log2 FC > 1). Pathway enrichment analysis was performed using the DAVID website (https://david.ncifcrf.gov/) to exam the enriched processes in different cell clusters. Expression of cytokines, chemokines and their receptors, immune checkpoints and their ligands were shown by dot plots. To identify the cells and genes potentially involved in the development of colon cancer, trajectory analysis was performed using Monocle 3. Kaplan-Meier curves were conducted use the Kaplan-Meier plotter website (https://kmplot.com/analysis) to determine the importance and role of genes (DEGs) in prognosis [8].

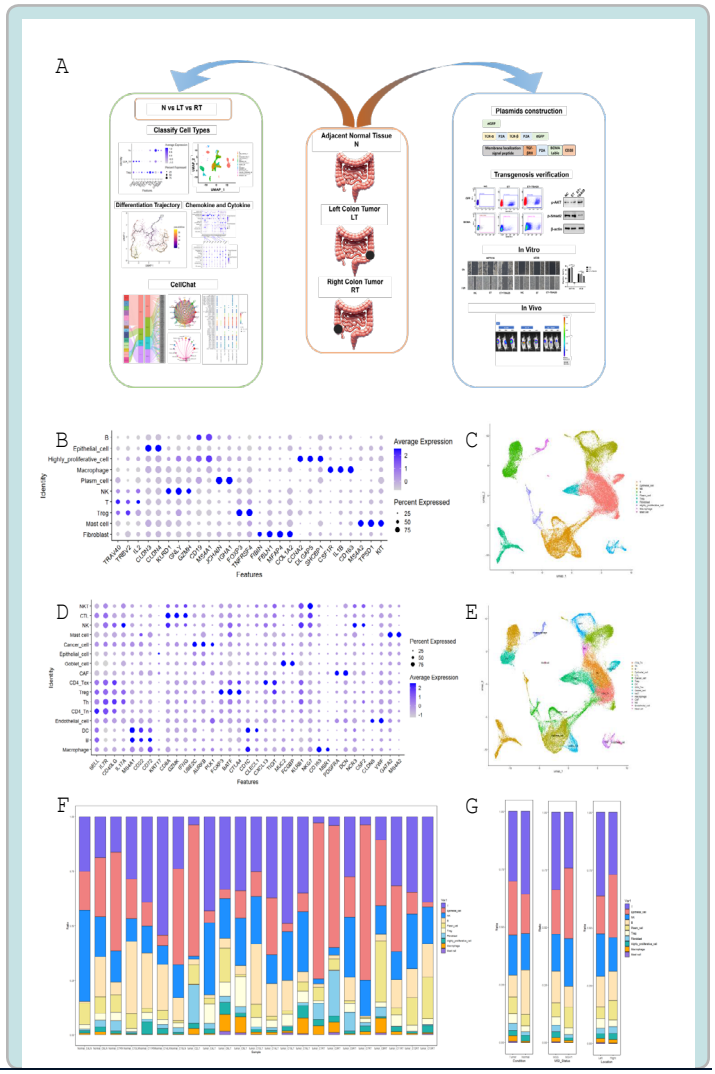
Statistical Analysis

All statistical analyses were carried out using GraphPad Prism software version 9.0.0. All grouped data were summarized as mean ± Standard Deviation (SD). An unpaired Student's t test and one-way analysis of variance (ANOVA) were used to determine the statistical significance when comparing two groups and more than two groups, respectively. Two-tailed p values less than 0.05 were considered to be statistically significant.

Results

Single-Cell Landscape of CRC Microenvironment

To investigate the differences between normal tissue, left colon cancer and right colon tissue, we analyzed single-cell sequencing data from 16 colorectal cancer patients from the GSE200997 dataset, including 8 patients with left-sided colon cancer, 8 patients with right-sided colon cancer, and 7 normal tissues from the paired normal tissue. High-resolution depiction of total 49589 cells were performed.



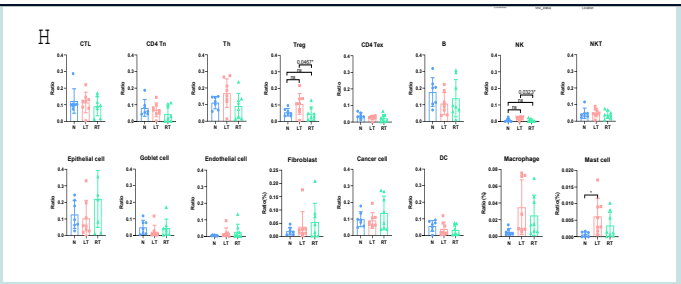


Figure 1: Overview of the single-cell landscape for CRC tumor tissues and paired normal tissues.

A) Schematic diagram of scRNA-seq analysis workflow and in vivo and in vitro experiments. The series GSE200997 was obtained from GEO (Gene Expression Omnibus) database. Droplet-based scRNA-seq (10X Genomics Single Cell 5′ Platform) were performed on 49,589 cells from 23 samples of 16 racially diverse, resectable treatment naive CRC patients, including 8 tumor tissue from the left side colon cancer (LT) and another 8 from the right colon cancer (RT) , with 7 paired para-carcinoma tissue (N). Data were collected for integration and analyzing differences in cellular components of the immune microenvironment and their possible causes.

B) Dotplot showed the percentage of expressed cells and average expression levels of canonical marker genes of the 10 cell types.

C) Uniform manifold approximation and projection (UMAP) plot Showed the transcriptome landscape consisting of 10 major cell types. Cells are colored by clusters.

D) Dotplot showed the percentage of expressed cells and average expression levels of canonical marker genes of the 16 cell types after further subdividing the T cells.

E) UMAP plot of 16 major cell types including further subdivided T cells.

F) Clustering of cell components in every sample.

G) Clustering of cell components in different conditions, MSI states and locations.

H) Histogram of the percentage of 16 cell subclusters in N, LT and RT.

As shown in Figure 1C, after normalization and integration, 10 major clusters, including T cells, B cells, Natural Killer cell (NK), regulatory T cells (Treg), plasm cell, epithelial cells, highly proliferative cell, macrophage, mast cells and fibroblast were distinguished by high-expressed marker genes (Figure 1B). Then T cells were further subdivided into Nature Killer T cells (NKT), Cytotoxicity T lymphocytes (CTL), naïve CD4 T cell (CD4 Tn), T helper cell (Th), Treg, exhausted CD4 T cells (CD4 Tex) by the marker genes (Figure 1D) in each cell clusters (Figure 1E).

Further analysis of the percentage of cells in each patient (Figure 1F) and different condition, MSI state and location showed a decrease in T cells and an increase in epithelial cells in tumor tissues, MSI-H state and right colon tissue compared to normal tissue, MSS and left tissue. But the statistical significance only showed in Treg, NK and mast cell (Figure 1H) indicating that differences in cell numbers may not be the key point between the right and left halves of colorectal cancer.

Most CD8 in RT are Effector Memory T Cells and are More Likely to Differentiate into Exhausted T Cells with High Expression of CXCL13 and MTRNR2L8 and Less Expression of Chemokines

We further characterized the CD8 cells in the 3 tissues further and described the trajectory of cell development using the Monocle3 R package. As can be seen (Figure 2A,2B) in N, C0 and C8 are two populations of initially activated T cells highly expressing CD27, and C1-C7 are cytotoxic T cells highly expressing NKG7 and granzyme. Eventually, they will differentiate into C4 which highly express CTLA4, PDCD1, BATF. In LT (Figure 2C, 2D), CD8 T cells were classified into 4 clusters C0_GZMH, C1_TMIGD2, C2_CRTAM, C3_TRDV2 which major in T cell activation and cytokine production. In RT, CD8 T cells were classified into naïve T, effect memory T (Tem) and exhausted T (Tex), but not CTL, suggesting of tumor immune escape which can lead to lymph node metastasis and prognosis of tumors [9].

The transition of naive CD8 T cells into effector memory T cells in RT is characterized by the high expression of key

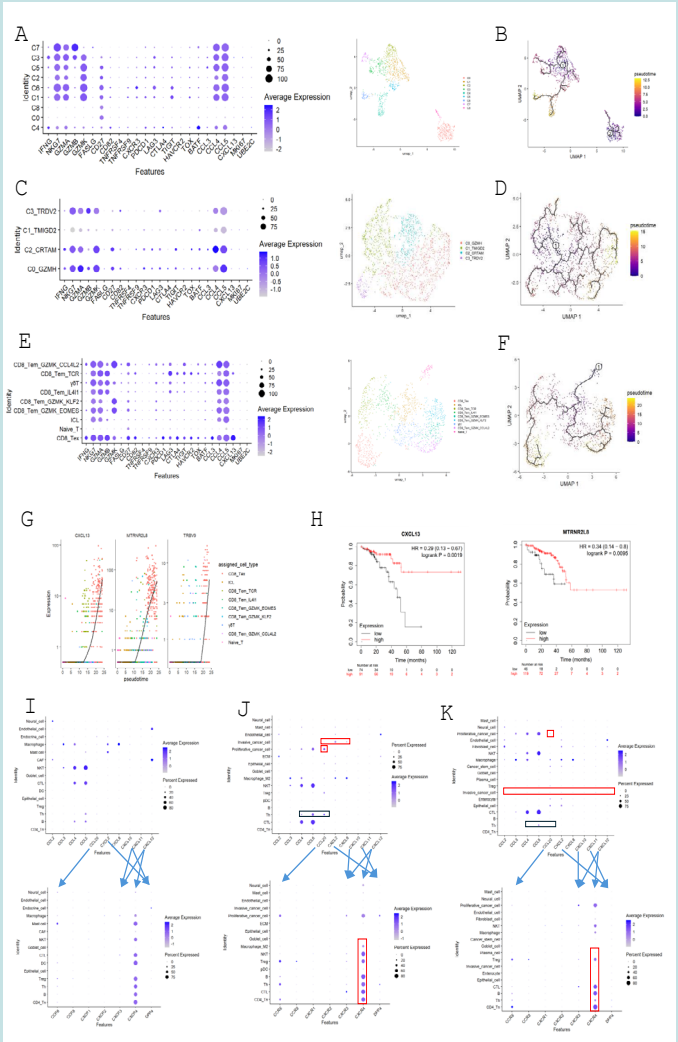


Figure 2: CD8 T cells in RT are predominantly effector memory CD8 T cells and tend to be more exhausted than those in N and LT. A, C, E UMAP plot of scRNA-seq profile from CD8+ T cells separated into subclusters according to their typical marker genes. Cells are colored according to different clusters. **B, D, F** Pseudotime trajectory of CD8+ T cell subclusters in N, LT and RT inferred by Monocle3. Trajectory is colored by the pseudotime. **G** Single-cell expression genes along pseudotime towards exhausted CD8+ T cell. **H** Kaplan-Meier plotter (KM plotter) illustrated the overall survival of rectum adenocarcinoma patients based on the TCGA dataset. **I, J, K** Dot plot showing average expression of chemokines and their paired receptors in the three TME.

genes such as TCR, IL4I1, GZMK, EMOMES, KLF2, and CCL4L2, alongside a progression toward an exhausted T cell phenotype with C-X-C Motif Chemokine Ligand 13 (CXCL13), MT-RNR2-Like 8 (MTRNR2L8) and TRBV9 involved in (Figure 2G). CXCL13 is a chemokine that plays a critical role in the immune response by attracting B cells and T Follicular Helper (Tfh) cells to lymphoid tissues to form Tertiary Lymphoid Structures (TLS) [10]. But is also a mark of dysfunctional CD8+ T cells which are often found in the tumor microenvironment [11]. The upregulation of MTRNR2L8 in exhausted T cells might be a compensatory response to the prolonged antigen stimulation [12] and cellular stress that characterizes tumor microenvironment [13]. The high expression of CXCL13 and MTRNR2L8 in CRC patients is associated with a good prognosis (Figure 2H). Further analysis on ligand-receptor pairs showed that chemokines on proliferative cancer cell, invasive cancer cell and Th was significantly decreased in RT than in N and LT (Figure 2I, 2J, 2K).

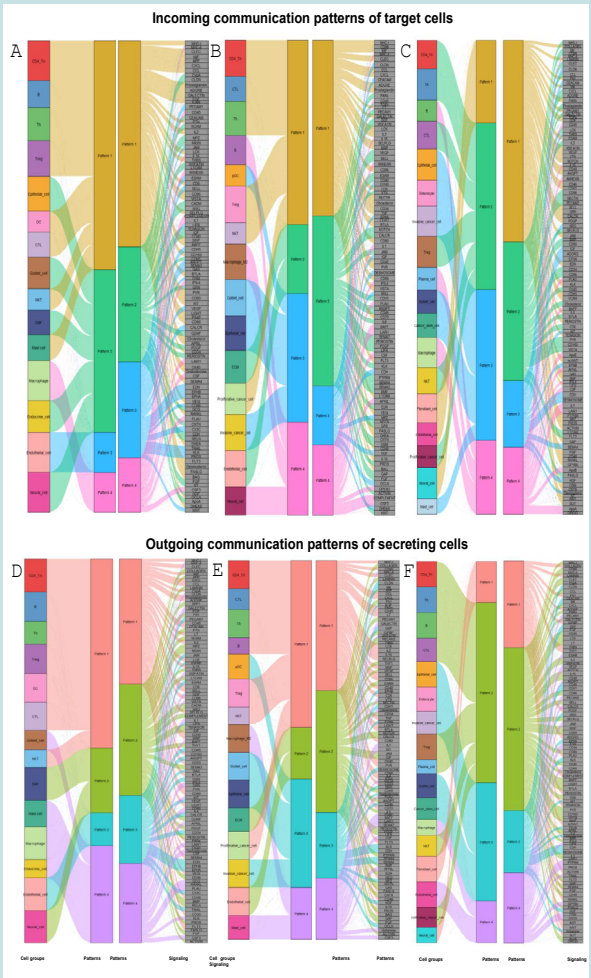


Figure 3: Cell-cell communication patterns.

A, B, C The inferred incoming communication patterns of target cells visualized by the alluvial plot in N (left), LT (middle) and RT (right) respectively. **D, E, F** The inferred outgoing communication patterns of secreting cells in N (left), LT (middle) and RT (right) respectively. The thickness of the flow indicates the contribution of the cell group or signaling pathway to each pattern. The height of each pattern is positive related to the number of its associated cell groups and signal pathways.

Invasive Cancer Cells and Epithelial Cells Shared the Same Communication Pattern and Differentiated from Epithelial Cells

To further explain why CD8 T cells in RT are more Tem than CLT, we analyzed cellular incoming (Figure 3A-C) and outgoing (Figure 3D-F) communication patterns in the 3 environments using the Cellchat R package. Both outgoing and incoming of cellular communication were dominated by pattern1 and 2 in N and LT, and pattern2 in RT. Proliferative cancer cellgs shared pattern1 with T cells, B cells and NKT, but invasive cancer cells and cancer stem cells shared communication pattern with Epithelial cell (Figure 3B, 3C, 3E, 3F), suggesting that cancer stem cell, invasive cancer cell and epithelial cell may have the same origin.

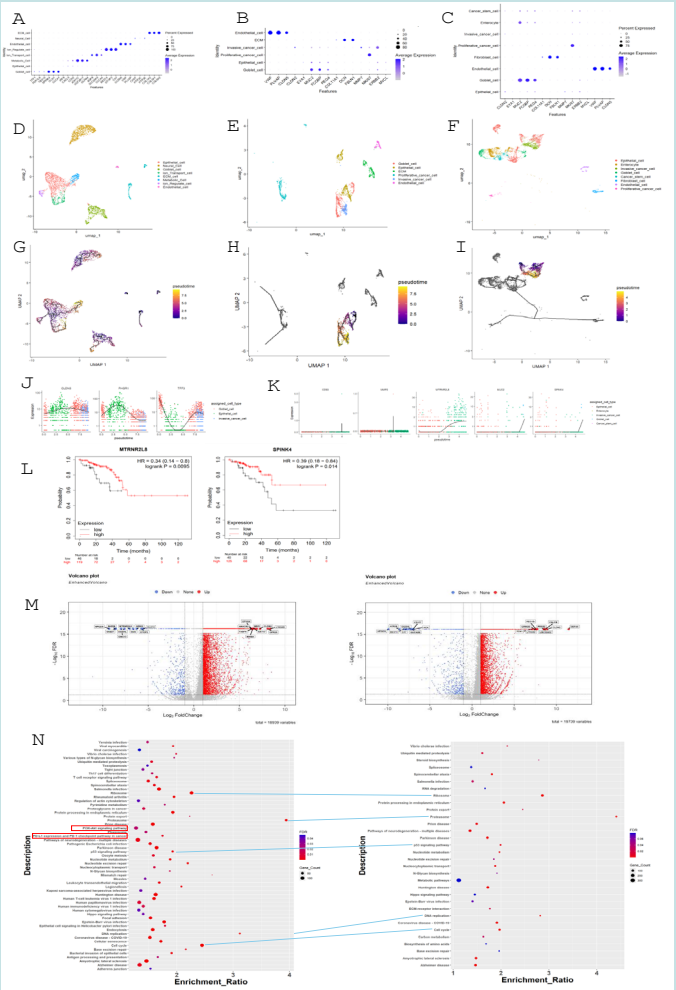


Figure 4: Molecular features and relative genes of epithelial cells.

A, B, C Dot plot showed canonical gene marker of each epithelial cell subpopulation in N (left), LT (middle) and RT (right) respectively. **D, E, F** UMAP visualization of subpopulations of epithelial cell in three TME. **G, H, I** Single cell trajectory of epithelial cells and tumor cells in the three TME. Each dot represents a single cell and colors represent pseudotime from yellow to blue. **J, K** Bridge plot showed the expression of genes related to carcinogenesis in LT and RT. **L** Kaplan Meier curves showed genes negatively related to OS. **M** Volcano plot showing the differentially expressed genes in LT (left) and RT (right) conditions. The filter criteria was set as FDR < 0.05 and |log2FC|> 0.05. FC fold change. **N** Pathways enriched in epithelial cells. Benjamini-Hochberg (BH) adjusted p value < 0.05. Odds ratio = Gene Ratio/Background Ratio.

Further selection of epithelial cells and tumor cells in the 3 environments (Figure 4A-F) and analysis of the developmental trajectories by Monocle3 (Figure 4G-I) showed that invasive cancer cells developed from Epithelial cells, and genes that were highly expressed in the developmental process were CLDN3, TFF3, PHGR1, CD93, MMP2, MTRNR2L8, MUC2, and SPINK4 (Figure 4J, 4K). Among them, TFF3 is negatively associated with the prognosis of CRC patients (Figure 4L). Volcano map (Figure 4M) and KEGG pathway analysis (Figure 4N) showed that Epithelial cell highly expressed genes in colorectal cancer tissues were enriched in tumor-associated signaling pathways such as PI3K-AKT, P53, Proteasome, DNA replication, Cell cycle and so on.

The TGFB Pathway was Highly Expressed on Proliferative and Invasive Cancer Cells in LT and on Epithelial and Endothelial Cells in RT.

To further explore cells in the tumor immune microenvironment that are responsible for differences in CLTs and the communication pathways between them, we used the CellChat R package to analyze the number and strength of the overall interactions between cells in the 3 environments (Figure 5A-C) and used each cell as a source to analyze the strength of his signals to the other cells. The strongest effects on CTL cells in N were endothelial cells, Treg and Epithelial cells (Figure 5D), and in LT it was proliferative cancer cell, Treg, invasive cancer cell, and epithelial cell (Figure 5E), in RT it was proliferative cancer cell, endothelial cell, Treg, and cancer stem cell (Figure 5F). TGFB-TGFBR was highly involved in proliferation cancer cell and invasive cancer cell comparing to the normal epithelial cell in LT (Figure 5H) and endothelial cell in RT (Figure 5I). Heatmaps of the TGFB pathway also showed that endothelial cell is the major sender and all the other cells including endothelial cells are influencer (Figure 5J).

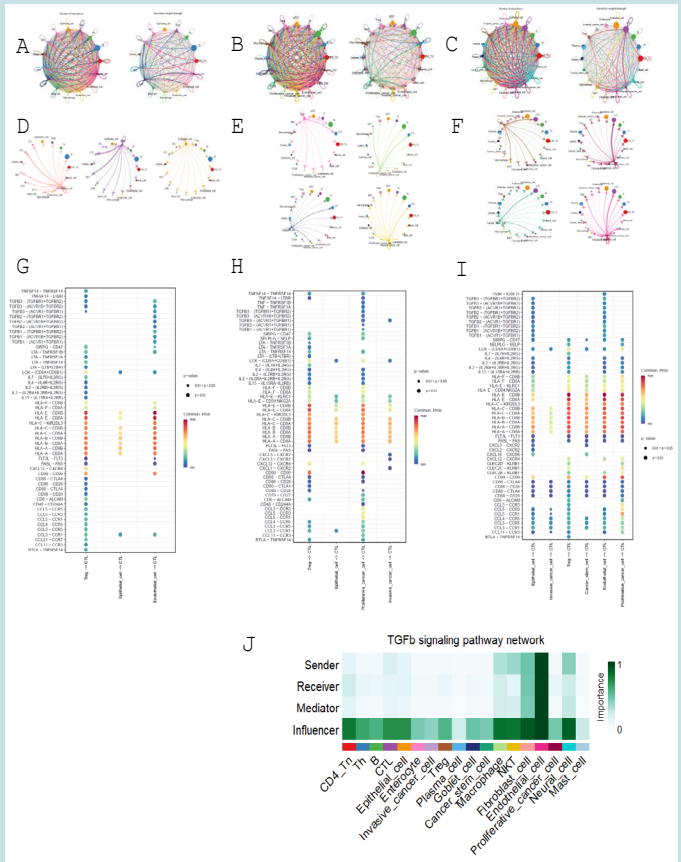


Figure 5: The cell-cell interaction between components in the TME of N, LT and RT. A, B, C NetVisual circle showed the number of interactios (left) and interaction weights (right) of cell components in N, LT and RT. **D, E, F** Shell plots show the strongest interaction strength between the source cells and CTL in N, LT and RT, separately. **G, H, I** Dot plot showing the crosstalk between CTL and source cells mentioned above in N, LT and RT, separately. **J** Heat map demonstrated the role of each cell in the TGFb pathway in RT. The color of heat map indicates the importance of cells from white (importance = 0) to dark green (importance = 1).

Discussion

Our findings reveal a significant divergence in the immune microenvironments between Right-Sided (RT) and Left-Sided (LT) colorectal cancer, particularly in the differentiation of CD8+ T cells. CD8+ T cells in Right-Sided Tumors (RT) are predominantly effector memory T cells with a tendency towards exhaustion, characterized by high expression of CXCL13 and MTRNR2L8. This contrasts with Left-Sided Tumors (LT) where a different cellular landscape was driven by interactions with specific cellular populations, including cancer stem cells, proliferative cancer cells, invasive cancer cells and Tregs. This underscores the importance of targeting T cell exhaustion pathways in CRC therapy, aligning with recent studies highlighting the role of TGF-β signaling in T cell dysfunction.

In solid tumors, Tumor Infiltrating Lymphocytes (TILs) have shown some potential in improving patient outcomes due to their ability to recognize and target tumor-specific antigens, especially when tumors are highly immunogenic or exhibit a high degree of immune infiltration [14-17]. However, the effectiveness of TIL therapy in CRC has been limited by several drawbacks. One of the major challenges is the highly immunosuppressive tumor microenvironment commonly found in CRC, particularly in right-sided tumors [17,18]. This environment often leads to TIL exhaustion and reduced efficacy due to the presence of Treg cells, cancer-associated fibroblasts, and other immunosuppressive factors that inhibit TIL function.

These limitations underscore the need for further optimization and enhancement of TIL therapies, including genetic modifications to improve their resistance to immunosuppressive signals and enhance their cytotoxic capabilities [12,18]. By co-expressing extracellular signaling peptide and intracellular costimulatory molecules, modified TILs are better equipped to resist the inhibitory signals mediated by tumorigenesis pathways and simultaneously enhance their activation and persistence, thereby improving their cytotoxic activity against tumor cells. This dual modification strategy aims to create a more robust and sustained antitumor response, addressing the challenge of immune evasion and potentially leading to more effective therapeutic outcomes in CRC patients.

But there are some flaws in our study. The data of scRNA-seq in this study were obtained from resectable treatment naive patients. If we can combine the scRNA-seq data of patients with advanced and distant metastatic colorectal cancer or treated patients, we can do a more fine-tuned immune and targeted therapy for colorectal cancer patients.

Author Contributions

All authors contributed to the study conception or design. Bioinformatics analysis were conducted by JL. YL and JY provided

original R code and software guidance. Bioinformatics servers and electronics are provided by ZK. The first draft of the manuscript was written by JL and all authors commented on previous versions of the manuscript. QX supervised the study and revised the manuscript. Authors read and approved the final manuscript.

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Data Availability

The datasets generated and analyzed during the study are available from the corresponding author on reasonable request.

Ethics approval

This study was approved by The Ethics Committees of Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai (Ethical code, SHSY-IEC-4.1/20-230/03). Participants were given informed consent to participate in the study before taking part.

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