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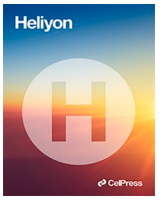

Research article

High infiltration of immune cells with lower immune activity mediated the heterogeneity of gastric adenocarcinoma and promoted metastasis

Hongpeng Lu [a], Zhihui Xu [b], Lihong Shao [a], Peifei Li [a], Yonghong Xia [b,*]

[a] Department of Gastroenterology, The First Affiliated Hospital of Ningbo University, Ningbo, 315010, China
[b] Department of Gastroenterology, Ninghai County Second Hospital, Ningbo, 315600, China

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ABSTRACT

Background: Gastric adenocarcinoma (GA) is a heterogeneous malignancy with high invasion and metastasis. We aimed to explore the metastatic characteristics of GA using single-cell RNA-sequencing (scRNA-seq) analysis.

Methods: The scRNA-seq dataset was downloaded from the GEO database and the "Seurat" package was used to perform the scRNA-seq analysis. The CellMarker2.0 database provided gene markers. Subsequently, differentially expressed genes (DEGs) were identified using the FindMarkers function and subjected to enrichment analysis with the "ClusterProlifer". "GseaVis" package was used for visualizing the gene levels. Finally, the SCENIC analysis was performed for identifying key regulons. The expression level and functionality of the key genes were verified by quantitative real-time PCR (qRT-PCR), wound healing and transwell assays.

Results: A total of 7697 cells were divided into 8 cell subsets, in which the Cytotoxic NK/T cells, Myeloid cells and Myofibroblasts had higher proportion in the metastatic tissues. Further screening of DEGs and enrichment analysis revealed that in the metastatic tissues, NK cells, monocytes and inflammatory fibroblasts with low immune levels contributed to GA metastasis. In addition, this study identified a series of key immune-related regulons that mediated the lower immune activity of immune cells. Further *in vitro* experiment verified that CXCL8 was a key factor mediating the proliferation and migration of GA cells.

Conclusion: The scRNA-seq analysis showed that high infiltration of immune cells with lower immune activity mediated heterogeneity to contribute to GA metastasis.

1. Introduction

Tumor heterogeneity brings about a great many challenges to accurate diagnosis and personalized treatment [1]. Gastric adenocarcinoma (GA) is a heterogeneous malignancy with high invasion and metastasis [2,3], imposing health threat to the patients worldwide [4]. According to the latest statistical report, the estimated new patients with GA and related death number will reach 26,890 and 10,880 in 2024, respectively [5]. The incidence and mortality of GA decrease slightly due to the application of accurate diagnosis and personalized treatment strategies [6,7]. In recent years, studies have been increasingly performed to analyze the molecular mechanisms of the heterogeneity in GA, for example, a series of gene mutations and signaling pathways associated with GA

* Corresponding author.
E-mail address: zjnbnhxiayonghong@163.com (Y. Xia).

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heterogeneity are discovered using high-throughput sequencing technology [8–10]. Zhang et al. described the cellular and differentiation heterogeneity among GA patients at single-cell level and indicated that the degree of tumor differentiation is a potential predictor of GA prognosis [11]. Histopathology-based method are simple for GA diagnosis and classification [12], but the pathology results of GA may vary due to the complex background factors and subjective discrimination [13]. Overall, elucidating the molecular basis features of GA could effectively help understand the tumor biological behaviors and develop effective intervention strategies.

Currently, surgery is still the primary treatment for GA patients [14]. Though surgery and adjuvant therapy have been advanced greatly [15], the 5-year survival of patients still remains lower than 30 % [16] due to a high post-surgery recurrence rate (40%–65 %) caused by metastasis [17]. Thus, illustrating the underlying molecular mechanisms involved in gastric cancer (GC) metastasis is crucial for improving the treatment outcome and promoting patients' survival [18,19]. Huang et al. used an *in vivo* metastasis assay model to study the effect of stress-inducible protein-1 (STIP1) on GC cell metastasis and found that STIP1 facilitates the spread of GC to other parts of the body by enhancing the expression of specific genes such as c-Myc and Cyclin D1 in the Wnt/β-catenin signaling pathway. This process is also linked to the movement of β-catenin into the cell nucleus [20]. Moreover, researchers also demonstrated that the forced expression of miR-206 inhibits the proliferation, colony formation, and tumorigenesis of SCG-7901 cells (a line of GC cells) in the xenograft. The anti-metastatic effects of miR-206 are possibly mediated through targeting metastasis-regulatory genes such as STC2, HDAC4, KLF4, IGF1R, FRS2, SFRP1, BCL2, BDNF, and K-ras. These genes are significantly downregulated by the stable expression of exogenous miR-206 in SCG-7901 cells [21]. However, the exact mechanism of these genes in GC metastasis awaits to be comprehensively analyzed. In addition, evidence showed that cell-cell interaction and immune cell infiltration state also play an important role in the tumor metastasis-related tumor microenvironment (TME). Fan el. revealed that a high level of epithelial SOX9 mediated the M2 macrophage repolarization (immune suppressive phenotype) to inhibit the function of T cells, which drive GA metastases through the regulation of paracrine LIF factor [22]. Therefore, understanding TME and its regulatory mechanisms is important for developing new therapeutic strategies.

Recently, scRNA-seq has become an useful tool to analyze the gene expression and intracellular subset heterogeneity at the single-cell level [23], classify novel cell subtypes and define the dynamic transformations in certain cell states and cell types [24,25]. In this work, we analyzed the GA heterogeneity and metastasis of primary and metastatic GA tissues using the scRNA-seq technology. Changes of infiltrated cells in the two types of tissues were explored and the immune activation state of immune cells was elucidated. Our findings provided a better understanding of immune cell infiltration heterogeneity in GA metastasis, contributing to the development of GA treatment and intervention strategies.

2. Material and methods

2.1. Single-cell RNA-seq data acquisition of metastatic tissues

The single-cell RNA-seq dataset (GSE234129), which included 6 primary gastric adenocarcinoma (GA), 1 ovarian and 1 liver metastasis samples, was downloaded from the Gene Expression Omnibus (GEO, https:/ww.ncbinlm.nih.gov/geo/) database [26] based on the 10x Genomics library-building platform and Illumina NovaSeq 6000 sequencing platform.

2.2. Preprocessing the scRNA-seq data

The Read10X function of "Seurat" R package was used to read the single-cell sequencing data, retaining cells expressing 200–6000 genes and containing proportion <15 % mitochondrial genes [27]. Then, the cell data were normalized by the SCTransform function and subjected to principal component analysis (PCA) using the RunPCA function. The sample batch effect was removed by the "harmony" package [27]. Further, the top 20 PCs were used for Uniform Manifold Approximation and Projection (UAMP) dimensionality reduction, followed by performing cell subpopulation clustering analysis (at resolution = 0.1) applying the FindNeighbors and FindClusters function [27]. Finally, the gene markers from the CellMarker2.0 database were used to annotate cell types [28].

2.3. Gene set enrichment analysis (GSEA)

Firstly, the FindMarkers function was used to calculate the log2FC value for the cell clusters and identify significant differentially expressed genes (DEGs) in metastatic group and primary tumor group [29]. After that, the gseGO and gseKEGG function in "ClusterProlifer" package was used to perform GSEA and visualize the biological process (BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathways [30]. Genes within the differentially enriched pathways were visualized by the "GseaVis" package [31].

2.4. SCENIC analysis

The gene regulatory networks (GRNs) composing of transcription factors (TFs) are decisive to the transcription specificity and cell heterogeneity. Thus, exploring the GRNs of single cell can help understand the biological significances behind the cell heterogeneity [23]. SCENIC is algorithm for single-cell GRNs analysis. The gene co-expression network could be inferred based on the motifs of TFs and highly reliable GRNs that mediated by the key TFs were identified [32]. Following the official guideline of SCENIC, the GENIE3 method was used to infer the potential target genes for each TF and the GRNs were constructed using the top10perTarget method [33]. Eventually, a series of highly reliable regulons (a gene set including one TF and multiple target genes) were obtained, and then the

Table 1
The primers of the target interest genes.

Genes	Forward (5'-3')	Reversed (5'-3')
CCL22	AGCGTCTGCTGCCGTGA	AGTCTGAGGTCCAGTAGAAGTGTTT
CD74	CCAGGCCACCACCGCCTA	TCTGCATGGGCCCCTGGG
CXCL8	CAGTTTTGCCAAGGAGTGCTAA	TTCCATCAGAAAGCTTTACAATAATTT
HLA-E	GCTACTACAATCAGAGCGAGGCC	TCTCCAGGTATTTGTGGAGCCA
HSPA1A	GGAAAGAAAAGAGAAAAGGGGG	AGTGCGGTCATCAGATTGGG
PLTP	AACGAAAAAGAAAAGGGAGGAA	AGTGCAGGGTCCGAGGTATT
CD1C	GAGATTCAAGACCATGCAAGTCAA	CCTGGAGATGGCACCCATG
SYK	TGTCAAGGATAAGAACATCATAG	CACCACGTCATAGTAGTAATTG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTG

AUCell function was used to calculate the regulon activity in each cell based on the gene expression level [33].

2.5. Cell culture and qRT-PCR

The human gastric adenocarcinoma (GA) cell line (HGC-27) with a highly metastatic nature and the AGS cancer cell line without metastasis were obtained from the COBIOER corp. (Nanjing, China). All the cells were cultured in RPMI-1640 medium that supplemented with 1 % penicillin/streptomycin, 10 % heat inactivated fetal bovine serum (FBS) at 37 °C in a 5 % CO_2 incubator (cells were generally passaged 1–3 times before experiments) [34]. Total RNA was extracted from these cultured cells using TRizol reagent (Invitrogen) and then cDNA synthesized with the use of PrimeScript Reverse Transcriptase (TaKaRa). qPCR detection was performed on a LightCycler 480 (Roche) according to the manufacturer's specification. GAPDH were the reference genes. Each sample were performed in triplicate. The $2^{-\Delta DDCt}$ method was used for analyzing the results. The specific primers are shown in (Table 1).

2.6. Wound healing and transwell assay

The si-CXCL8 was purchased from the Sigon corp. (Suzhou, China) and its reagent was dissolved and configured to work concentrations for silencing the cells based on the manufacturer's specification. To measure cell migration using wound healing assay, HGC-27 cells were seeded in 6-well plates at a density of 2×10^4 cells per well and incubated until confluent. Then a rectilinear scratch was generated using 100 μL pipette tip. After incubating the cells for 24 h (h), 4 % paraformaldehyde was used for cell fixation for 15 min (min) and 0.1 % crystal violet was used for cell staining for another 15 min at room temperature. An inverted microscope was used for observing wound closure [34]. For invasion assays, a total of 5×10^4 cells were inoculated into the upper chamber of 6-well plates with 8-μm pore chamber inserts and 100 μL FBS-free medium, while the lower chamber contained 600 μL DMEM. After 24 h, the 4 % paraformaldehyde and 0.1 % crystal violet were used to fix and stain the cells for 15 min, respectively. The migrating cells were imaged under an inverted microscope [34].

2.7. Statistical analysis

Statistical analysis and data visualization were performed in the R software (version 3.6.0). The Wilcoxon rank-sum test was used to assess the difference between two sets of continuous variables. *$p < 0.05$ was considered as statistically significant.

3. Results

3.1. Single-cell landscape of primary and metastatic GA

After cell screening, SCT normalization and dimensionality reduction clustering, a total of 7697 cells were obtained and mainly consisted of 8 cell subpopulations, namely, B cells, Cytotoxic NK/T cells, Myeloid cells, Regulatory T cells, Plasma B cells, Mast cells, Myofibroblasts and Endothelial cells (Fig. 1A). The expression of their marker genes was shown in Fig. 1B and C. Subsequently, we calculated the proportion of different cell type in primary tumor, liver metastasis and ovary metastasis groups and found that the proportion of Cytotoxic NK/T cells, Myeloid cells, Myofibroblasts and Endothelial cells were higher in the metastasis groups than in the primary tumor groups (Fig. 1D), which indicated that immune resistance to tumor were enhanced in metastatic tissues.

3.2. High infiltration of cytotoxic NK/T cells with lower immune activity in metastatic tissues

The single-cell landscape revealed that a variety of immune cells, such as Cytotoxic NK/T cells and Myeloid cells, had higher infiltration in metastatic tissues. Thus, we calculated the log2FC value for Cytotoxic NK/T cells and examined the biological process of the cells in two types of tissues. The GSEA results showed that in metastatic tissues, Cytotoxic NK/T cells had higher activated pathways including superoxide anion production and integrin activation, while the activity of these adaptive immunity and cell cycle-related pathways including T cell activation and differentiation, T cell receptor signaling and NF-kappaB and chromosome segregation pathways were inhibited (Fig. 2A). In Cytotoxic NK/T cells in metastatic tissues, the expression of most genes related to superoxide

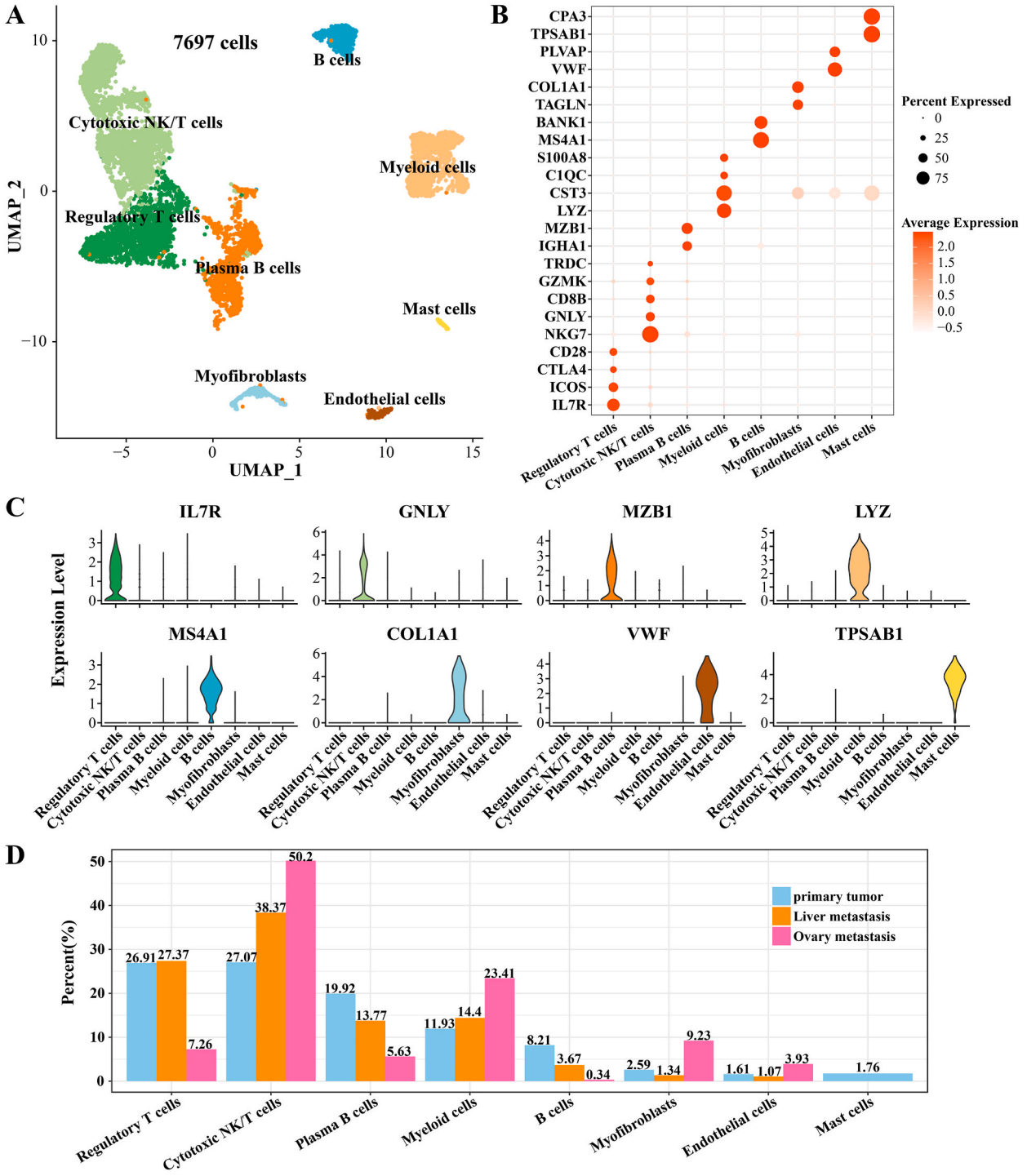

Fig. 1. Single cell landscape of primary and metastatic gastric adenocarcinoma.
(A) UMAP plot of the annotated cell clusters. (B) The bubble plot of marker genes expression in different cell type. (C) The violin plot of marker genes expression in different cell type. (D) The cell proportion in primary tumor, liver metastasis and ovary metastasis groups.

anion generation were upregulated (Fig. 2B), while that of a large number of genes related to T cell activation (Fig. 2C), T cell differentiation (Fig. 2D), immune response activation (Fig. 2E) and chromosome segregation (Fig. 2F) were downregulated. This suggested that the high infiltration of Cytotoxic NK/T cells was accompanied with a lower immune activity in the metastatic tissues.

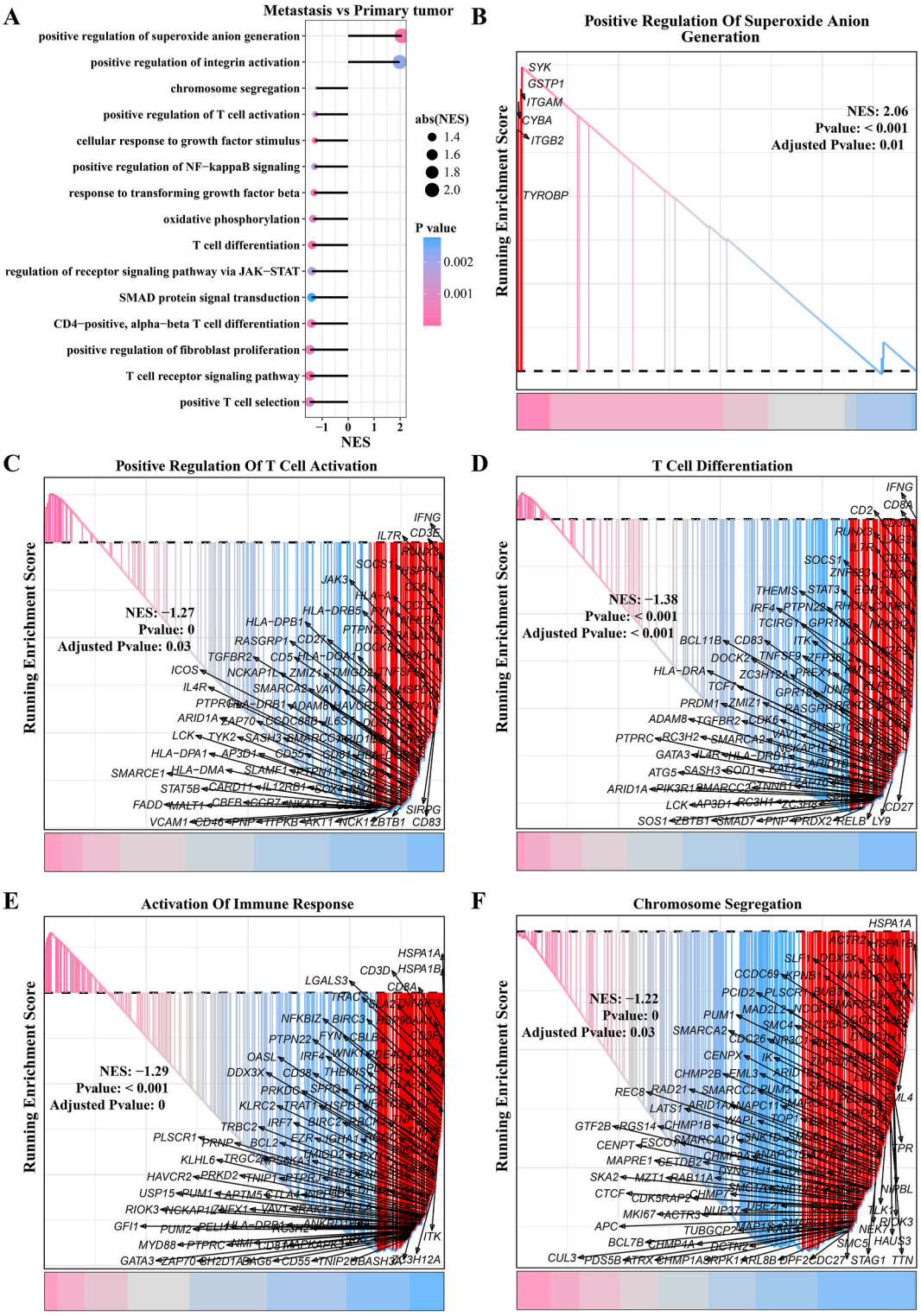

Fig. 2. The pathway enrichment of Cytotoxic NK/T cells in primary tumor and metastasis groups.
(A) The biological process scores of Cytotoxic NK/T cells in primary tumor and metastatic tissue. (B) The superoxide anion generation-related genes of Cytotoxic NK/T cells in metastatic tissue. (C) The T cell activation-related genes of Cytotoxic NK/T cells in metastatic tissue. (D) The T cell differentiation-related genes of Cytotoxic NK/T cells in metastatic tissue. (E) The immune response activation-related genes of Cytotoxic NK/T cells in metastatic tissue. (F) The chromosome segregation-related genes of Cytotoxic NK/T cells in metastatic tissue.

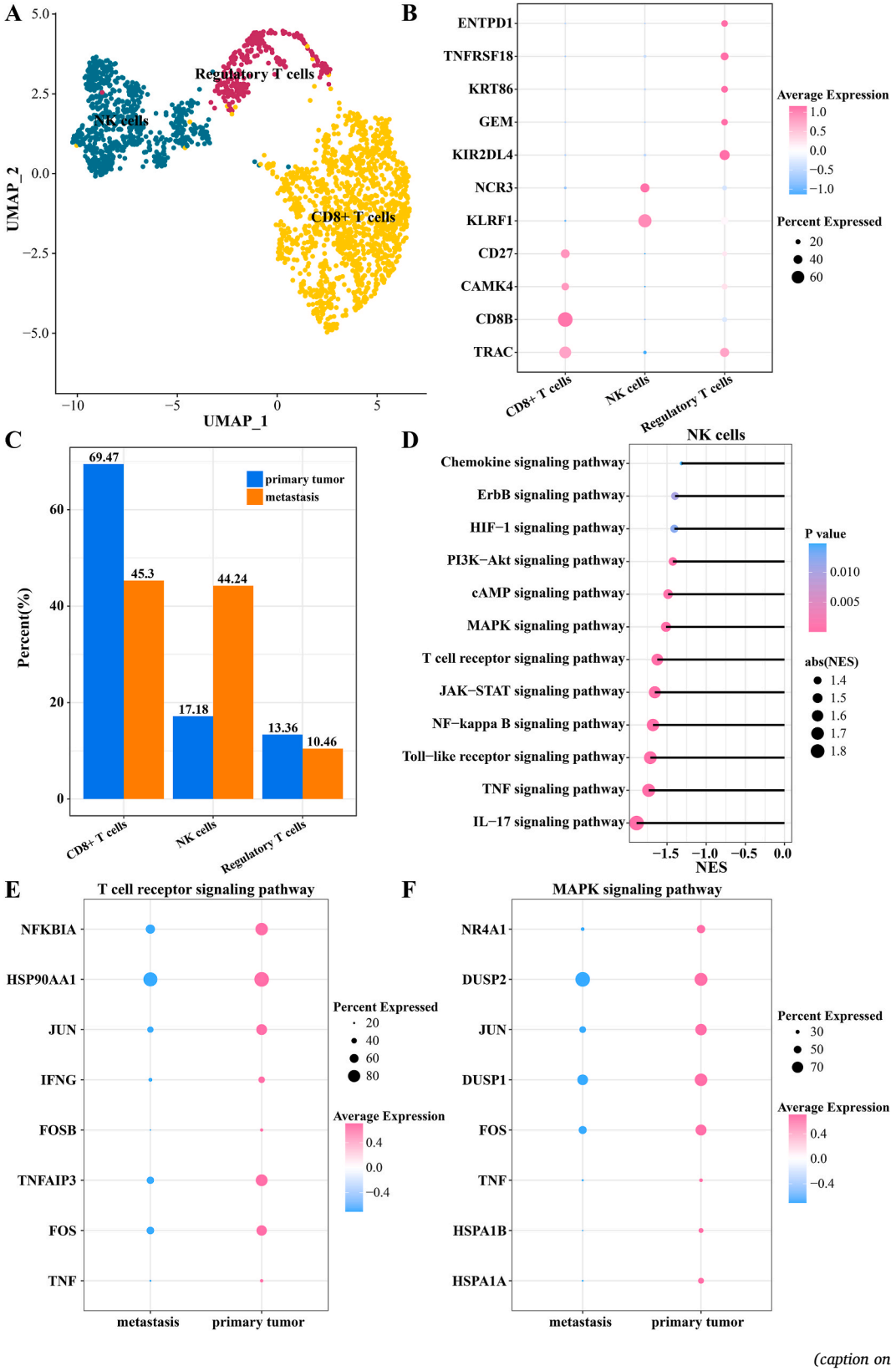

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Fig. 3. The heterogeneous characteristics of Cytotoxic NK/T cells.
(A) UMAP plot of Cytotoxic NK/T cells. (B) The expression of maker genes of $CD8^+$ T cell, NK cell and regulatory T cell. (C) The cell proportion of $CD8^+$ T cell, NK cell and regulatory T cell in metastatic and primary tumor tissue. (D) Pathway enrichment analysis of NK cell in metastatic tissue. (E) The expression of T cell receptor signaling genes in metastatic and primary tumor tissue. (F) The expression of MAPK signaling genes in metastatic and primary tumor tissue.

3.3. High infiltration of NK cells with lower immune activity in the metastatic tissues

To further elucidate the heterogeneous characteristics of Cytotoxic NK/T cells, these cells were subdivided into the $CD8^+$ T cells, NK cells and regulatory T cells (Fig. 3A) based on the expression of marker genes (Fig. 3B). Interestingly, we found that the NK cells had higher proportion in the metastatic group compared to that in the primary tumor group (Fig. 3C), indicating NK cells with lower immune activity were involved in the metastatic process of GA. Subsequently, we examined the KEGG pathway enrichment difference of NK cells in the metastatic and primary tumor group. The GSEA results demonstrated that the activity of most immune response pathways in metastatic group, such as MAPK and T cell receptor signaling and IL-17 signaling pathway, were inhibited (Fig. 3D), and that the expression of NFKBIA, HSP90AII, TNFAIP3 and FOS genes in T cell receptor signaling pathway (Fig. 3E) as well as DUSP (1, 2), JUN and FOS in MAPK signaling pathway (Fig. 3F) were downregulated. This indicated that the immune activity of NK cells was inhibited in the metastatic group, and that NK cells had high infiltration and low immunoreactivity in the metastatic GA tissues, which may be an immune escape mechanism for developing the metastatic TME in GA.

3.4. High infiltration of myeloid cells with lower immune activity in the metastatic tissues

Similarly, the same method was used to explore the immune characteristics of Myeloid cells in the metastatic tissues. GSEA results revealed that the activity of ATP biosynthetic process, Fc receptor signaling and oxidative phosphorylation pathway were enhanced, whereas the activity of MAPK cascade, inflammatory, IL-1 and chemokines response and angiogenesis were inhibited in the metastatic group (Fig. 4A), suggesting that the Myeloid cells were in an immunosuppressive state. Most genes related to Fc-gamma receptor signaling pathway (including CD47, PTPRC, and SYK) were up-regulated in Myeloid cells in the metastatic group, suggesting that they were involved in cell signaling and immune response pathways (Fig. 4B). A number of genes associated with angiogenesis (Fig. 4C), positive regulation of MAPK cascade (Fig. 4D), migration of neutrophils (Fig. 4E) and regulation of inflammatory response in Myeloid cells (Fig. 4F) were downregulated in the metastatic group. These results indicated that Myeloid cells enhanced cancer cell proliferation and survival by supporting tumor growth and inhibiting the immune system's attack on the tumor, facilitating the tumor cells to escape from immune surveillance.

3.5. High infiltration of monocytes with lower immune activity in the metastatic tissues

To elucidate the heterogeneous characteristics of Myeloid cells in the metastatic tissues, the cells were further subdivided into monocytes, macrophages and dendritic cells (Fig. 5A) based on the expression of marker genes (Fig. 5B). Further analysis revealed that only the proportion of monocytes was higher in the metastatic tissues than that in the primary tumor (Fig. 5C), indicating that monocytes were the main type of Myeloid cells involved in mediating GA metastasis. The GSEA results of monocytes revealed that only the activity of oxidative phosphorylation pathway was enhanced in the metastatic group, while the activity of immune response and receptor signaling pathways (Fig. 5D) as well as the expression of JUN, CEBPB, TNFAIP3, NFKBLA, SOCS3 and IL1B in TNF signaling pathway (Fig. 5E), the expression of CDKN1A, OSM, EREG, SGK1 and YWHAH in PI3K-Akt signaling pathway (Fig. 5F) were inhibited in the metastatic group. This suggested that the high infiltration of monocytes with lower immune activity contributed to the metastasis in GA.

3.6. High infiltration of myofibroblasts with lower immune activity in the metastatic tissues

Myofibroblasts are a crucial regulator of anti-tumor immune response and are activated by the paracrine signals derived from macrophages and lymphocytes [35]. The immune-active state of Myofibroblasts in metastatic and primary tumor tissues was analyzed. The GSEA results revealed that the activities of cytoplasmic translation, collagen fibril and basement membrane organization pathway were enhanced, but the activities of antigen treatment and presentation, T cell and B cell activation pathways were significantly inhibited (Fig. 6A) and the expression of most antigen processing and presentation genes was downregulated (Fig. 6B). This indicated that the ability of immune activation of myofibroblasts in the metastatic tissues was inhibited. Next, the myofibroblasts were subdivided into inflammatory fibroblasts, muscle-like fibroblasts, and secretory fibroblasts (Fig. 6C) based on the expression of maker genes (Fig. 6D). Inflammatory fibroblasts had higher proportion in the metastatic tissues compared to that in the primary tumor tissues (Fig. 6E), but the expression of its antigen presentation genes (such as HLA-B and PSME1) was downregulated (Fig. 6F), suggesting that high infiltration of Myofibroblasts with lower immune activity in the metastatic tissues was an important contributor to GA metastasis.

3.7. The downregulated immune-related TFs mediated the lower immune activity of immune cells

Finally, we performed the SCENIC analysis to identify the key immune activation-related regulons in monocytes, NK cells and

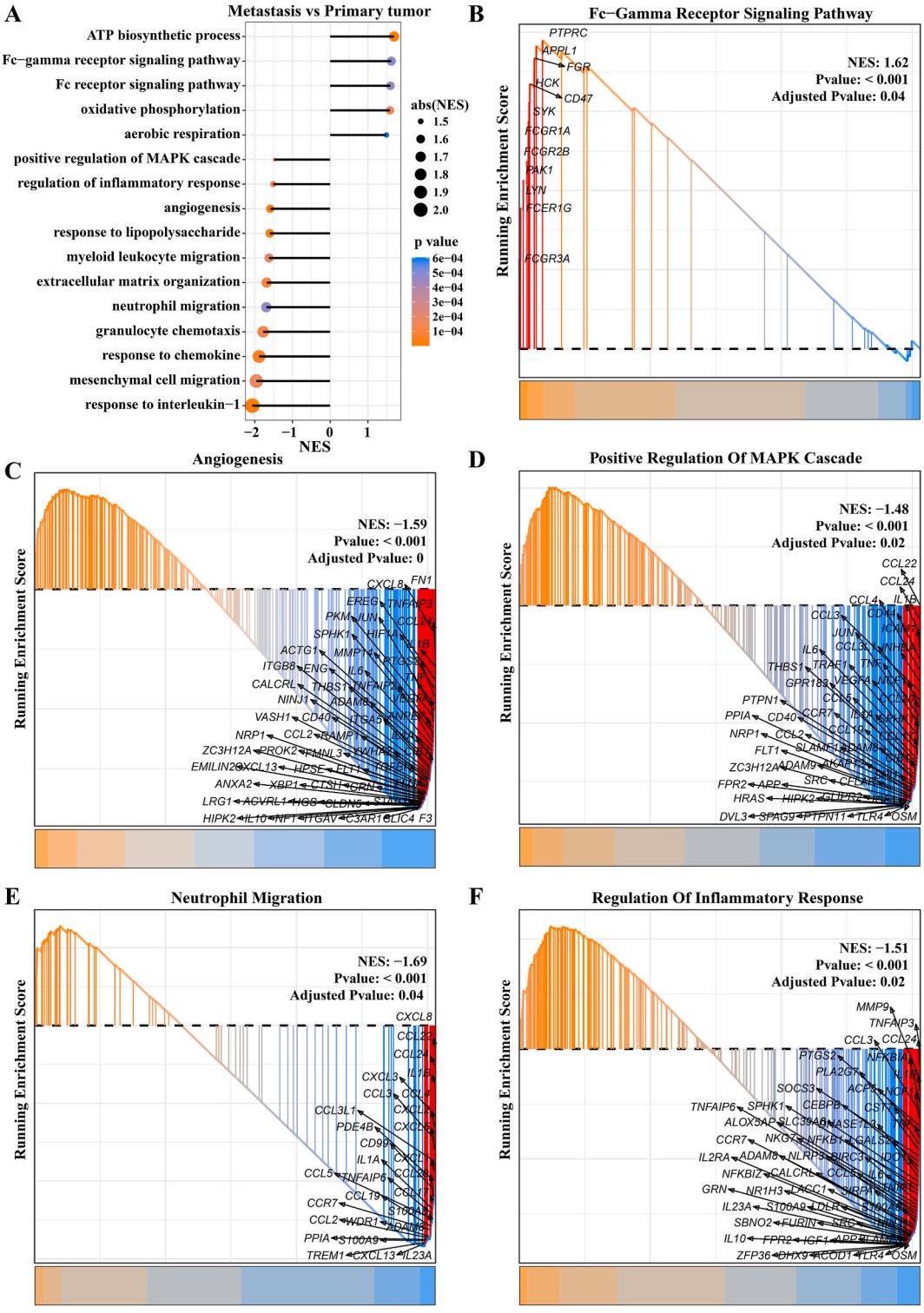

Fig. 4. The immune activity of Myeloid cell in metastatic tissue.
(A) Pathway enrichment analysis of Myeloid cell in metastatic tissue. (B) The Fc-gamma receptor signaling genes level in metastatic tissue. (C) The angiogenesis genes level in metastatic tissue. (D) The positive regulation of MAPK cascade gene level in metastatic tissue. (E) The neutrophil migration genes level in metastatic tissue. (F) The regulation of inflammatory response genes level in metastatic tissue.

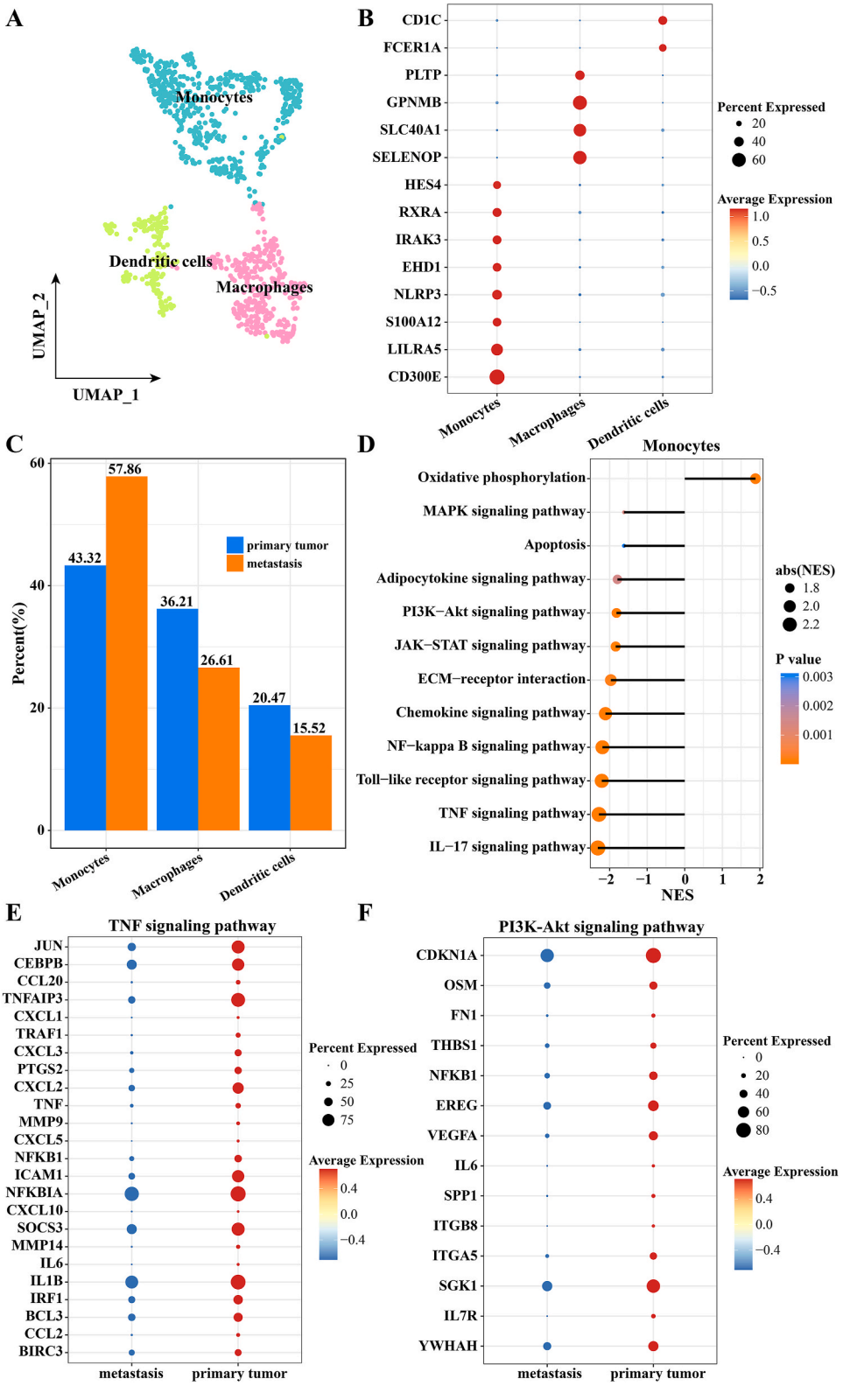

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Fig. 5. The heterogeneous characteristics of Myeloid cells.
(A) UMAP plot of Myeloid cell. (B) The marker gene expression in monocyte, macrophage and dendritic cell. (C) The cell proportion of monocyte, macrophage and dendritic cell in metastatic and primary tumor tissue. (D) Pathway enrichment analysis of monocyte in metastatic tissue. (E) The expression of TNF signaling genes in metastatic and primary tumor tissue. (F) The expression of PI3K-Akt signaling genes in metastatic and primary tumor tissue.

inflammatory fibroblasts. The AUCell algorithm was used to evaluate the activity of each regulons in the metastatic and primary tumor tissues. The results showed that the expressions of ATF3, BCL3, SNA11, NR3C1 and JUN in monocytes ($p < 0.05$, Fig. 7A), and IRF1, RELB, ATF3 and REL in NK cells ($p < 0.05$, Fig. 7B), and IRF7, FL11, ZNF771 and ETV5 in inflammatory fibroblasts were significantly downregulated in the metastatic tissues compared to that in the primary tumor tissues ($p < 0.05$, Fig. 7C). These results demonstrated that the downregulated immune-related TFNs mediated the lower immune activity of immune cells.

3.8. CXCL8 was a key factor mediating the proliferation and migration of GA cells

Based on importance of method validation in the analysis of biomarker [36], we detected the expression level of several genes with different expression levels in the metastatic and primary cells, and observed that genes including the CCL22, CD74, CXCL8 and HLA-E and HSPA1A were significantly high-expressed in the HGC27 cells ($p < 0.05$, Fig. 8A–E), while genes including the PLTP, CD1C and SYK were significantly inhibited in HGC27 cells ($p < 0.05$, Fig. 8F–H), moreover, their expression level was consistent with the results of scRNA-seq analysis. As CXCL8 was a chemokine with the highest expression level in the metastatic cells, its role in promoting proliferation and migration of HGC-27 cells was further analyzed. After si-CXCL8 silencing, the migration ability of tumor cell was significantly inhibited, the wound closure ratio was reduced by more than 1 fold (Fig. 8I and J), and fewer cells were invasive and stained with purple (Fig. 8K and L), indicating that CXCL8 was a key factor mediating the proliferation and migration of GA cells.

Subsequently, we explored the impact of high and low CXCL8 expression levels on patient survival using the GSE234129 dataset. As shown in Fig. S1A, it could be observed that GA patients with a high expression level of CXCL8 had significantly worse prognosis than those with low-expressed CXCL8. In addition, we found that GA patients with high-expressed CXCL8 were more sensitive to some common chemotherapeutic agents including 5-Fluorouracil 1073, Cisplatin 1005, Docetaxel 1007, and Docetaxel 1819 than those with low CXCL8 expression level ($p < 0.05$, Figs. S1B–E).

4. Discussion

GA is the most frequently detected subtype of GC, accounting for 95 % of all GC cases [37,38]. Considerable death cases of GA are resulted from the post-surgery recurrence mediated by metastasis [17], which represents a major obstacle to the successfully treatment in GA. To complete the metastatic cascade, tumor cells have to detach from primary tissue and intravasate into circulatory systems, escape immune attack and proliferate in distant organs [39]. Metastatic cells construct a TME that facilitates angiogenesis and proliferation to promote the development of macroscopic, malignant secondary tumors [40]. Although 90 % of cancer deaths are caused by systemic metastasis, most cancer studies do not delve into the *in vivo* state of metastasis [41]. Here, this study performed a systematic scRNA-seq analysis to analyze the cellular heterogeneity in GA metastasis, and we found that multiple immune cell had a high infiltration level in the metastatic tissues, while their immune activity was suppressed by different pathways to form a suppressive TME for GA metastasis.

The proportion of Cytotoxic NK/T cells, myeloid cells and myofibroblasts was increased in the metastatic tissues, suggesting that high immune cell infiltration enhanced the immune resistance to tumor cells. Since Rudolf Virchow observed the connection between tumor cells and their surrounding TME [42], TME has been considered to play a decisive role in determining cancer behaviors through epigenetic or genetic makeups of tumor cells as well as the interactive surrounding milieu of tumor cells for growth, proliferation, survival, metastasis and chemoresistance [43]. The cytotoxic effects and immune regulation of NK cells are precisely regulated through a balance of signals generated by a set of activating and inhibitory receptors on the NK cells. Meanwhile, metastatic tumor cells evade attacks by immune cells using various strategies [44]. Some researchers indicated that antibody-mediated depletion of NK cell function or the use of NK cell-deficient hosts could promote metastasis, suggesting that NK cells play a crucial role in anti-metastasis [45]. Correia et al. found that NK cells maintain cancer dormancy and therefore control liver metastasis in breast cancer [46]. These observations validated that NK cells play a crucial role in mediating antimetastatic effects in some clinical settings. This concept has also been closely associated with cancer therapy and the prevention of tumor metastasis. In addition, previous study identified four mouse myeloid cell subpopulations closely associated with lung metastasis in breast cancer, and found that several tumor-promoting pathways were responsible for such a process, including angiogenesis, immunosuppression, and tumor growth and metastasis. This suggested that myeloid cell subpopulations fulfill key functions in the formation and development of metastatic ecological niches [47]. Fibroblasts contribute to the advancement of metastatic tumors by restructuring and adding to the extracellular matrix. This process enhances the malignant characteristics of tumor cells and promotes the resistance of these metastasizing tumors to the existing therapies. Additionally, fibroblasts influence other cells within the TME [48]. Fibroblasts associated with liver metastasis from colorectal cancer do not express CD45 (a marker found in various types of white blood cells) as well, which suggested that they do not derive from the bone marrow [49,50]. Researchers developed a chimeric mouse model to explore pancreatic ductal adenocarcinoma and their results validated the hypothesis. They transplanted tdTomato-marked bone marrow into mice subjected to radiation. Interestingly, fibroblasts associated with metastasis in liver metastases did not exhibit the tdTomato marker [51]. The results

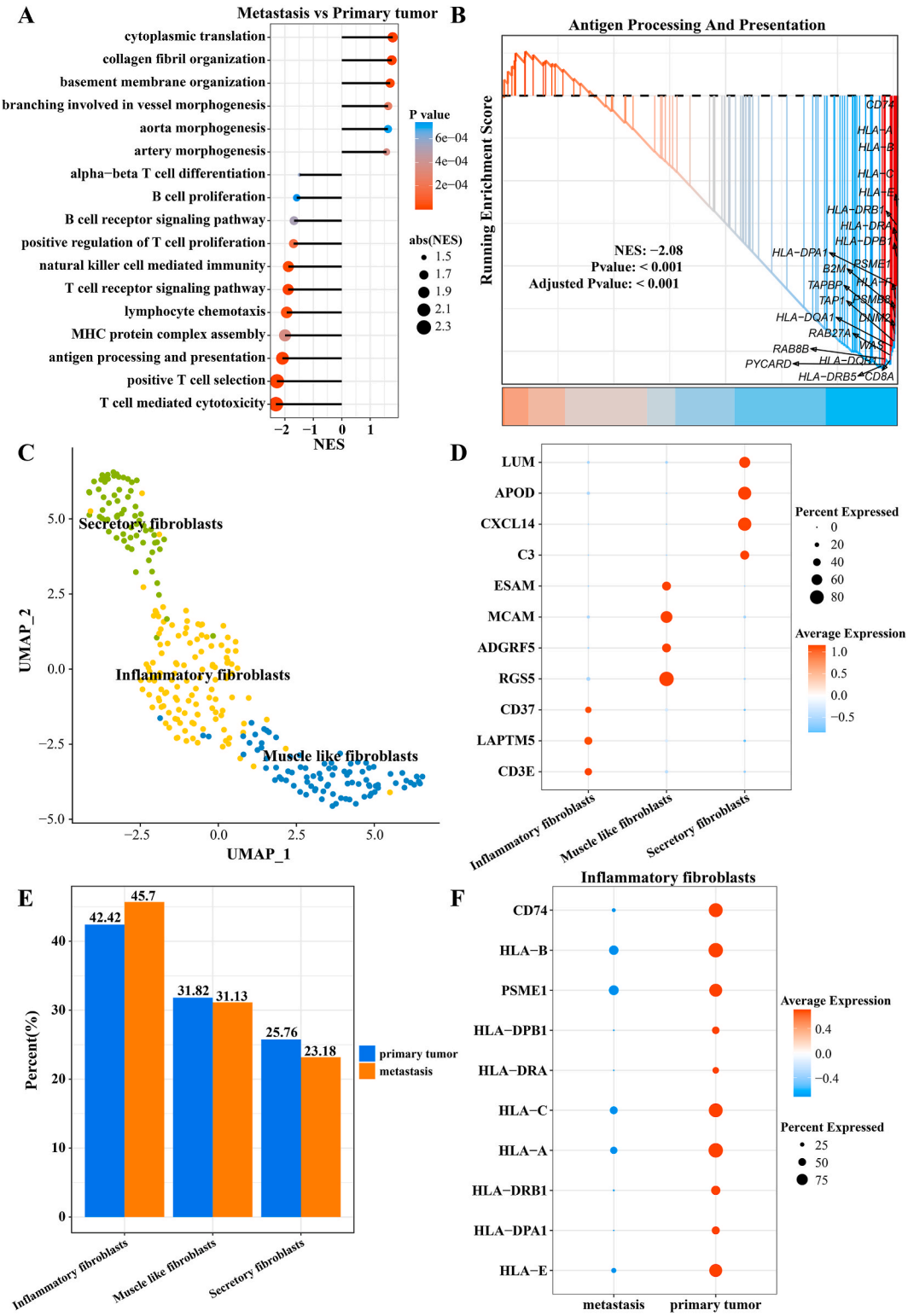

Fig. 6. The immune activity of Myofibroblasts in metastatic tissue.
(A) Pathway enrichment analysis of Myofibroblasts in metastatic tissue. (B) The antigen processing and presentation genes level in metastatic tissue. (C) UMAP plot of Myofibroblasts. (D) The marker genes expression of inflammatory fibroblasts, muscle like fibroblasts, and secretory fibroblasts. (E) The cell proportion of inflammatory fibroblasts, muscle like fibroblasts, and secretory fibroblasts. (F) The expression of antigen processing and presentation genes.

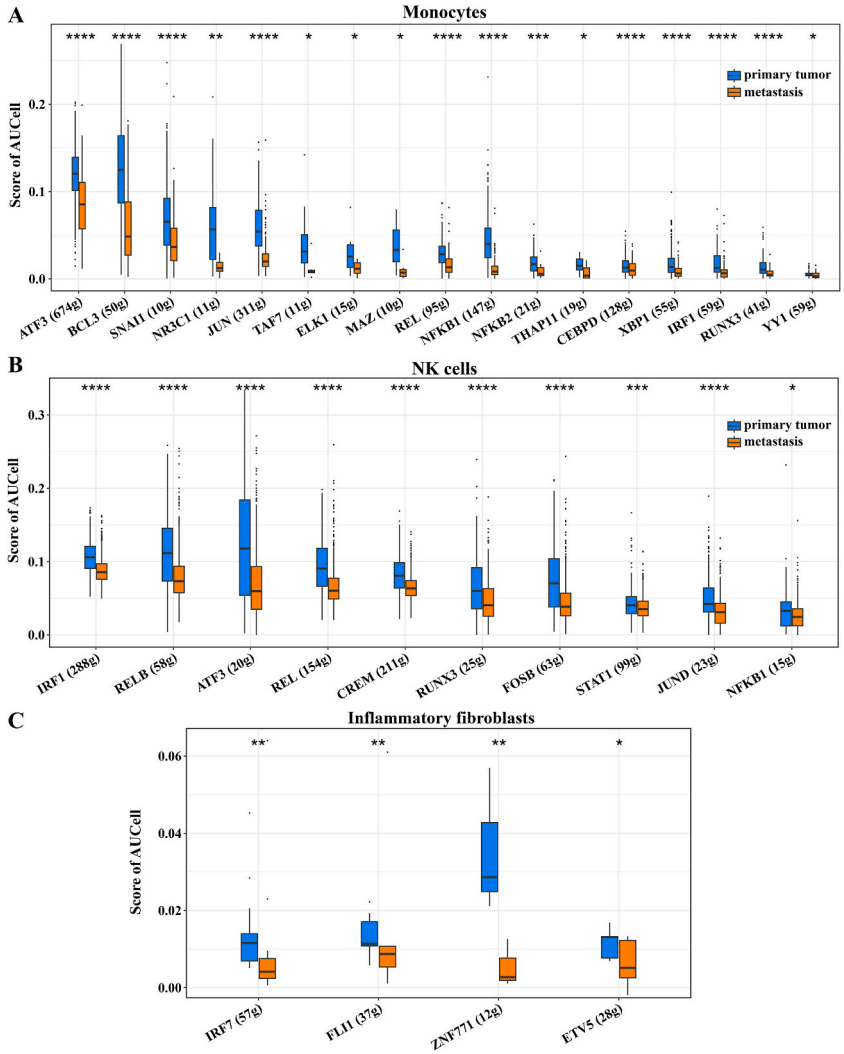

Fig. 7. The expression of immune-related regulons.
(A) The score of key regulons of monocytes in metastatic and primary tumor tissue. (B) The score of key regulons of NK cell in metastatic and primary tumor tissue. (C) The score of key regulons of inflammatory fibroblasts in metastatic and primary tumor tissue. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

supported our current findings that these cell subpopulations and their interactions in the TME had important implications for the development of GA metastatic phenotype.

Meanwhile, monocytes, NK cells and inflammatory fibroblasts were the primary cell clusters that contributed to the metastasis and heterogeneity of GA. However, the downregulated immune response pathways suggested that these cell clusters with a low level of immune activity as immunosuppressive cells shape an immunosuppressive environment for tumor progression [52] due to persistent antigenic stimulation and immune response activation that mediates immune depletion and remodeling. It is known that macrophages have two polarized states from anti-tumorigenic (M1) to pro-tumorigenic (M2) properties [53]. In the early stage of cancer, the antitumorigenic M1 type retains APC properties, such as phagocytotic, high-expressed MHCII and a high tumor-killing activity [54]. In contrast, the pro-tumorigenic M2 type is immunosuppressive and is characterized by a low expression of inhibitory molecules including PD-L1 and Tim3 at late stage of immune response [55,56]. Studies demonstrated that the protective and pathogenic effect of immune cells is depended on the cell subset, microenvironment and disease stage [57]. For instance, in pulmonary metastasis of breast cancer mouse, recruited monocytes convert into a $Ly6C^{hi}CD11b^{hi}$ cell population that produces CCL2 to recruit metastasis-facilitating macrophages [58]. The NK cells are effective anti-tumor cells that could be suppressed by tumor-derived molecules and tumor-educated stromal cells, eventually contributing to cancer progression during metastasis [59], for example, a past study found that the metastatic lung adenocarcinoma had low level infiltration of CD16 NK cells and formed a NK cell-excluded TME [60]. Sun el. reported that the inflammatory myofibroblasts induced by CXCL3 promote pancreatic cancer metastasis [61]. Inflammatory myofibroblasts consist of the “reactive stroma” and mediate epithelial-mesenchymal transition for cancer metastasis [62]. We analyzed the

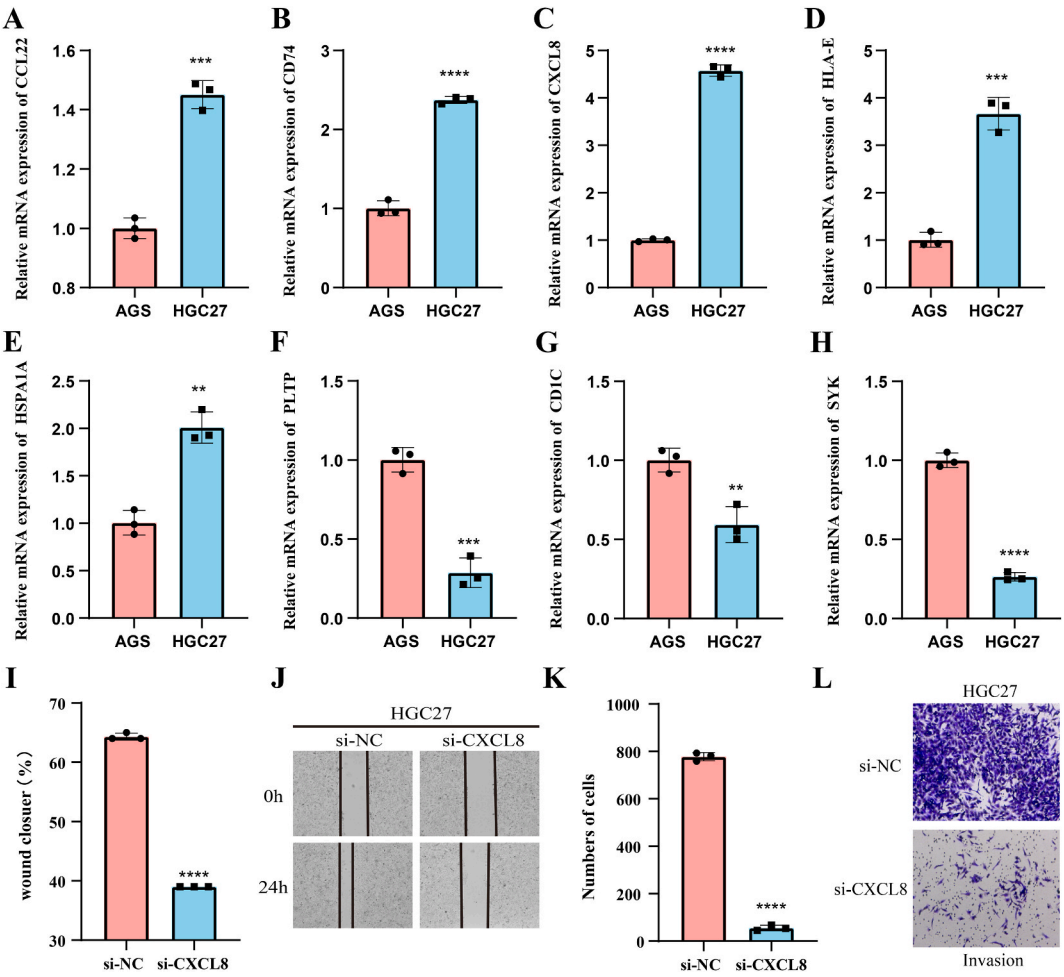

Fig. 8. The gene expression analysis *in vitro* experiment.
(A) The CCL22 expression analysis in AGS and HGC27 cells. (B) The CD74 expression analysis in AGS and HGC27 cells. (C) The CXCL8 expression analysis in AGS and HGC27 cells. (D) The HLA-E expression analysis in AGS and HGC27 cells. (E) The HSPA1A expression analysis in AGS and HGC27 cells. (F) The PLTP expression analysis in AGS and HGC27 cells. (G) The CD1C expression analysis in AGS and HGC27 cells. (H) The SYK expression analysis in AGS and HGC27 cells. (I) The wound healing ration of siNC and si-CXCL8 cells. (J) The wound closure analysis of siNC and si-CXCL8 cells. (K) The cell numbers analysis of siNC and si-CXCL8 cells in trans-well assay. (L) The images of siNC and si-CXCL8 cells in trans-well assay. (*p < 0.05, **p < 0.01, ***p < 0.001).

single-cell profile of GA with metastasis and elucidated the heterogeneous characteristics of GA metastasis. The chemokine CXCL8 was a key factor that mediated the proliferation and migration of GA cells. However, current results were mainly obtained using scRNA-seq technique, which requires additional histological data as well as functional experiments for validation and to more comprehensively analyze the interactions of different cell types in the TME. In addition, the importance of molecular signatures in clinical prognostic prediction and treatment demands a larger clinical sample size to assess these molecular signatures for clinical applications. Finally, although this study revealed the distribution and properties of different immune cell subpopulations in the metastatic tissues of GA applying the scRNA-seq, it failed to elucidate how these cell subpopulations specifically affected the metastatic phenotype of GA. In the future, we will explore the specific mechanisms through which immune cell subpopulations regulated the metastatic phenotype of GA through cytokines, chemokines and related signaling pathways by performing *in vitro* and *in vivo* experiments and by combining multi-omics data and functional analyses.

5. Conclusion

In summary, this study elucidated the heterogeneous characteristics of GA metastasis and revealed that high infiltration of immune cells with lower immune activity mediated the heterogeneity to promote metastasis in GA. The current findings provided a basis for the development of GA treatment and intervention strategies.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available in the GSE repository [GSE234129] (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE234129).

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CRediT authorship contribution statement

Hongpeng Lu: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Funding acquisition, Data curation. **Zhihui Xu:** Validation, Supervision, Software, Project administration, Data curation. **Lihong Shao:** Visualization, Resources, Investigation, Data curation, Conceptualization. **Peifei Li:** Visualization, Supervision, Investigation, Funding acquisition. **Yonghong Xia:** Writing – review & editing, Visualization, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37092.

Abbreviations

GA	Gastric adenocarcinoma
scRNA-seq	single cell RNA-seq
DEGs	differentially expressed genes
EMT	epithelial-mesenchymal transition
GEO	Gene Expression Omnibus
UAMP	Uniform Manifold Approximation and Projection
GSEA	Gene Set Enrichment Analysis
BP	biological process
KEGG	Kyoto Encyclopedia of Genes and Genomes
GRNs	gene regulatory networks
TFs	transcription factors
APC	antigen presenting cells
CTL	cytotoxic T lymphocytes
TME	tumor microenvironment

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