Original Article

Multi-omics profiling identifies C1QA/B$^+$ macrophages with multiple immune checkpoints associated with esophageal squamous cell carcinoma (ESCC) liver metastasis

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Background: Esophageal squamous cell carcinoma (ESCC) is a highly lethal malignant tumor lacking effective treatments; 20% of ESCC patients develop liver metastasis with an extremely short survival time of ≈5 months. The tumor microenvironment (TME) plays a crucial role in tumor homeostasis, but the relationship between the ESCC TME and liver metastasis is still unknown.

Methods: To identify potential cell populations contributing to ESCC liver metastasis, single-cell RNA (scRNA) sequencing data were analyzed to identify the major cell populations within the TME. Each of the major cell populations was re-clustered to define detailed cell subsets. Thereafter, the gene set variation analysis (GSVA) score was calculated for the bulk RNA-seq data based on the gene signatures of each cell subset. The relationship between the GSVA score of each cellular subset and clinical outcome was further analyzed to identify the cellular subset associated with ESCC liver metastasis, which was validated by multiplex immunohistochemistry.

Results: C1QA/B$^+$ tumor-associated macrophages (TAMs) acted as the central regulator of the ESCC TME, closely associated with several key cell subsets. Several immune checkpoints, including CD40, CD47 and LGALS9, were all positively expressed in C1QA/B$^+$ macrophages, which may exert central regulatory control of immune evasion by ESCC via these immune checkpoints expressions.

Conclusions: Our results comprehensively revealed the landscape of tumor-infiltrating immune cells associated with ESCC prognosis and metastasis, and suggest a novel strategy for developing immunotherapies for ESCC liver metastasis by targeting C1QA/B$^+$ TAMs.

Keywords: Esophageal squamous cell carcinoma (ESCC); C1QA/B$^+$ tumor-associated macrophages (C1QA/B$^+$ TAMs); multi-omics profiling

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Introduction

Esophageal cancer is one of the most serious diseases threatening human health (1) and esophageal squamous cell carcinoma (ESCC) is the predominant histological type, accounting for ≈90% of esophageal cancers worldwide (2). The etiology of ESCC mainly comprises of cigarette smoking, alcohol drinking, hot food and beverages, pickled vegetables, radiation damage, and genetic factors (3). Generally, ESCC is diagnosed at an advanced stage due to the low sensitivity and efficiency of endoscopy and barium swallow techniques (4). The 5-year overall survival (OS) rates of ESCC are <20%, due to tumor recurrence,

extensive invasion and metastasis (5). The liver is the major distal metastasis site of ESCC and ≈20% of ESCC patients develop liver metastasis.

Extraordinary advances have been made in the diagnosis and treatment of ESCC. PD-1 antibodies such as pembrolizumab and nivolumab have been used in clinical trials to treat patients with advanced ESCC (6), but the prognosis of ESCC remained poor, and only subtle improvement was achieved in OS compared with traditional chemotherapy (7); for the liver metastasis patients especially, the median survival time is only 5 months. Therefore, the tumor microenvironment (TME) of ESCC to profile the immune status for elucidation the mechanism of ESCC liver metastasis, and development of innovative immunotherapies for ESCC.

Single-cell RNA sequencing (scRNA-seq) is widely utilized for analysis of the heterogeneity of complex biological systems (8). Currently, single-cell transcriptomic analysis provides a strategy for synthetically elucidating intercellular relationships in complex TMEs (9). The types and status of tumor-infiltrating immune cells have been well distinguished and dissected by scRNA-seq in lung cancer (10), hepatocellular carcinoma (11) and breast cancer (12). In this study, high-dimensional scRNA-seq obtained from a Gene Expression Omnibus (GEO) dataset combined with an RNA-Seq dataset were used to describe the immune landscape of ESCC. Furthermore, we identified gene signatures by gene set variation analysis (GSVA) score to explore the relationship between specific cell subsets and survival probability.

Briefly, major cell populations such as B and T lymphocytes, granulocytes, natural killer (NK) cells and macrophage (MΦ) were clustered and redefined. We found several unique cell subsets correlated to patient prognosis and liver metastasis, and the C1QA/B$^+$ tumor-associated macrophages (TAMs) were likely to be the central regulator in the ESCC TME. Furthermore, analysis indicated that the key molecules in immune regulation included TNFSF9, CD40, and CD47, mediated by the C1QA/B$^+$ TAMs. Therefore, our results comprehensively revealed the landscape of tumor-infiltrating immune cells associated with ESCC prognosis and metastasis, and provided a novel strategy for developing immunotherapies for ESCC liver metastasis targeting C1QA/B$^+$ TAMs. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5351/rc).

Highlight box

Key findings

- C1QA/B$^+$ tumor-associated macrophages (TAMs) acted as the central regulator of the esophageal squamous cell carcinoma (ESCC) liver metastasis.

What is known and what is new?

- The tumor microenvironment (TME) plays a crucial role in tumor homeostasis;
- C1QA/B$^+$ TAMs acted as the central regulator of the ESCC TME.

What is the implication, and what should change now?

- A novel strategy for developing immunotherapies for ESCC liver metastasis by targeting C1QA/B$^+$ TAMs.

Methods

scRNA-seq data analysis

Raw sequencing data were preprocessed by Cell Ranger 3.0.1, then unique molecular identifier (UMI) count data were generated by inputting the fastq files into the Cellranger count. Single-cell data analysis was conducted using R package Seurat (http://satijalab.org/seurat) (13). Briefly, high-quality cells (>200 genes and <6,000 genes/cell, <10% mitochondrial genes) were kept for downstream analysis. Datasets were integrated using the Seurat package to eliminate batch effects, followed by principal component analysis, and Uniform Manifold Approximation and Projection (UMAP) visualization. Graph-based clustering was applied to identify clusters using default parameters.

The Cancer Genome Atlas (TCGA) data analysis

ESCC RNAseq data were downloaded from TCGA database, accompanied by corresponding clinical information. GSVA was performed based on the cluster-specific gene sets derived from scRNA-seq to assign cell type signature scores per sample ranging from –1 to +1 (14).

Multiplex immunohistofluorescence (mIHC) assay

Tissues samples were obtained from the patients with ESCC liver metastasis from Fujian Cancer Hospital between January 2018 to August 2020. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Fujian Cancer Hospital (No. 10104700) and informed

consent was taken from all individual participants. Multiplex staining of primary ESCC tissues and their corresponding liver metastasis was performed using Opal™ 7-color multiplex IHC kit (Akoya Biosciences, NEL861001KT, USA) according to the manufacturer's instructions (15). Briefly, the slides were incubated for approximately 1 h at 68 ℃ followed by deparaffinization and rehydration. For each staining cycle, the slides were treated with retrieval of antigen, blocking, and primary antibodies incubation, followed by horseradish peroxidase-conjugated secondary antibody incubation and Opal tyramide signal generation (detailed information of antibodies and corresponding Opal fluorophores are given in Table S1). The slides were stripped with retrieval solution as required before the next round of staining. The above process was repeated until all markers were completed. Once all markers were labeled, slides were counterstained with DAPI (Akoya Biosciences) and scanned using the PerkinElmer Vectra3® Polaris™ platform. The multispectral images obtained were unmixed using the inForm Advanced Image Analysis software (inForm 2.4.1; Akoya Biosciences). The antibodies and reagents are listed in Table S1.

Statistical analysis

Kaplan-Meier survival plots were generated by survival and survminer package in R. The log-rank test was used to calculate the statistical significance between the survival curves. $P<0.05$ was considered significant. No data point was excluded from analysis. No blinding or randomisation was performed for statistical analysis. Sample size was determined to ensure the proper comparison between different groups.

Results

Experimental scheme and single-cell transcriptomic landscape of ESCC

To clarify the potential effect of different cell subpopulations, the immune landscape of ESCC was established. The workflow of scRNA-seq analysis is shown in *Figure 1A*. First, the major cell subpopulations were clustered and annotated, and each major cell population was re-clustered (*Figure 1A*). The signature marker genes for each cell subset were identified using the Seurat toolkit (16). Next, the RNA-seq dataset for ESCC was acquired from TCGA database (*Figure 1B*), and the GSVA score of the gene signature specific to each cellular subset was calculated for each ESCC RNA-seq data set based on the gene signatures of each cellular subset (*Figure 1C*) to estimate the correlation between GSVA score and survival probability (*Figure 1D*).

The scRNA-seq cluster numbers were assigned from the largest cell population (Cluster 0) to the smallest (Cluster 9) (*Figure 1E*). The characteristic gene markers in each cluster are shown in *Figure 1F*. The cell distribution Frequency is shown in *Figure 1G*.

The largest single-cell constituent Cluster 0 cells were T lymphocytes with specific gene expression including *BATF*, *IL32* and *CTLA4* (*Figure 1F*) (17-19). Cluster 1 cells were MΦ, expressing *SPP1*, *CXCL2* and *CTSB* (*Figure 1F*) (20-22). Cluster 2 represented B lymphocytes with high expression of *IGHG1*, *IGHG2* and *IGKC* (*Figure 1F*) (23,24). Cluster 3 represented NK cells that expressed *GNLY*, *NKG7* and *KLRD1* (*Figure 1F*) (25-27). Cluster 4 was tissue stem cells with special genes including *HST1H4C*, *STMN1* and *TUBA1B* (*Figure 1F*) (28,29). Cluster 5 cells were granulocytes with special genes including *PI3*, *CXCL8* and *SLPI* (*Figure 1F*) (30-32). Cluster 6 cells were DC cells expressing *HLA-DRA*, *HLA-DPA1*, and *CCL17* (*Figure 1F*) (33-35). Cluster 7 represented mast cells expressing *TPSAB1*, *TPSAB2* and *CPA3* (*Figure 1F*) (36-38). Cluster 8 cells appeared to be a pDC (plasmacytoid dendritic cell) cell subset expressing *IRF4*, *TCL1A* and *AREG* (*Figure 1F*) (39,40). Cluster 9 cells represented endothelial cell expressing *COL1A1*, *COL6A2* and *S100A2* (*Figure 1F*) (41-43). In addition, the distribution frequency of each major cluster is showed in *Figure 1G* among the different patients and no cellular cluster was derived from a single patient. Overall, the major cellular clusters forming the ESCC TME were profiled.

Exhausted CD4⁺ T (ExhCD4T) cells correlated with poor prognosis and liver metastasis

T cells regulate the ESCC TME by secreting specific cytokines and interacting with other immune cells (44,45), so they were further re-clustered to investigate the effect of the T cell subsets in the prognosis and liver metastasis of ESCC patients.

There were 6 T-cell subsets from Cluster 0 to Cluster 5 (*Figure 2A,2B*). UMAP of scRNA-seq data visualizing the special gene markers is showed in *Figure 2C*). Cluster 0 cells were cytotoxic T (CytT) cells expressing *CCL5*, *NKG7* and

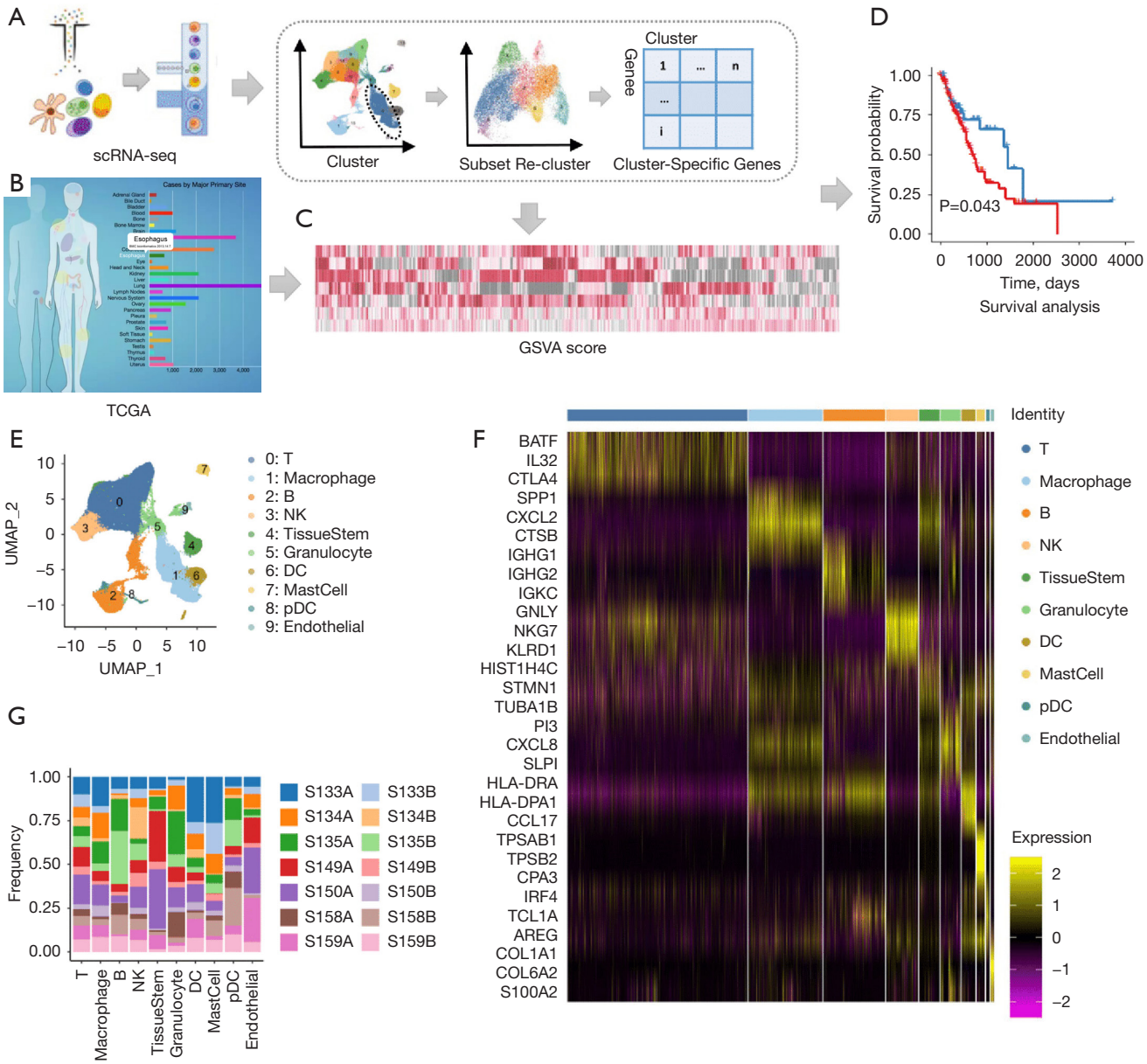

Figure 1 scRNA-seq clustering analysis for ESCC. (A) Overview of the workflow; (B) TCGA dataset analysis; (C) heatmap of the distribution of the assigned gene sets by GSVA scores; (D) survival curves for Low/High GSVA score groups; (E) UMAP of scRNA-seq data visualizing 10 cell clusters marked 0–9; (F) heatmap of differentially expressed genes in each cluster, yellow to dark purple: high to low expression; (G) distribution frequency of cluster cells. scRNA-seq, single-cell RNA sequencing; TCGA, The Cancer Genome Atlas; GSVA, gene set variation analysis; UMAP, Uniform Manifold Approximation and Projection; NK, natural killer; DC, dendritic cell; pDC, plasmacytoid dendritic cell; ESCC, esophageal squamous cell carcinoma.

GZMB (46,47) (*Figure 2B*), and *CD8A* (*Figure 2* C3) (48). Cluster 1 cells represented regulatory T cells (Tregs) with *TNFRSF4*, *BATF* and *FOXP3* expression (*Figure 2B*, *Figure 2* C4) (49-51), and *CD4* (*Figure 2* C2) (52). Cluster 2 cells were resident CD4 T (ResCD4$^+$ T) cells with *IL7R*, *KLRB1* and *FOS* expression (*Figure 2B*) (53-55). Cluster 3 cells were naïve CD4$^+$ T cells expressing *SELL*, *PASK* and *LEF1* (*Figure 2B*) (56), and *CCR7* (*Figure 2* C6) (57). Cluster 4

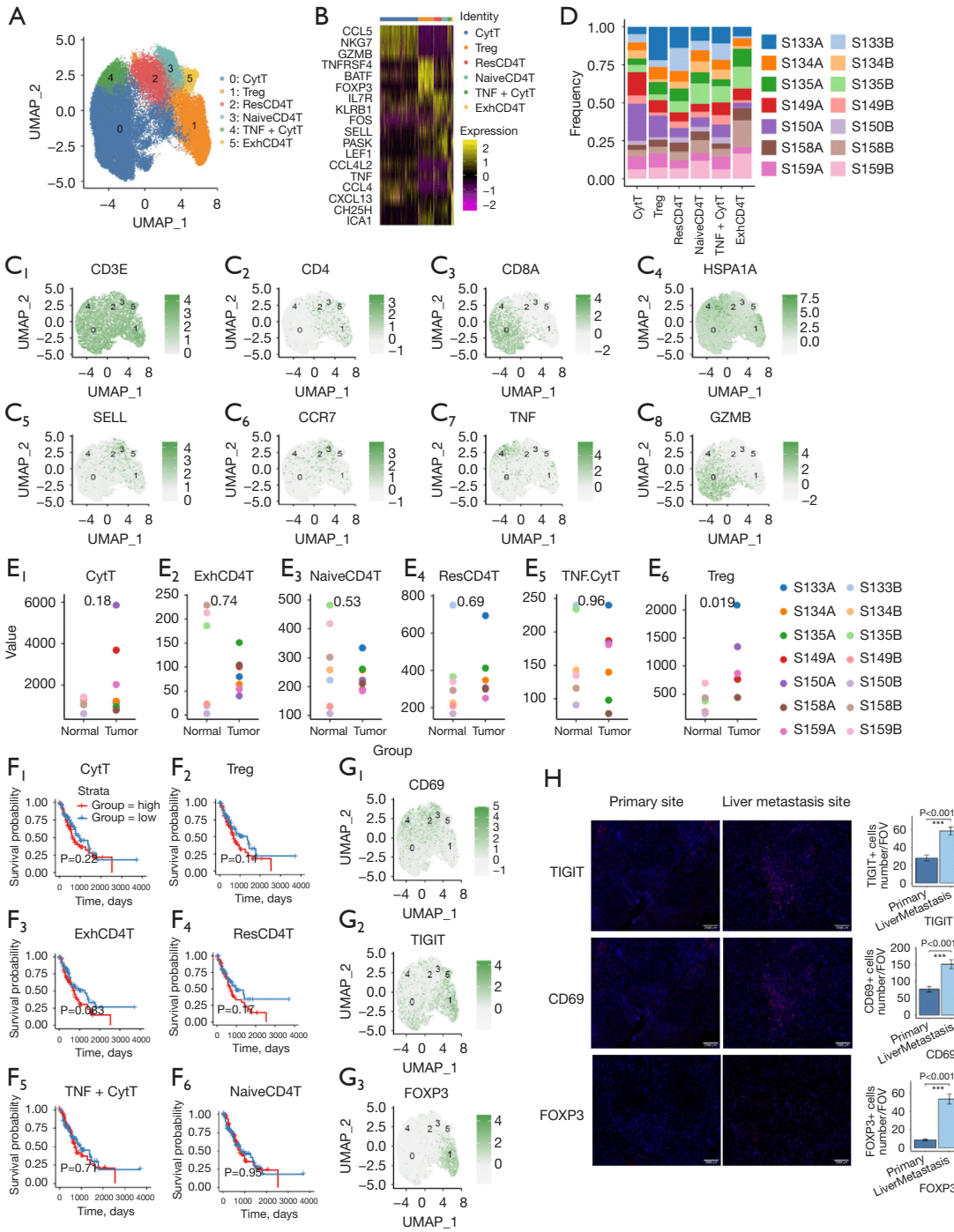

Figure 2 Analysis of cell subset of T cells. (A) UMAP of scRNA-seq data visualizing 6 cell clusters marked 0–5; (B) heatmap of differentially expressed genes in each cluster, Yellow to dark purple: high to low expression; (C) UMAP of scRNA-seq data visualizing the special gene markers; (D) distribution frequency of cluster cells; (E) expression values of cluster cells in normal and tumor groups; (F) survival curves for low/high expression group; (G) feature plot of CD69 (resident T cell marker), TIGIT (exhausted T cell marker), and FoxP3 (regulatory T cell marker), shown in UMAP; (H) multiplex immunohistofluorescence assays for TIGIT, CD69 and FOXP3. Scale bar =250 μm. ***, P<0.001. UMAP, Uniform Manifold Approximation and Projection; CytT, cytotoxic T; Treg, regulatory T cell; NaiveCD4T, naive CD4$^+$ T cell; TNF, tumor necrosis factor; ExhCD4T, exhausted CD4$^+$ T cell; ResCD4T, resident CD4$^+$ T cell; FOV, field of view; scRNA-seq, single-cell RNA sequencing.

cells were TNF$^+$ CytT cells with specific genes including *CCL4L2*, *TNF* and *CCL4* (*Figure 2B*, *Figure 2* C7) (58,59). Cluster 5 cells were ExhCD4$^+$ T cells expressing *CXCL13*, *CH25H* and *ICA1* (*Figure 2B*) (60,61). We found that the frequency of Cluster 0 (CytT) and Cluster 1 (Tregs) was higher in the tumor group than in the normal group (*Figure 2D*,*2E*).

Next, we calculated the T-cell subset specific score based on the feature genes belonging to each subset. We found the CytT-specific and Treg-specific GSVA scores did not correlate with patients' prognosis. Only the Cluster 5 (ExhCD4$^+$ T) cell subset was negatively related to the ESCC patients' prognosis (*Figure 2F*). Furthermore, mIHC staining was used to confirm some of the marker genes representing the key cellular subsets. *CD69*, *TIGIT* and *FoxP3* were not only detected in the specific subsets based on scRNA-seq data (*Figure 2G*), these genes were signature markers as well. *CD69* is the key marker for resident T cells (62). *TIGIT* could be used to identify exhausted T cells (63) and *FoxP3* is the master transcription factor for Tregs (64).

We found there were more TIGIT$^+$ ExhCD4$^+$ T cells, CD69$^+$ ResCD4$^+$ T cells and FoxP3$^+$ Tregs in the ESCC liver metastasis site than in the primary site and the quantification was also added for key cell signatures (*Figure 2H*). Thus, we identified key T-cell subsets associated with the prognosis and liver metastasis of ESCC patients.

Increased TolPlaB cells in liver metastasis associated with poor prognosis of ESCC patients

B lymphocytes are primarily responsible for basic functions such as antibody production (65). However, the phenotypic and functional diversity of B lymphocytes also results in independent regulatory roles in the immune response (65). To explore the role of B-cell subpopulations in ESCC patients' prognosis and liver metastasis, B cells were re-clustered. Firstly, the B cells were divided into 4 cell subsets (*Figure 3A*,*3B*). UMAP of scRNA-seq data visualizing the special gene markers is showed in *Figure 3C* Cluster 0 cells were active B (ActB) cells expressing *CD69*, *CD83* and *HLA-DRA* (*Figure 3B*, *Figure 3* C3) (66,67). Cluster 1 cells were plasma B (PlaB) cells with *IGHG1*, *IGHG2* and *IGKV3-20* expression (*Figure 3B*, *Figure 3* C1, C2). Cluster 2 cells were proliferating B (ProlB) cells expressing *TUBA1B*, *H2AFZ*, *HIST1H4C* and *MKI67* (*Figure 3B*, *Figure 3* C5). Cluster 3 cells were TCL1A$^+$ tolerance plasma B (TolPlaB) cells expressing *TCL1A*, *LRMP* and *PRPSAP2* (*Figure 3B*, *Figure 3* C4) (68,69). The results showed that the number of Cluster 1 (PlaB) and Cluster 3 (TolPlaB) cells were upregulated in the tumor group compared with the normal group (*Figure 3D*,*3E*), although only the TCL1A$^+$ TolPlaB-specific GSVA score was associated with poor prognosis (*Figure 3F*). We found TCL1A$^+$ TolPlaB cells were also increased in the liver metastasis site compared with the ESCC primary site (*Figure 3G*). Thus, increased numbers of TolPlaB cells (Cluster 3) in the ESCC liver metastasis site was associated with poor prognosis.

Contribution of CMTM2$^+$ neutrophil cell subset in ESCC liver metastasis site to shorter survival time in ESCC patients

Granulocytes participate in a series of tightly controlled molecular processes to regulate tumor immunity (70). To analyze the effect of granulocyte cell subsets in ESCC and its clinical significance, the cell populations were re-clustered and analyzed. The granulocyte cells included 3 cellular subsets (*Figure 4A*,*4B*). UMAP of scRNA-seq data visualizing the special gene markers is showed in *Figure 4C*. Cluster 0 cells were monocyte cells with *CCL3L1*, *PI3* and *CCL3* expression (71,72) (*Figure 4B*). Cluster 1 cells represent S100A10$^+$ neutrophil cells with *S100A9*, *S100A2* and *SFN* expression (*Figure 4B*, *Figure 4* C3, C4) (73,74). Cluster 2 cells were CMTM2$^+$ neutrophil cells with *CMTM2*, *S100A9* and *S100A12* expression (*Figure 4B*, *Figure 4* C1, C2, C5).

Please note that myeloid cells, including monocytes and neutrophils, in the tumor site are also referred to as myeloid-derived suppressor cells (MDSCs) (75). Therefore, MDSCs can be categorized as monocytic MDSCs (M-MDSC) and polymorphonuclear MDSCs (PMN-MDSCs) (75). Neutrophils and PMN-MDSCs share the same origin and many morphological and phenotypic features (75). Thus S100A9$^+$ neutrophil cells defined here may also represent the so-called MDSCs, which could perform their immune-suppressive activity through S100A8 and S100A9 (76).

The counts of Cluster 0 (monocyte) and Cluster 1 (S100A10$^+$ neutrophils) were elevated in the tumor group compared with the normal group (*Figure 4D*,*4E*), but only the CMTM2$^+$ neutrophil-specific GSVA score was associated with patients' poor prognosis. The monocytes and S100A10$^+$ neutrophil-specific GSVA scores were not correlated with patients' prognosis (*Figure 4F*). Furthermore, we found there were more CMTM2$^+$

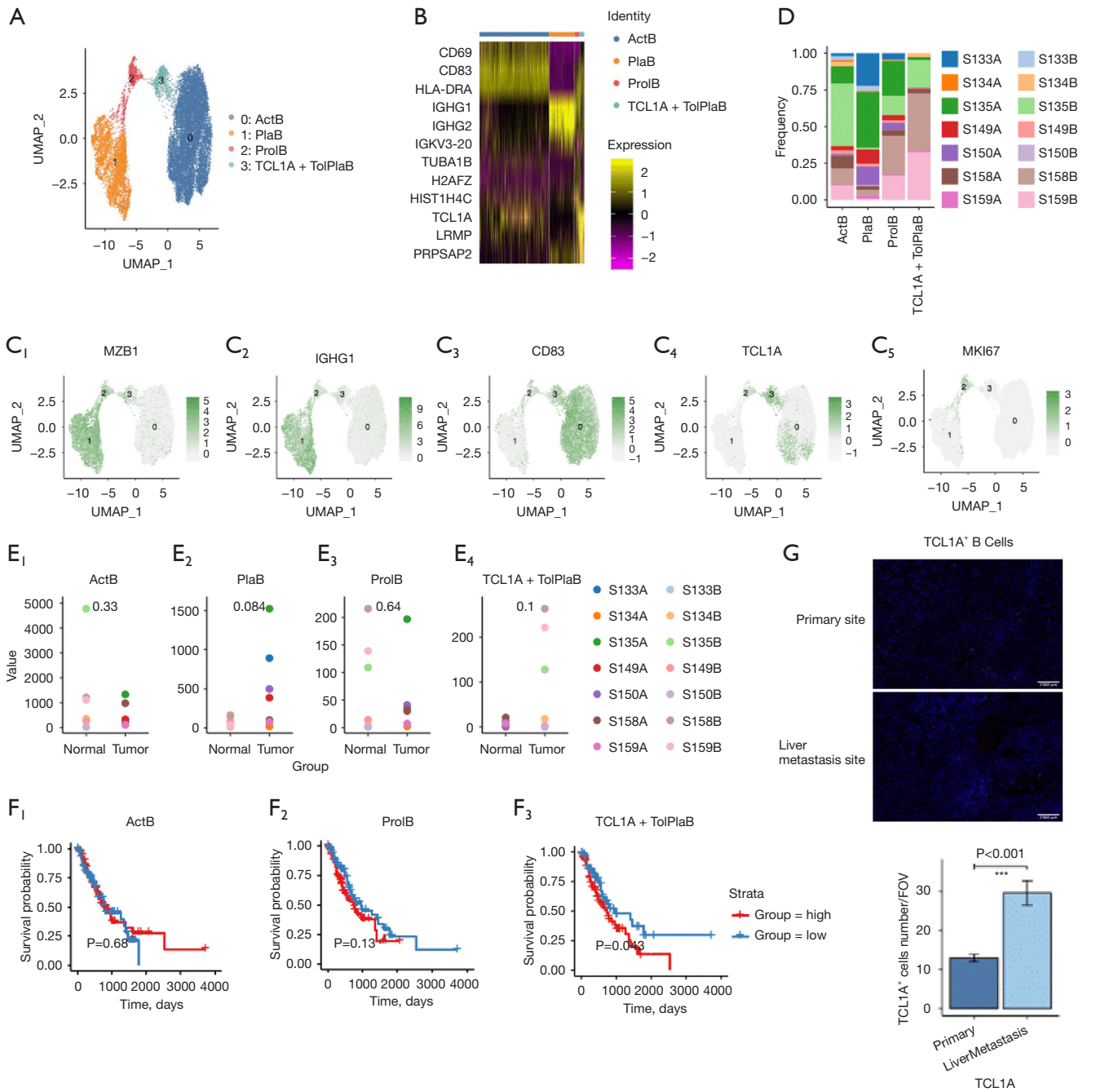

Figure 3 Analysis of cell subset of B cells. (A) UMAP of scRNA-seq data visualizing 4 cell clusters marked 0–3; (B) heatmap of differentially expressed genes in each cluster. Yellow to dark purple: high to low expression; (C) UMAP of scRNA-seq data visualizing the special gene markers; (D) distribution frequency of cluster cells; (E) expression values of cluster cells in normal and tumor group; (F) survival curves for low/high expression groups; (G) multiplex immunohistofluorescence assay for TCL1A$^+$ B cells. Scale bar =250 μm. ***, P<0.001. UMAP, Uniform Manifold Approximation and Projection; ActB, active B; PlaB, plasma B; ProlB, proliferating B; TolPlaB, tolerance plasma B; FOV, field of view; scRNA-seq, single-cell RNA sequencing.

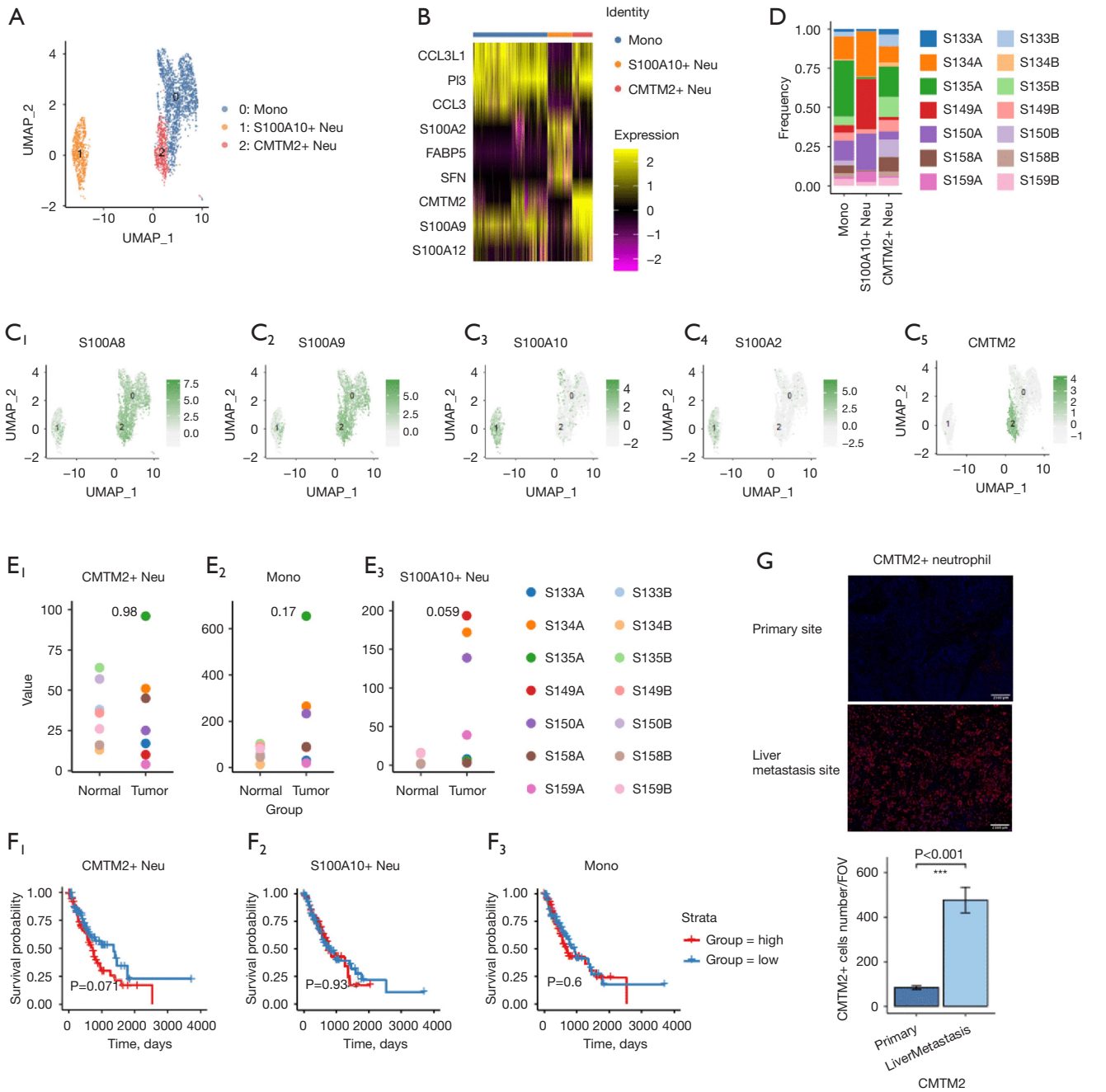

Figure 4 Analysis of cell subset of granulocytes. (A) UMAP of scRNA-seq data visualizing 3 cell clusters marked 0–2; (B) heatmap of differentially expressed genes in each cluster. Yellow to dark purple: high to low expression; (C) UMAP of scRNA-seq data visualizing the special gene markers; (D) distribution frequency of cluster cells; (E) expression values of cluster cells in normal and tumor groups; (F) survival curves for low/high expression groups; (G) multiplex immunohistofluorescence assay for CMTM2+ neutrophils. Scale bar =250 μm. ***, P<0.001. UMAP, Uniform Manifold Approximation and Projection; Mono, monocyte; Neu, neutrophil; FOV, field of view; scRNA-seq, single-cell RNA sequencing.

neutrophil cells in the liver metastasis site than in the ESCC primary site (*Figure 4G*). Therefore, the increased CMTM2$^+$ neutrophil cell proportion in the liver metastasis may contribute to shorter survival time and worse prognosis.

Effect of elevated natural killer T (NKT) cell subset in the primary site on prognosis in ESCC patients

NK cells, as cytotoxic lymphocytes of the innate immune system, can effectively kill cancerous cells. However, they are highly heterogeneous in the TME (77). Hence, the cell subset of NK cells was re-clustered to explore the role of the diverse NK cell subpopulation in ESCC.

The NK cells were re-clustered to 5 subsets (*Figure 5A,5B*). UMAP of scRNA-seq data visualizing the special gene markers is showed in *Figure 5C*. Cluster 0 cells were NK cells expressing *KH2D1B*, *HIR2DL3* and *INC02446* expression (*Figure 5B*) (78,79). Cluster 1 cells were ILC3b cells with *KRT81* expression (*Figure 5B*) (80). Cluster 2 cells were NKT cells with *PRSS23*, *FGFBP2* and *GZMH* expression (*Figure 5B*) (81). Cluster 3 cells were spondin 2 (SPON2)$^+$ NK cells with *SPON2* expression (*Figure 5B*) (81). Cluster 4 cells were γδNKT cells expressing *TRDV2*, *TRGV9* and *CD3E* (*Figure 5B*, *Figure 5* C4) (82,83). In addition, the distribution frequency of each NK cell subsets was shown in *Figure 5D* among the different patients and no subset was derived from a single patient. However, we did notice NKT and SPON2$^+$ NK subsets were quite obvious in patient S134 para-tumoral site (*Figure 5D*).

Cluster 2 (NKT), Cluster 3 (SPON$^+$ NK) and Cluster 4 (γδNKT) cells were higher in the tumor group than in the normal group (*Figure 5E*), but it seemed that only Cluster 2 (NKT) cells were associated with better prognosis of ESCC patients (*Figure 5F*).

In the liver metastasis site, there were also fewer Cluster 2 (NKT) cells than in the primary ESCC site (*Figure 5G*), which suggested that an increased NKT cell subset in the primary site may induce a better prognosis in ESCC patients.

Effect of elevated C1QA/B$^+$ MΦ subset in liver metastasis site on prognosis in ESCC patients

As an essential innate immune population, TAMs perform diverse functions to maintain homeostasis and ward off foreign pathogens. They are also pivotal for driving tumor progression, metastasis, and resistance to therapy (84). To elucidate the special function of different subsets, the MΦ subset was re-clustered. The results showed there were 8 cell subsets from Cluster 0 to Cluster 7 (*Figure 6A,6B*). UMAP of scRNA-seq data visualizing the special gene markers is showed in *Figure 6C*. Cluster 0 cells were infiltrating MΦ (InfMΦ) with *IL1B*, *CCL20* and *G0S2* expression (*Figure 6B*) (85,86). Cluster 1 cells were C1QA/B$^+$ resident MΦ (C1QA/B$^+$ ResMΦ) cells with *C1QA*, *C1QB* and *APOE* expression (*Figure 6B*, *Figure 6* C1, C2) (87,88). Cluster 2 cells were CD206$^+$ MΦ cells with *CD206* and *GPR183* expression (*Figure 6B*) (89). Cluster 3 cells were CTSK$^+$ resident MΦ (CTSK$^+$ ResMΦ) cells with *CTSK*, *APOC1* and *CCL18* expression (*Figure 6B*, *Figure 6* C3) (90,91). Cluster 4 cells were IDO1$^+$ MΦ cells with *IDO1*, *CXCL10*, *ISG15* and *APOBEC3A* expression (*Figure 6B*) (92,93). Cluster 5 cells were SPP1$^+$ MΦ cells with *SPP1*, *CXCL10*, *ISG15* and *APOBEC3A* expression (*Figure 6B*, *Figure 6* C4). Cluster 6 cells were Mono cells with *CXCL8* and *PI3* expression (*Figure 6B*) (94,95). Cluster 7 cells were proliferating MΦ (ProMΦ) with *TUBA1B*, *STMN1* and *HIST1H4C* expression (*Figure 6B*, *Figure 6* C5).

The Cluster 0 (C1QA/B ResMΦ), Cluster 1 (CD206$^+$ MΦ), Cluster 2 (CTSK$^+$ MΦ) and Cluster 7 (Spp1+ MΦ) cells were higher in the tumor group than in the normal group (*Figure 6D*, *Figure 6E*). The GSVA score was calculated using bulk RNA-Seq data obtained from TCGA based on MΦ subset feature genes. The correlation between the subset-specific GSVA score and patients' prognosis was investigated. We found survival time was shorter in the High group than in the Low group based on the GSVA score of the C1QA/B$^+$ MΦ subset (*Figure 6F*). We also observed more C1QA/B MΦ in the liver metastasis site than in the primary ESCC site (*Figure 6G*). The results revealed that an elevated C1QA/B$^+$ MΦ subset was associated with liver metastasis and poor prognosis in ESCC patients.

Role of C1QA/B$^+$ TAMs in ESCC

To identify the key cell subset regulating the ESCC TME, cell-cell interactions were analyzed. The results showed that the C1QA/B$^+$ MΦ subset was closely associated with other cell subsets (*Figure 7A*), and the absolute value of the correlation coefficient was maximum for C1QA/B$^+$ MΦ (*Figure 7B*).

To visualize the interrelationships among the different cell subsets, we first calculated the correlation coefficiency between any two subsets within TCL1A$^+$ TolPlaB cells,

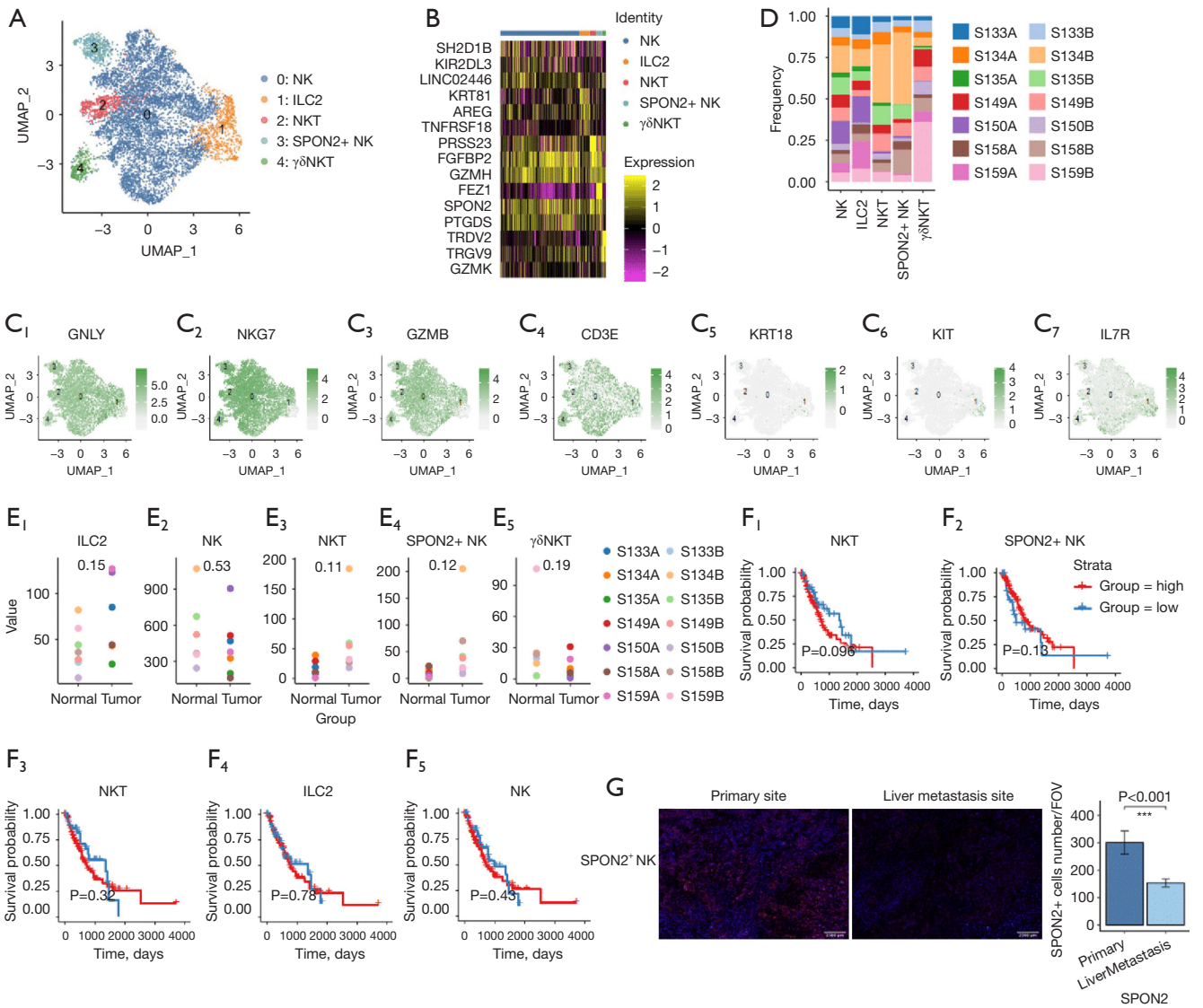

Figure 5 Analysis of NK cell subset. (A) UMAP of scRNA-seq data visualizing 5 cell clusters marked 0–4; (B) heatmap of differentially expressed genes in each cluster. Yellow to dark purple: high to low expression; (C) UMAP of scRNA-seq data visualizing the special gene markers; (D) distribution frequency of cluster cells; (E) expression values of cluster cells in normal and tumor groups; (F) survival curves for low/high expression groups; (G) multiplex immunohistofluorescence assay for SPON2$^+$ NK cells. Scale bar =250 μm. ***, P<0.001. UMAP, Uniform Manifold Approximation and Projection; ILC2, type 2 innate lymphoid cells; NK, natural killer; NKT, natural killer T; SPON2, spondin 2; FOV, field of view; scRNA-seq, single-cell RNA sequencing.

CMTM2$^+$ neutrophils, C1QA$^+$/B$^+$ MΦ, NKT cells, SPON2$^+$ NK cells, Tregs, ResCD4$^+$ T cells and ExhCD4$^+$ T cells. A circular chord diagram was constructed to visualize the correlation ecoefficiency weight between the different cell subsets, and the results showed that C1QA/B$^+$ MΦ had more connections with other cell subsets (*Figure 7C*). Other than C1QA/B$^+$ MΦ, CMTM2$^+$ neutrophils also had multiple connections, indicating that CMTM2$^+$ neutrophils could also mediate important immune-suppressive roles in the ESCC TME, associated with worse prognosis.

We wanted to investigate the interaction between C1QA/B$^+$ MΦ and other cell subpopulations. Firstly, the expression of myeloid immune checkpoints was portrayed in C1QA/B$^+$ MΦ. The results showed that *CD274*, *PDCD1LG2*,

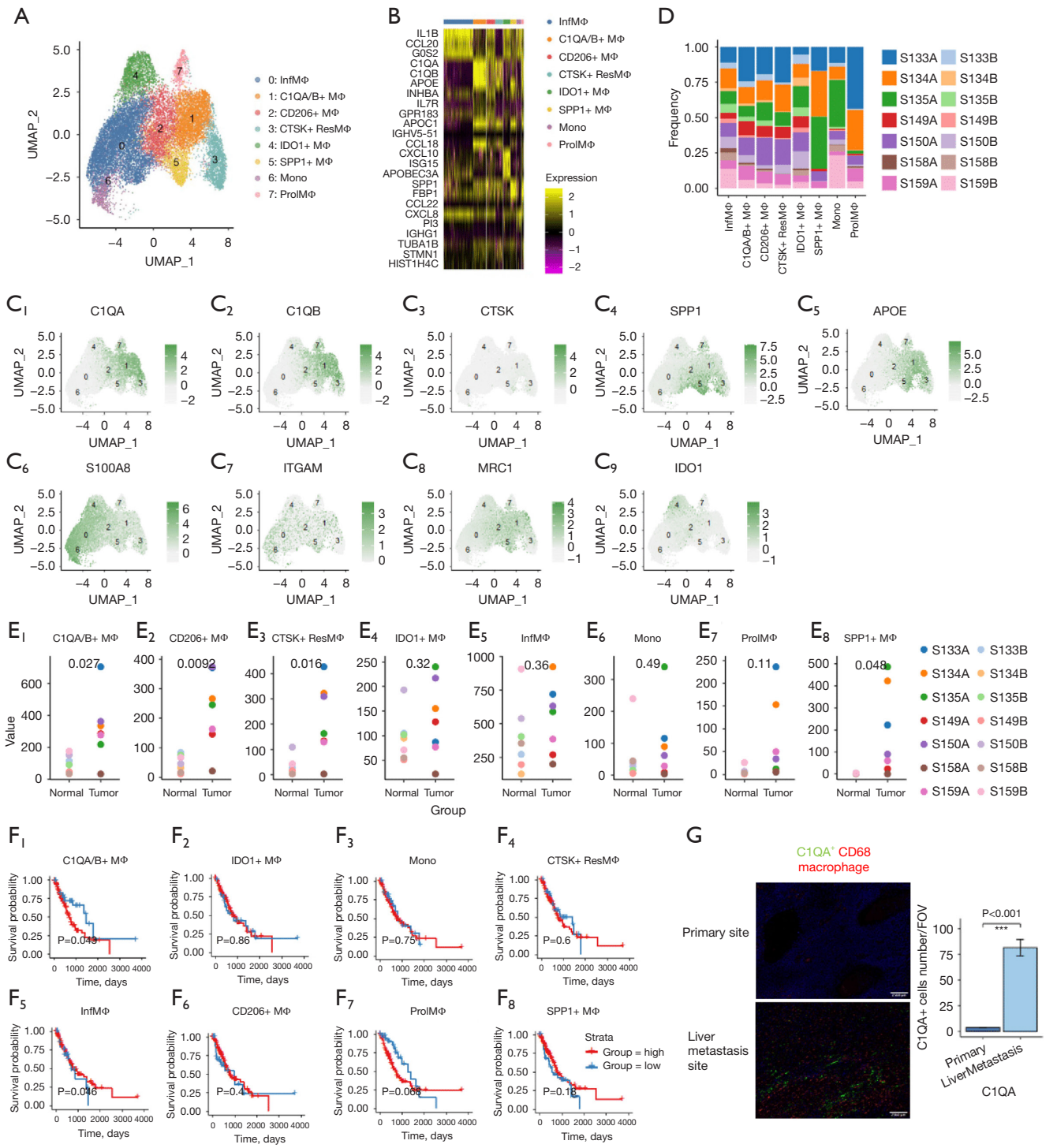

Figure 6 Analysis for cell subset of macrophages. (A) UMAP of scRNA-seq data visualizing 8 cell clusters marked 0–7; (B) heatmap of differentially expressed genes in each cluster. Yellow to dark purple: high to low expression; (C) UMAP of scRNA-seq data visualizing the special gene markers; (D) distribution frequency of cluster cells; (E) expression value of cluster cells in normal and tumor groups; (F) survival curves for low/high expression groups; (G) multiplex immunohistofluorescence assay for C1QA/B$^+$ CD68 macrophages. Scale bar =250 μm. ***, P<0.001. UMAP, Uniform Manifold Approximation and Projection; InfMΦ, infiltrating macrophages; MΦ, macrophage; Mono, monocyte; ResMΦ, resident macrophage; ProlMΦ, proliferating macrophage; FOV, field of view; scRNA-seq, single-cell RNA sequencing.

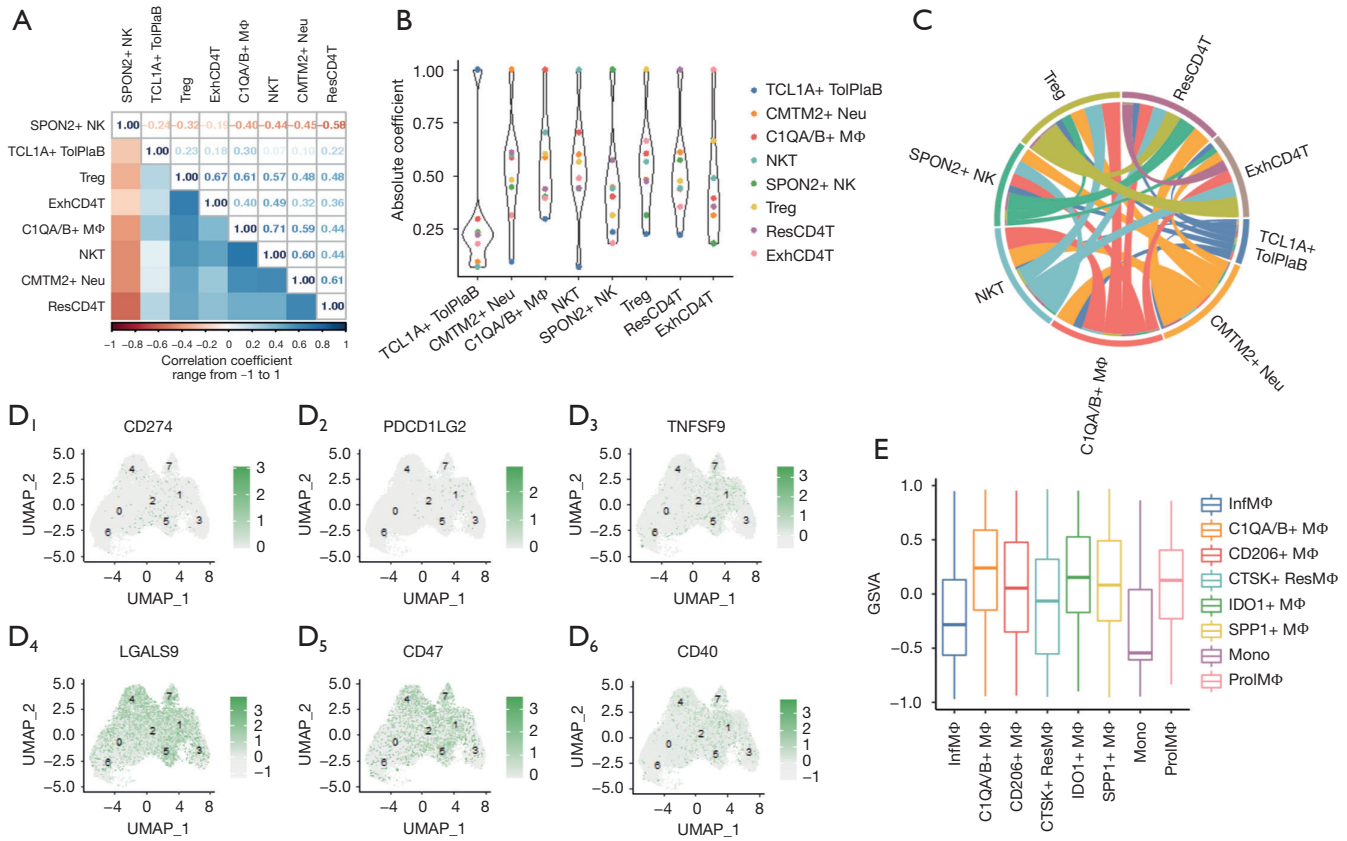

Figure 7 Role of C1QA/B+ tumor-associated macrophages in esophageal squamous cell carcinoma. (A) Principal component analysis; (B) absolute value of correlation coefficient; (C) circular chord diagram to describe the correlation; (D) UMAP of scRNA-seq data visualizing the special gene markers; (E) GSVA scores for different cell subsets. UMAP, Uniform Manifold Approximation and Projection; SPON2, spondin 2; NK, natural killer; NKT, natural killer T; Treg, regulatory T cell; ExhCD4T, exhausted CD4+ T; Neu, neutrophil; ResCD4T, resident CD4+ T; Mono, monocyte; GSVA, gene set variation analysis; scRNA-seq, single-cell RNA sequencing; InfMΦ, infiltrating macrophage; MΦ, macrophage; ResMΦ, resident macrophage; ProlMΦ, proliferating macrophage.

TNFSF9, *CD40*, *LGALS9* and *CD47* positively correlated to Cluster 8 (C1QA/B+ MΦ) (*Figure 7D*). Next, the gene signature was established based on these myeloid immune checkpoints, and the GSVA score was calculated. The score is the highest in the C1QA/B+ MΦ cell subset (*Figure 7E*), which indicated that C1QA/B+ MΦ may play a central role by expressing myeloid immune checkpoints to mediate the immune microenvironment of ESCC, and thereby influence patients' prognosis.

Discussion

The TME is complex and continuously evolving, which plays a crucial role in tumor homeostasis (96). In this study, multi-omics profiling of the ESCC TME was performed using scRNA-seq and RNA-Seq data. The gene signatures were established, and the specific GSVA scores were calculated. Thereafter, the GSVA score was utilized to investigate the correlation between cell subset abundance and survival probability, and the crucial cell subsets were screened and identified. Generally, single-cell transcriptome analyses were conducted to assess the cellular heterogeneity in normal and tumor tissues. In this study, 10 cell populations were identified such as T lymphocytes, B lymphocytes, granulocytes, NK cells, and MΦ. Major cell populations were re-clustered, the specific gene signature was described and the survivorship curve was draw to investigate the role of the cell subset in ESCC.

Tregs play a critical role in the maintenance of self-tolerance and suppressing aberrant immune responses.

Treg infiltration promotes tumor growth and invasion in ESCC (97). Accumulating evidence suggests that the removal of Tregs could evoke and enhance an anti-tumor immune response (98). Our results also indicated that the higher the proportion of Tregs, the shorter survival time of the patient. The percentage of $CD4^+$ T cells is an indicator of cellular immunity and they have a unique role in promoting tumor eradication, which implies $CD4^+$ T cells could influence the prognosis of ESCC (99). However, our results showed that upregulation of the $ResCD4^+$ T and $ExhCD4^+$ T cell subpopulations resulted in worse prognosis in ESCC patients, which may identify the unique function of specific CD4 T-cell subsets.

Tumor-infiltrating B lymphocytes play a pivotal role in shaping tumor development and promoting/suppressing tumor growth (100). Previous study indicated that enhanced *TCL1A*, an oncogene, results in an aggressive cellular and clinical phenotype (101). *TCL1A* is positively associated with various hematological malignancies by its effect on the infiltration of B cells and dendritic cells (102,103). *TCL1A* also plays an important role in promoting multi-modal tumor resistance (104). In this study, we also found the ESCC patients' prognosis was worse when the proportion of $TCL1A^+$ TolPlaB cells was significantly upregulated.

Granulocytes, as the most abundant leukocytes in human blood, are involved in the immune response against cancer (70). Previous study showed that *CMTM2* down-regulation induces hepatocellular carcinoma metastasis by promoting the epithelial-mesenchymal transition process (105). In our study, the survival time was shorter when the $CMTM2^+$ neutrophil cell proportion increased, which was in accord with previous reports conducted to predict the progression of ESCC.

TAMs promote tumor growth and metastasis by enhancing cancer cell proliferation, immunosuppression, and angiogenesis (106). In the ESCC TME, MΦ infiltration could promote tumor vascularity and chemoresistance via monocyte chemoattractant protein - 1 (MCP-1) and IL34, respectively (107,108). Macrophages could also contribute to ESCC tumor progression through the AKT and ERK signaling pathways via GDF15 (109) and the CCL3-CCR5 axis (110). In addition, MΦ could also promote proliferation and invasion of ESCC via EGF (epidermal growth factor) production (111).

C1QA plays a significant role in the innate immune response by counteracting the C1Q receptor (112). Additionally, related research has revealed that C1QA and C1QB are potential indicators of the tumorigenesis and development of osteosarcoma (113). Interestingly, our results suggested that $C1QA/B^+$ TAMs were the central regulator of the ESCC TME, closely associated with several key cell subsets. In terms of mechanism, several immune checkpoints, including CD40, CD47 and LGALS9, showed positive expression in $C1QA/B^+$ MΦ. The CD47 protein plays a pivotal role in tumors by delivering a "don't eat me signal", and targeting CD47 regulates the cancer cell fate (114). Therefore, we speculate that $C1QA/B^+$ TAMs may exert a central regulatory effect in immune evasion of ESCC via multiple immune checkpoint expressions. In addition, a previous report showed that tumor cells could hijack MΦ-produced complement C1Q molecules to promote tumor growth (115), and such a mechanism might also exist in the ESCC TME. Current immunotherapy mainly depends on the PD-1/PD-L1 axis, which targets T cell exhaustion. Immunotherapy relies on MΦ targets is still lacking. Here, we identified the $C1QA^+$ MΦ as the central interacting immune cells during immunosuppression loop formation. $C1QA^+$ MΦ carries many immune checkpoints, and future immunotherapy could target $C1QA^+$ MΦ as an alternative option when T cells are spared in the tumor immune microenvironment.

We mainly applied scRNA-seq, mIHC and transcriptomic to study ESCC. Epigenetic landscape is very important for the ESCC study; however, our current tools setting is not able to capture the epigenetic landscape. In addition, $C1QA^+$ MΦ subset distribution and contribution at various ESCC stages were not revealed since only few pairs of tissues were included for scRNA-seq analysis.

Conclusions

Our results indicated that $C1QA/B^+$ MΦ maybe a potential immunotherapy target for ESCC. Deletion of this cell subset may increase the efficacy of immunotherapy by mediating myeloid immune checkpoint expressions, and thereby influence the ESCC patients' prognosis.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5351/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5351/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5351/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 (as revised in 2013). The study was approved by the ethics committee of Fujian Cancer Hospital (No. 10104700) and informed consent was taken from all individual participants.

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Supplementary

Table S1 List of antibodies for multiplex immunohistofluorescence assay

Antibodies	Source	Identifier
CD3	Cell signaling technology	Cat#: 85061s
CD11b	Abcam	Cat#: ab13357
Pan cytokeratin	BioLegend	Cat#: 914704
CD68	BioLegend	Cat#: 916104
CD138	Invitrogen	Cat#: 362900
CD4	Abcam	Cat#: ab133616
CD8	BioLegend	Cat#: 372902
CD69	Abcam	Cat#: ab234511
Foxp3	Cell Signaling Technology	Cat#: 12653s
CD57	BD	Cat#: 555618
Ki67	BD	Cat#: 556609
GAMMADELTA	Novus Biologicals	Cat#: NBP2-62225

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