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Association of CD8⁺TILs co-expressing granzyme A and interferon- γ with colon cancer cells in the tumor microenvironment

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Abstract

CD8⁺T cells secreting granzyme A (GZMA) can induce pyroptosis in tumor cells by effectively cleaving gasdermin B (GSDMB), which is stimulated by interferon- γ (IFN- γ). However, the interaction between GZMA-expressing CD8⁺T cells and GSDMB-expressing tumor cells in colon cancer remains poorly understood. Our research employed multi-color immunohistochemistry (mIHC) staining and integrated clinical data to explore the spatial distribution and clinical relevance of GZMA- and IFN- γ -expressing CD8⁺ tumor-infiltrating lymphocytes (TILs), as well as GSDMB-expressing CK⁺ cells, within the tumor microenvironment (TME) of human colon cancer samples. Additionally, we utilized single-cell RNA sequencing (scRNA-seq) data to examine the functional dynamics and interactions among these cell populations. scRNA-seq analysis of colorectal cancer (CRC) tissues revealed that CD8⁺TILs co-expressed GZMA and IFN- γ , but not other cell types. Our mIHC staining results indicated that a significant reduction in the infiltration of GZMA⁺IFN- γ ⁺CD8⁺TILs in colon cancer patients ($P < 0.01$). Functional analysis results indicated that GZMA⁺IFN- γ ⁺CD8⁺TILs demonstrated enhanced activation and effector functions compared to other CD8⁺TIL subsets. Furthermore, GSDMB-expressing CK⁺ cells exhibited augmented immunogenicity. Correlation analysis highlighted a positive association between GSDMB⁺CK⁺ cells and GZMA⁺IFN- γ ⁺CD8⁺TILs ($r = 0.221$, $P = 0.033$). Analysis of cell-cell interactions further showed that these interactions were mediated by IFN- γ and transforming growth factor- β (TGF- β), the co-stimulatory molecule ICOS, and immune checkpoint molecules TIGIT and TIM-3. These findings suggested that GZMA⁺IFN- γ ⁺CD8⁺TILs modulating GSDMB-expressing tumor cells, significantly impacted the immune microenvironment and patients' prognosis in colon cancer. By elucidating these mechanisms, our present study aims to provide novel insights for the advancement of immunotherapeutic strategies in colon cancer.

Keywords Granzyme A, GSDMB, Colon cancer, Interferon- γ , CD8⁺T cells

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Introduction

The incidence of colon cancer has increased steadily in recent years, with a notable shift toward younger populations [1, 2]. The advent of immunotherapy has revolutionized the treatment landscape for colon cancer, particularly through the employment of immune checkpoint inhibitors (ICIs) [3]. Researches have indicated that programmed cell death 1 (PD-1) monoclonal antibodies, pembrolizumab and nivolumab, are effective in treating metastatic colon cancer in patients who exhibit mismatch-repair deficiency (dMMR) and high microsatellite instability (MSI-H) [4, 5]. In contrast, these therapeutic agents are not effective in patients with proficient mismatch-repair (pMMR) and microsatellites that are either stable (MSS) or show low microsatellite instability (MSI-L) [3, 4]. The underlying mechanisms of this immune resistance are believed to include a low tumor mutational burden and inadequate immune cell infiltration [4]. Moreover, challenges such as limited therapeutic applicability, variable patient response, and severe side effects, such as cytokine release syndrome (CRS) persist [3]. This underscores the urgent need for exploration into novel immunotherapeutic approaches to develop innovative treatment strategies for colon cancer.

A high infiltration rate of CD8⁺TILs within the colon cancer TME correlates with improved patient prognosis [6]. CD8⁺TILs play a critical role in surveilling and eliminating colon cancer cells that spread from the primary tumor site, significantly impacting the prevention of metastasis [7]. Additionally, the presence of CD8⁺TILs in the TME is linked to a better response to ICIs therapy [8], highlighting their potential as predictive biomarkers and effective therapeutic targets in colon cancer [6]. Granzymes (GZMs) from CD8⁺T cells or natural killer (NK) cells can enter target cells through pores formed by perforin, subsequently cleaving GSDM proteins into N-terminal and C-terminal domains [9, 10]. The N-terminal domain forms channels in the cell membrane, causing cytoplasmic swelling, cell membrane rupture, and the release of inflammatory factors and danger signals, which elicit a strong antitumor immune response [11]. Furthermore, GZMs indirectly promote the cleavage of GSDM proteins by activating caspases, which in turn activate GZMs, creating a positive feedback loop that enhances the overall effect [11].

Researches have indicated that GZMA can specifically cleave GSDMB at the Lys244 or Lys229 residues in tumor epithelial cells, thereby inducing pyroptosis [12, 13]. This mechanism may present a promising approach for tumor immunotherapy [14, 15]. Concurrently, IFN- γ , also originating from CD8⁺TILs, upregulates the transcription of GSDMB mRNA, thereby enhancing this cell pyroptosis pathway [12]. IFN- γ enhances the motility of antigen-specific CD8⁺T cells towards their

target antigens and downregulates the expression of vascular endothelial growth factor A (VEGFA), leading to decreased blood flow within tumor tissues [16]. In the CT26 model treated with PD-1, the K229A/K244A double mutants at the GSDMB loci resulted in the most pronounced increases in both tumor volume and weight [12]. Conversely, GSDMB has been identified as playing a pivotal role in the metastasis of colon cancer cells to distant organs by modulating cell adhesion and migration processes [17, 18]. Additionally, GSDMB contributes to the development of resistance against chemotherapy and targeted therapies by promoting protective autophagy [19]. In summary, it is crucial to further explore how CD8⁺TILs co-expressing GZMA and IFN- γ regulate GSDMB in colon cancer epithelial cells and their impact on patient prognosis. Moreover, investigating the effects of GZMA⁺IFN- γ ⁺CD8⁺TILs and GSDMB⁺ tumor epithelial cells on signaling pathways within the colon cancer TME is essential. Understanding these mechanisms could enable strategic manipulation of their infiltration ratios to enhance antitumor responses.

In this study, we utilized mIHC to examine the spatial distribution and clinical significance of GZMA, IFN- γ , and GSDMB within TME of human colon cancer patients. Leveraging scRNA-seq data from normal colorectal and CRC tissues, we explored the effector functions and interactions among these cellular groups. Our results reveal that CD8⁺TIL co-expressing GZMA and IFN- γ are crucial in shaping the immune microenvironment, particularly affecting tumor cells that express GSDMB. By elucidating these mechanisms, our study offers new insights that could enhance immunotherapeutic strategies in the management of colon cancer.

Materials and methods

Patients and tissue specimens

The human colon cancer tissue microarray (TMA, catalog: HCoLA180Su21) was provided by Outdo Biotech Co., Ltd., Shanghai, China. A total of 94 patients (46 males and 48 females, aged 29 to 86 years) who underwent surgery from July 2012 to April 2014 were enrolled in this study. This study, which involved human participants, was reviewed and received approval from the Clinical Research Ethics Committee at Outdo Biotech (Shanghai, China, SHYJS-CP-1,910,009).

Multi-color immunohistochemical staining

The mIHC analysis was performed utilizing the PANO 7-plex IHC kit (catalog No. 0004100100, Panovue, Beijing, China) based on the manufacturer's instructions to identify the different cell subsets expressing GZMA, IFN- γ , GSDMB, CD8, cytokeratin (CK) on colon cancer and normal colon TMA. CK served to pinpoint malignant epithelial cells, while 4',6-diamidino-2-phenylindole

(DAPI, SIGMA-ALDRICH) highlighted the nucleus. Tissue chips were deparaffinized with xylene, rehydrated with graded ethanol, rinsed in distilled water, and soaked in 10% buffered formalin. Antigen retrieval entailed microwave heating of slides in a citric acid solution, initially at high power, followed by low power for 15 min, with subsequent cooling to ambient temperature. Post-drying, a blocking solution was applied for 10 min at room temperature, succeeded by the incubation with primary antibodies for 1 h. The primary antibodies used were as follows: anti-GSDMB (1:500 dilution, catalog No. ab235540, Abcam, Cambridge), anti-GZMA (1:500 dilution, catalog No. ab209205, Abcam, Cambridge), anti-CD8 (1:400 dilution, catalog No. CST 70,306, Cell signaling technology, USA), anti-IFN- γ (1:800 dilution, catalog No. ab231036-40, Abcam, Cambridge), and anti-CK (1:800 dilution, catalog No. CST4545T, Cell signaling technology, USA). This step was followed by the application of horseradish peroxidase (HRP)-conjugated secondary antibodies for 10 min. Subsequently, the slides were stained with PPD-520 dye (1:100 dilution) for 10 min, washed with TBST, and, if required for additional markers, subjected once more to microwave-mediated antigen retrieval. The final step involved DAPI staining, incubation at room temperature, TBST wash, and mounting with an antifade mounting medium.

Image analysis

For multispectral imaging, stained slides were scanned using the PanoVIEW VS200 slide scanner (Panovue, Beijing, China) with Olympus 20X lens to create composite stack images. Data was imported into OlyVIA software for spectral unmixing of the multispectral scans, isolating single-channel fluorescence signals. The DAPI staining functioned as an essential navigational tool, facilitating the accurate distinction among the nucleus, cytoplasm, and cell membrane of each cell. The expressions of biomarkers such as CK, CD8, GZMA, GSDMB, and IFN- γ were synthesized with the DAPI staining to produce binary masks. These masks were specifically designed to illuminate cells expressing the aforementioned biomarkers. In particular, the CK binary mask was scrutinized to count local tumor cells effectively. For the purpose of tissue identification, Pan-CK was utilized to differentiate stromal nuclei from epithelial tumor tissue areas, thereby enhancing the precision of tissue recognition.

Survival analysis and univariate Cox's model analysis

The fluorescence intensity of each protein across different patients was cut off by `surv_cutpoint` function of R package `survminer`, and patients were divided into two groups according to cut off value. Then, Kaplan-Meier survival analysis and univariate Cox's model analysis were performed using GraphPad Prism 9.5.0 software.

Single-cell RNA sequencing data analysis

scRNA-seq data from primary, treatment-naïve colorectal cancer (GSE178341) [20] were obtained from the Gene Expression Omnibus (GEO) database. The gene expression profiles and sample metadata were integrated using the Seurat package to construct a Seurat object. Log-normalized data was achieved with the `NormalizeData` function, and highly variable genes were identified through the `FindVariableFeatures` function. Data standardization was conducted using the `ScaleData` function, preparing it for principal component analysis (PCA) with the `RunPCA` function. Subsequently, uniform manifold approximation and projection (UMAP) was applied for non-linear dimensionality reduction and clustering, emphasizing the leading 20 principal components (PCs) through the `RunUMAP` function. The clustering analysis on these PCs was conducted using `FindNeighbors` and `FindClusters` functions, leveraging the shared nearest neighbor (SNN) algorithm. Visualization of the results was generated using the graphical tools integrated within Seurat.

Differentially expressed genes and gene sets enrichment analysis

Gene sets were downloaded from the MSigDB database, a comprehensive collection of annotated gene sets available at the website (<https://www.gsea-msigdb.org/gsea/msigdb/>). Subsequently, based on the expression levels of *GZMA* and *IFNG* on CD8⁺TILs, these cells were further stratified into four subgroups: *GZMA*⁻IFN- γ ⁻CD8⁺TILs, *GZMA*⁺IFN- γ ⁻CD8⁺TILs, *GZMA*⁻IFN- γ ⁺CD8⁺TILs, and *GZMA*⁺IFN- γ ⁺CD8⁺TILs. To assess the functional activities of these subgroups, the `AddModuleScore` function was employed to calculate gene set scores for the Hallmark, Reactome, GOBP, and KEGG datasets. The top 20 pathways showing differential activities were then selected and visualized using the `DoHeatmap` function to identify key functional differences.

Additionally, epithelial cells were categorized based on the expression of *GSDMB* into *GSDMB*⁻ and *GSDMB*⁺ populations. The `FindMarkers` function was used to identify differentially expressed genes (DEGs) between these two epithelial cell populations. The `clusterProfiler` package was then utilized to perform Gene Set Enrichment Analysis (GSEA) on these DEGs, encompassing both KEGG and GO enrichment analyses. The top 15 pathways showing enriched pathways were then selected and visualized using the `dotplot` function.

Cell-cell interactions analysis

Gene expression matrices, derived from quality-controlled and standardized single-cell RNA sequencing data, were extracted for CellPhoneDB analysis. The preprocessed data were then subjected to intercellular

interaction analysis through the `cpdb_statistical_analysis_method` function's statistical analysis module. Then, the `ktplots` package was utilized for the visualization of the results. By integrating the interaction scores and statistical metrics generated by CellPhoneDB into `ktplots`, heatmaps and interactive network diagrams were created for visualizations.

Spatial transcriptome sequencing data analysis

Spatial transcriptome (ST) data from CRC patients [21] were obtained from the Zenodo website (<https://zenodo.org/records/7760264>) We employed the `Load10X_Spatial` function to generate Seurat objects from the data. The data were then normalized by the `SCTransform` function with `Log1p` normalization and identification of highly variable genes through a Poisson regression model. To address potential batch effects, we integrated multiple datasets using the `IntegrateData` function. The integrated datasets were then processed using the standard scRNA-seq analysis protocol. We conducted Spearman correlation analysis to investigate the associations among *EPCAM*, *GSDMB*, *CD8A*, *GZMA*, and *IFNG*. For visualizations, we utilized the `SpatialFeaturePlot` function and the `pheatmap` package.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5.0 software. Log-rank survival analysis was used to predict the overall survival (OS) of the patients. Mann-Whitney test was used to analyze the proportion of cells expressing *GZMA*, *GSDMB*, *IFN-γ*, *CK* and *GSDMB* in colon cancer and normal colon tissues. Spearman correlation analysis was used to determine the correlation between different cells in the colon cancer microenvironment. $P < 0.05$ was considered statistically significant. Specific details about the significance tests can be found in the accompanying figure legends.

Results

GSDMB⁺ epithelial cells regulated by *GZMA*⁺*IFN-γ*⁺*CD8*⁺TILs were enhanced in the TME of CRC

To clarify and refine the expression of *GSDMB*, *GZMA*, and *IFN-γ* in CRC and normal colorectal tissues, we analyzed scRNA-seq data of both CRC and normal colorectal samples obtained from the GEO database. According to the official standard protocol, 371,223 cells were mainly categorized into seven clusters: epithelial cells, stromal cells, mast cells, myeloid cells, T cells, B cells, and plasma cells (Fig. 1A). We observed that *GSDMB* was mainly expressed on epithelial cells (Fig. 1B), whereas *GZMA* was predominantly expressed on T cells (Fig. 1C). This differential expression pattern implied that T cells might play a significant role in modulating function of *GSDMB*⁺ epithelial cells by secreting *GZMA*. Comparative analysis

between CRC and normal colorectal tissues revealed a marked increase in *GZMA* within TILs, along with a similar upregulation of *GSDMB* in tumor cells (Fig. 1D). This pattern suggests an enhanced regulatory interaction between *GZMA*⁺TILs and *GSDMB*⁺ epithelial cells within the TME. Considering previous findings that *IFN-γ*, primarily secreted by effector T cells, modulates *GSDMB* expression in epithelial cells, we evaluated the expression levels of *GZMA* and *IFN-γ* among various T cell subsets [22]. Our findings showed that *GZMA* and *IFNG* were notably co-expressed on *CXCL13*⁺*CD8*⁺T cells (Fig. 1E). In addition, we utilized spatial transcriptomic data to explore their spatial distribution (Fig. 1F and G). Our findings indicate that *GZMA* and *IFNG* were co-localized within *CD8*⁺ spots, while *GSDMB* was found within *EPCAM*⁺ spots. Moreover, we observed co-localization of *GZMA* and *IFNG* with *GSDMB*, suggesting that *GZMA*⁺*IFN-γ*⁺*CD8*⁺T cells might co-localize with *GSDMB*⁺ epithelial cells. These data demonstrated that *GSDMB*⁺ epithelial cells, regulated by *GZMA*⁺*IFN-γ*⁺*CD8*⁺T cells, exhibit enhanced activity in the TME of CRC.

Expression and localization of *GSDMB*, *GZMA* and *IFN-γ* in colon cancer and normal colon tissues

To spatially confirm the co-expression of *GZMA* and *IFN-γ* in *CD8*⁺T cells and the expression of *GSDMB* in epithelial cells, we performed mIHC staining on both colon cancer and normal colon TMA (Fig. 2A: *CK* (green), *CD8* (indigo), *GZMA* (yellow), *GSDMB* (purple) and *IFN-γ* (red)). mIHC staining results revealed that the elevated frequencies of *CK*⁺ and *GSDMB*⁺ cells were detected in colon cancer tissues relative to normal colon tissues (Fig. 2B and C, $P < 0.001$). In contrast, the expression of *GZMA* and *IFN-γ* in normal colon tissues were significantly upregulated compared to those in colon cancer tissues (Fig. 2D and E, $P < 0.001$). Additionally, the proportion of *CD8*⁺TILs was also higher in normal colon tissues than that in colon cancer tissues (Fig. 2F, $P < 0.01$).

Cytotoxic *CD8*⁺TILs, as the primary cell type responsible for combating tumors, can serve as predictors of clinical outcomes in CRC patients. To confirm the prognostic significance of *CD8*⁺TILs in colon cancer, we assessed the clinical outcome values associated with *CD8*⁺TILs, *GZMA*⁺ cells and *IFN-γ*⁺ cells. Survival analysis results indicated that a higher proportion of *CD8*⁺TILs in colon cancer was associated with a better prognosis of the patients (Fig. 2G, $HR = 0.381$, 95% *CI*: 0.184–0.792, $P = 0.018$). Similarly, higher frequency of *GZMA*⁺TILs was significantly associated with better clinical outcomes (Fig. 2H, $HR = 0.439$, 95% *CI*: 0.212–0.909, $P = 0.028$). However, we found no significant association between the frequency of *IFN-γ*⁺ cells in colon cancer tissues and OS (Fig. 2I, $HR = 0.546$, 95% *CI*: 0.257–1.161, $P = 0.136$).

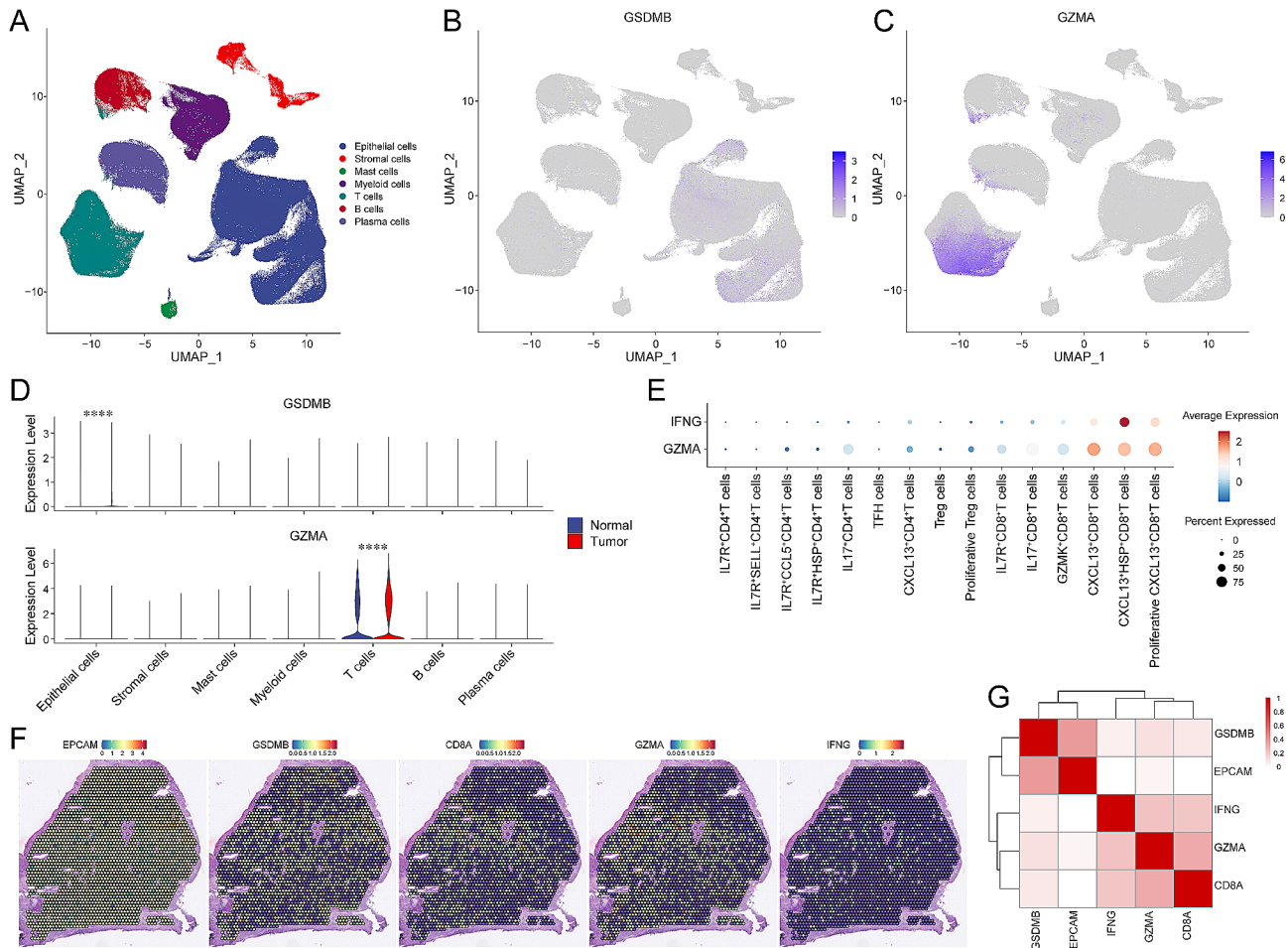


Fig. 1 GSDMB⁺ epithelial cells regulated by GZMA⁺IFN- γ ⁺CD8⁺TILs were enhanced in TME of CRC. **(A)** UMAP displaying the seven major clusters in normal and CRC tissues: epithelial cells, stromal cells, mast cells, myeloid cells, T cells, B cells, and plasma cells. **(B-C)** UMAP showing the expression of *GSDMB* (in **B**) and *GZMA* (in **C**). **(D)** Violin plots compared the expressions of *GSDMB* and *GZMA* across the seven major clusters between normal and CRC tissues. **(E)** Dot plot displaying the expressions of *GZMA* and *IFNG* on CD8⁺TILs. **(F)** SpatialFeaturePlots showing the expressions of *EPCAM*, *GSDMB*, *CD8A*, *GZMA*, and *IFNG*. **(G)** Heat map displaying Spearman correlation coefficients across the expressions of *EPCAM*, *GSDMB*, *CD8A*, *GZMA*, and *IFNG*.

These data suggest that CD8⁺TILs expressing GZMA could be serve as an independent prognosis predictor in colon cancer patients.

mIHC staining of CD8⁺TILs expressing GZMA and/or IFN- γ in human colon cancer tissues

Since GZMA⁺IFN- γ ⁺CD8⁺T cells may regulate GSDMB expression on epithelial cells in the TME, Fig. 3A specifically focused on the co-expression of GZMA and IFN- γ in CD8⁺TILs within colon cancer TMA sections. Then, the prognostic significance of GZMA⁺IFN- γ ⁺CD8⁺TILs in human colon cancer tissues was assessed. In comparison to colon cancer tissues, normal colon tissues exhibited a higher frequency of GZMA⁺CD8⁺TILs (Fig. 3B, $P < 0.001$). Likewise, the proportion of IFN- γ ⁺CD8⁺TILs and GZMA⁺IFN- γ ⁺CD8⁺TILs were significantly elevated in normal colon tissues than those in colon cancer tissues (Fig. 3C and D, $P < 0.01$). In addition, the proportions of

GZMA⁺IFN- γ ⁺CD8⁺TILs among CD8⁺TILs or among GZMA⁺CD8⁺TILs were higher in normal colon tissues than those in colon cancer tissues (Fig. 3E and F, $P < 0.01$).

The proportion of CD8⁺TILs co-expressing GZMA and IFN- γ in scRNA-seq data

Expression of IFN- γ in CD8⁺TILs showed no significant correlation with OS, but its expression on GZMA⁺CD8⁺TILs was associated with a better prognosis. Consequently, we further explored the relationship between IFN- γ ⁺ cells and GZMA⁺ cells, as well as between IFN- γ ⁺CD8⁺TILs and GZMA⁺CD8⁺TILs. Spearman correlation analysis revealed a significant positive correlation between IFN- γ ⁺ cells and GZMA⁺ cells (Fig. 4A, $r = 0.656$, $P < 0.001$). Similarly, a positive correlation was observed between the proportions of IFN- γ ⁺CD8⁺TILs and GZMA⁺CD8⁺TILs (Fig. 4B, $r = 0.673$, $P < 0.001$). Leveraging the expression profiles of IFN- γ

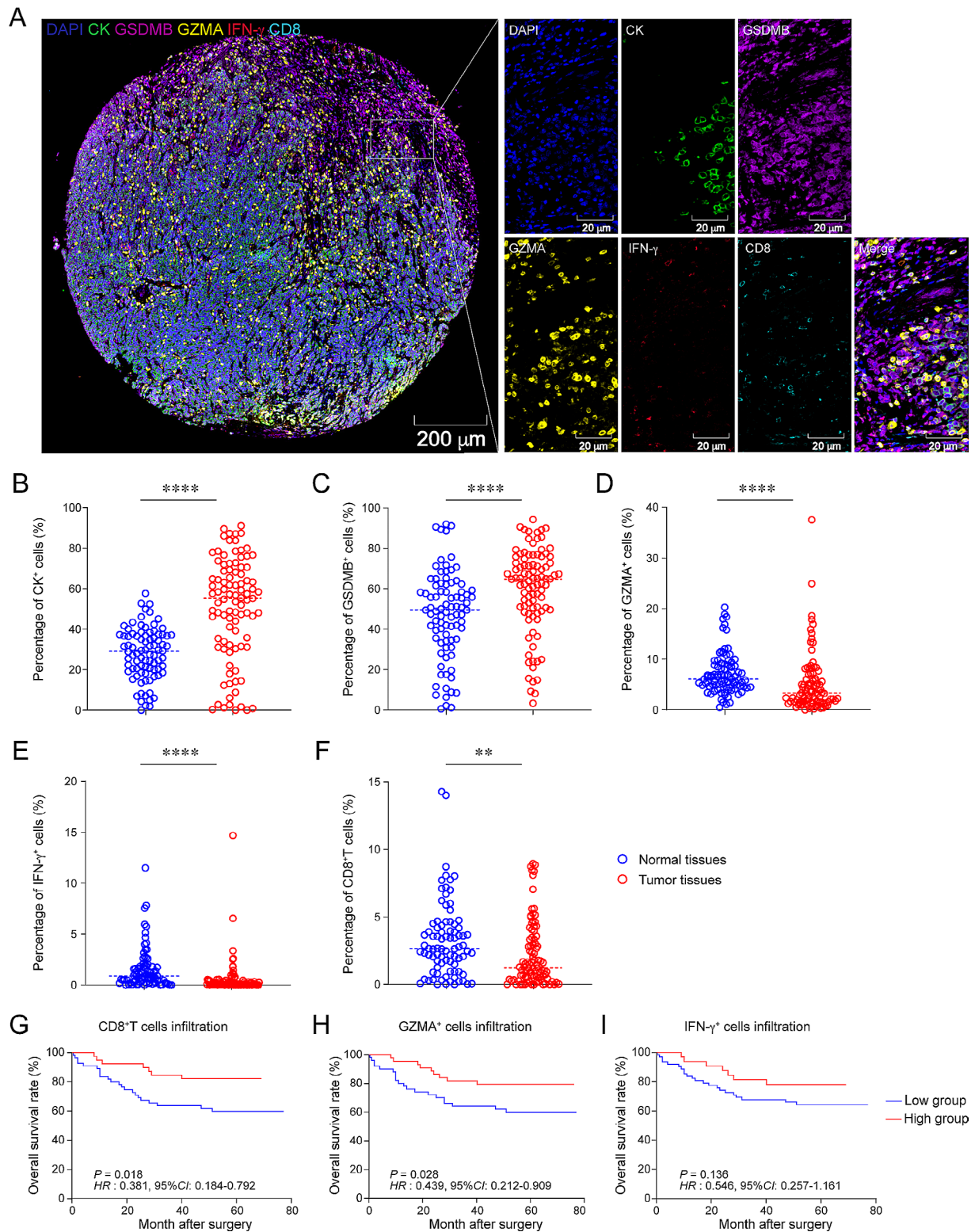


Fig. 2 Expression and localization of GSDMB, GZMA and IFN- γ in colon cancer and normal colon tissues. **(A)** mIHC and single-color images were stained in the colon cancer TMA. CK: green, CD8: indigo, GZMA: yellow, GSDMB: purple, IFN- γ : red. **(B-F)** Bar plots compared the proportions of cells between normal and tumor tissues. GZMA⁺ cells in **(B)**, IFN- γ ⁺ cells in **(C)**, GSDMB⁺ cells in **(D)**, CK⁺ cells in **(E)** and CD8⁺T cells in **(F)**. **(G-I)** Kaplan-Meier test analyzed the proportions of CD8⁺T cells **(G)**, GZMA⁺ cells **(H)**, IFN- γ ⁺ cells **(I)** in colon cancer patients. The *P* values in **(G-I)** were calculated by using the Kaplan-Meier test, whereas *HR* and 95%*CI* using univariate Cox's regression. Statistical analyses in **(B-F)** were performed using an unpaired, two-tailed Student's *t*-test. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001

A

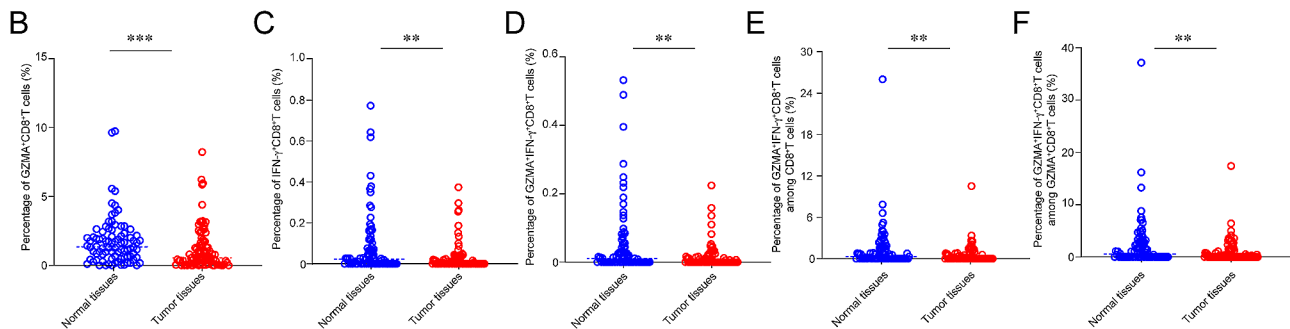
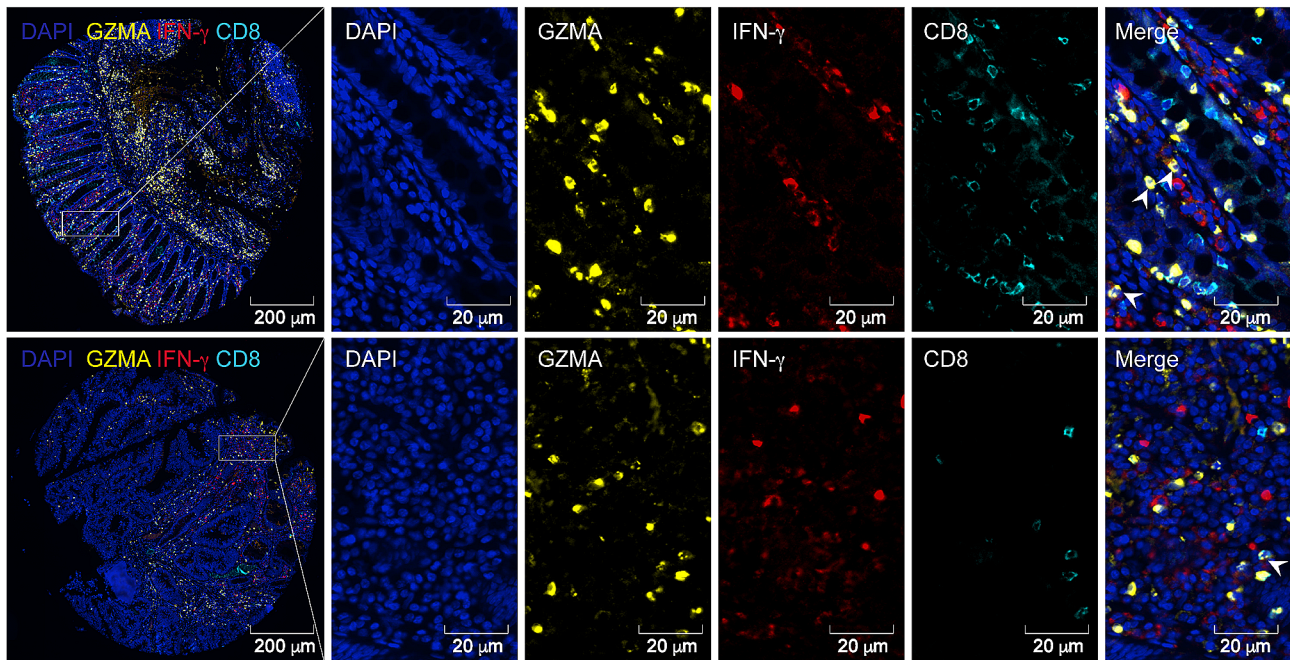


Fig. 3 mIHC staining of CD8⁺TILs expressing GZMA and/or IFN- γ in human colon cancer tissues. **(A)** mIHC and single-color images were stained for GZMA⁺IFN- γ ⁺CD8⁺T cells in the colon cancer TMA, and the arrows correspond to the GZMA⁺IFN- γ ⁺CD8⁺T cells. CD8: indigo, GZMA: yellow, IFN- γ : red. **(B–D)** Bar plots compared the proportions of cells between normal and tumor tissues. GZMA⁺CD8⁺T cells in **(B)**, IFN- γ ⁺CD8⁺T cells in **(C)**, GZMA⁺IFN- γ ⁺CD8⁺T cells in **(D)**. **(E and F)** Bar plots compared the proportions of cells between normal and tumor tissues. The ratio of GZMA⁺IFN- γ ⁺CD8⁺T cells among CD8⁺T cells in **(E)**, and the ratio of GZMA⁺IFN- γ ⁺CD8⁺T cells among GZMA⁺CD8⁺T cells in **(F)** between normal colon tissues and colon cancer tissues. Statistical analyses in **(B–F)** were performed using an unpaired, two-tailed Student's *t*-test. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001

and GZMA, we stratified CD8⁺TILs from scRNA-seq data into four categories: GZMA⁻IFN- γ ⁻, GZMA⁺IFN- γ ⁻, GZMA⁻IFN- γ ⁺ and GZMA⁺IFN- γ ⁺ (Fig. 4C). Statistical analyses indicated a high frequency and proportion of GZMA⁺IFN- γ ⁺CD8⁺TILs within the total CD8⁺TILs population (Fig. 4D and E).

The function of GZMA⁺IFN- γ ⁺CD8⁺TILs in TME

To further investigate the functional characteristics of GZMA⁺IFN- γ ⁺CD8⁺TILs, we conducted GSEA on the total population of CD8⁺TILs in CRC. We sourced gene sets from public databases such as Reactome, Hallmark, KEGG, and GOBP, and computed enrichment scores at the single-cell level. Compared to GZMA⁻IFN- γ ⁺CD8⁺TILs, our study identified a

pronounced augmentation in the IFN- γ signaling pathway within GZMA⁺IFN- γ ⁺CD8⁺TILs, signifying their crucial role in mediating IFN- γ -dependent immune responses (Fig. 5A and D). We also observed upregulation of molecules associated with major histocompatibility complex class II (MHC-II), implying an enhanced capability in antigen presentation and immune surveillance (Fig. 5A, C and D). Furthermore, an increase in the expression of cell cycle-related molecules was detected, possibly reflecting the proliferative dynamics of TILs within the TME (Fig. 5A and C). Metabolic pathways, including glycolysis, oxidative phosphorylation, and fatty acid metabolism, were notably enriched, indicating increased metabolic and energetic adaptations of these cells to the tumor environment (Fig. 5B and C).

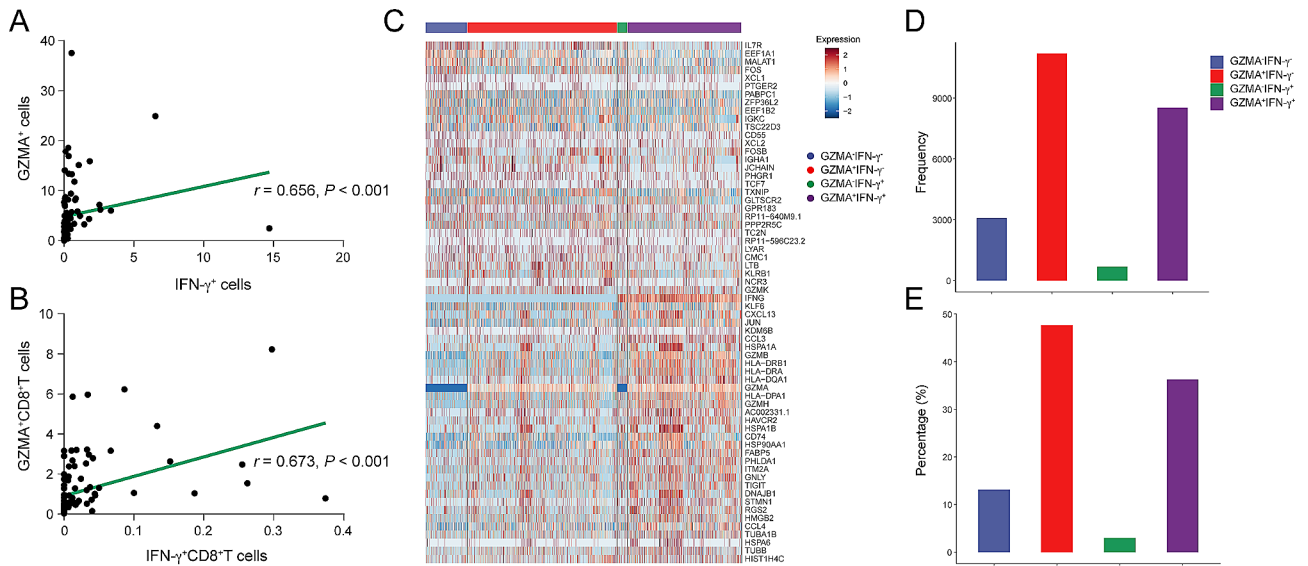


Fig. 4 The proportion of CD8⁺TILs co-expressing GZMA and IFN- γ in scRNA-seq data. **(A)** The Spearman correlation for GZMA⁺ cells and IFN- γ ⁺ cells. **(B)** The Spearman correlation for GZMA⁺CD8⁺T cells and IFN- γ ⁺CD8⁺T cells. **(C)** Heat map displaying the marker genes of each cluster in CD8⁺TILs, which were categorized by the expression profiles of IFN- γ and GZMA. **(D-E)** Bar plots showing the frequencies **(D)** and percentages **(E)** of CD8⁺TIL subsets in **(C)**

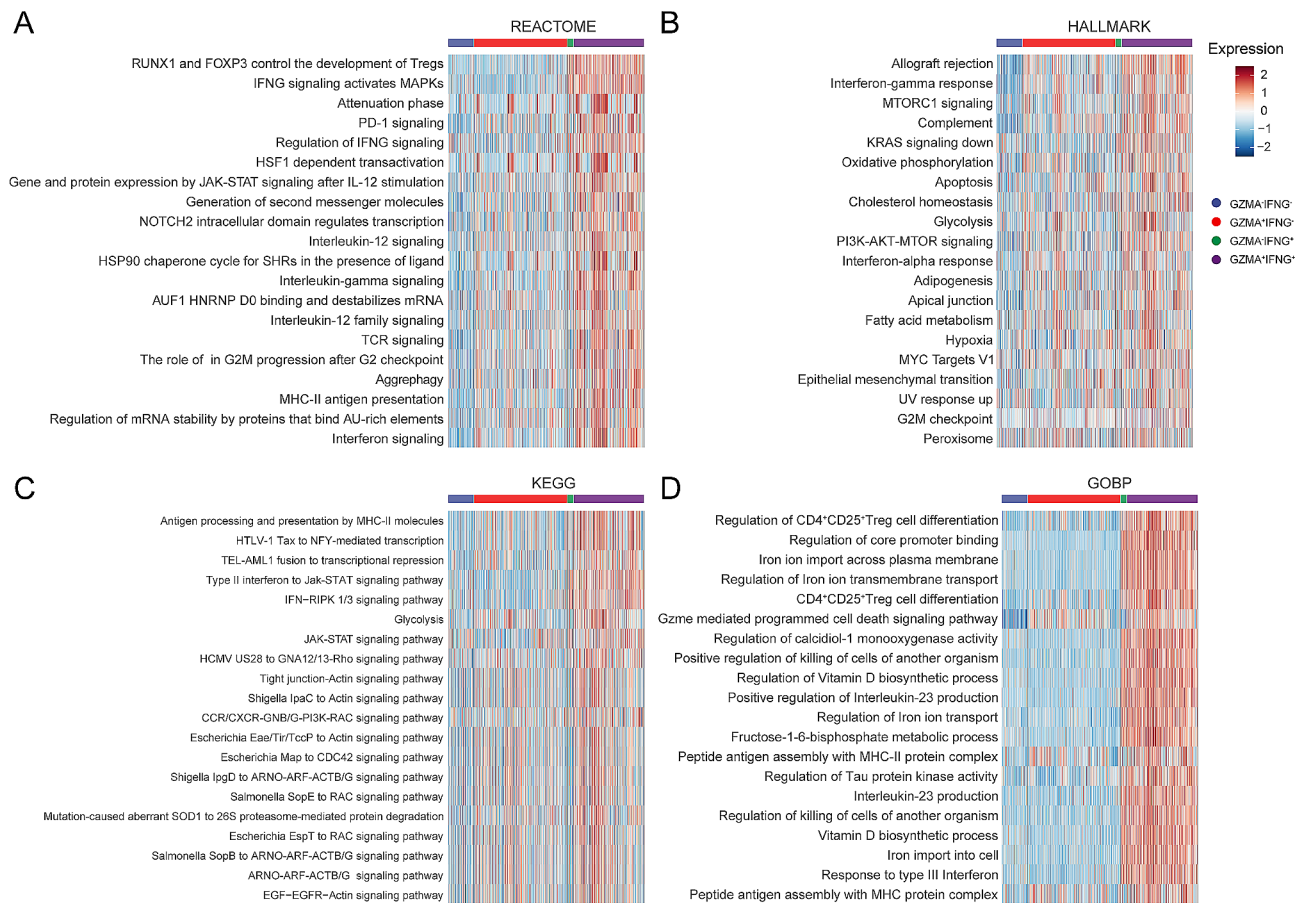


Fig. 5 The function of GZMA⁺IFN- γ ⁺CD8⁺TILs in TME. **(A-D)** Heat maps displaying the top 20 pathways of GSEA results. Reactome **(A)**, Hallmark **(B)**, KEGG **(C)**, and GOBP **(D)**

Through Reactome pathway analysis, we found significant enrichment in IL-12 signaling, TCR signaling, and PD-1 signaling pathways, highlighting their integral role in modulating GZMA⁺IFN- γ ⁺CD8⁺TILs functionality (Fig. 5A). Particularly, the enrichment in IL-12 and PD-1 signaling pathways may be pivotal in modulating the immune responsiveness and evasion from the immunosurveillance [23, 24]. In the analysis of the Hallmark pathway, we noted significant enrichment of the PI3K-AKT-mTOR axis in TCR downstream signaling, suggesting active metabolic regulation in signal transduction and reinforcing their dynamic involvement in the TME [25, 26] (Fig. 5B). Altogether, our comprehensive analyses shed light on the intricate functional roles and regulatory mechanisms of GZMA⁺IFN- γ ⁺CD8⁺TILs in the tumor microenvironment, underscoring their potential impact on anti-tumor responses.

Prognostic value of GSDMB⁺CK⁺ cells in human colon cancer

To verify the impact of epithelial cell expressed GSDMB on patients with colon cancer, we calculated the proportion of GSDMB⁺CK⁺ cells and evaluated the prognostic value of GSDMB⁺CK⁺ cells in human colon cancer tissues (Fig. 6A). We found that higher frequency of GSDMB⁺CK⁺ cells was observed in colon cancer tissues compared with normal colon tissues (Fig. 6B, $P < 0.001$). However, we found no significant association between the frequency of GSDMB⁺CK⁺ cells in colon cancer tissues and overall survival (OS) ($HR = 0.517$, 95% CI : 0.233–1.149, $P = 0.056$, Fig. 6C).

To elucidate the functional differences between GSDMB⁻CK⁺ and GSDMB⁺CK⁺ cells, we analyzed their differential gene expression profiles and conducted GO and KEGG enrichment analyses (Fig. 6D and E). The results indicated significant enrichment of genes associated with antigen processing and presentation, as well as cell-cell adhesion, in GSDMB⁺CK⁺ cells. These findings suggested that GSDMB⁺CK⁺ cells may play a pivotal role in the pathogenesis of colon cancer, particularly in the immunoregulation of the tumor microenvironment.

Correlation among IFN- γ ⁺CD8⁺TILs, GZMA⁺CD8⁺TILs and GSDMB⁺CK⁺ cells in the TME of colon cancer

To further investigate the associations among IFN- γ ⁺ cells, GZMA⁺ cells and GSDMB⁺ cells, we conducted correlation analysis. Our results revealed no significant correlation between the infiltration levels of GZMA⁺ cells and that of GSDMB⁺ cells ($r = 0.067$, $P = 0.520$, Fig. 7A). Conversely, a notable positive correlation was observed between the counts of IFN- γ ⁺ cells and GSDMB⁺ cells ($r = 0.257$, $P = 0.013$, Fig. 7B), as well as between GZMA⁺IFN- γ ⁺ cells and GSDMB⁺ cells ($r = 0.267$, $P = 0.009$, Fig. 7C). Moreover, our analysis

showed that neither the proportion of GZMA⁺CD8⁺TILs nor IFN- γ ⁺CD8⁺TILs correlated significantly with the ratio of GSDMB⁺CK⁺ cells ($r = 0.170$, $P = 0.102$, Fig. 7D; $r = 0.175$, $P = 0.092$, Fig. 7E). However, a positive correlation was detected between the number of GZMA⁺IFN- γ ⁺CD8⁺TILs and GSDMB⁺CK⁺ cells within the colon cancer microenvironment ($r = 0.221$, $P = 0.033$, Fig. 7F). Considering the impact of these factors on colon cancer patients' survival, it suggested that the signaling pathways involving these markers may play crucial roles in enhancing long-term prognosis for colon cancer patients.

To clarify the intricate interplays between GZMA⁺IFN- γ ⁺CD8⁺TILs and GSDMB⁺CK⁺ cells, we utilized the cellphoneDB package, a tool that delineates the interaction networks in cellular communication [27]. Our analysis highlighted a pronounced interaction between GZMA⁺IFN- γ ⁺CD8⁺TILs and GSDMB⁺CK⁺ cells, exceeding that in other CD8⁺T cell subsets (Fig. 7G). Notably, GZMA⁺IFN- γ ⁺CD8⁺TILs emerged as key regulators of GSDMB⁺CK⁺ cells, with IFN- γ and TGF- β acting as essential mediators in this intercellular dialogue. Additionally, a stronger interaction was observed between FasL produced by GZMA⁺IFN- γ ⁺CD8⁺TILs and the Fas receptor on GSDMB⁺CK⁺ cells, enhancing their interconnection. The expression of ICOSL on GSDMB⁺CK⁺ cells also appeared to increase ICOS activation on GZMA⁺IFN- γ ⁺CD8⁺TILs, indicating a more vigorous immunologic synergy [28]. Importantly, the immunosuppressive capacity of GSDMB⁺CK⁺ cells on GZMA⁺IFN- γ ⁺CD8⁺TILs was amplified, especially through interactions with TIGIT and TIM-3 (*HAVCR2*), underscoring these pathways as pivotal in immune evasion mechanisms [29–31] (Fig. 7H). These detailed insights not only enrich our comprehension of the bidirectional interactions between GZMA⁺IFN- γ ⁺CD8⁺TILs and GSDMB⁺CK⁺ cells but also provide a strong theoretical basis for developing targeted immunotherapies against these cell interactions.

Discussion

Research findings demonstrated that GZMA released by CD8⁺TILs can effectively cleave GSDMB within tumor epithelial cells, culminating pyroptosis [12]. Moreover, IFN- γ produced by CD8⁺TILs can bolster GSDMB expression, further intensifying this cascade [12]. Our findings revealed a pronounced expression of GSDMB in colon cancer cells, with GZMA and IFN- γ frequently co-expressed within CD8⁺TILs. Spatial transcriptomics and mIHC analysis revealed that CD8⁺TILs co-expressing GZMA and IFN- γ may localize near GSDMB-expressing tumor cells, suggesting potential interactions between these cell types. Cell-cell interactions analysis results revealed that GZMA⁺IFN- γ ⁺CD8⁺TILs form stronger interactions with GSDMB⁺CK⁺ cells compared to other

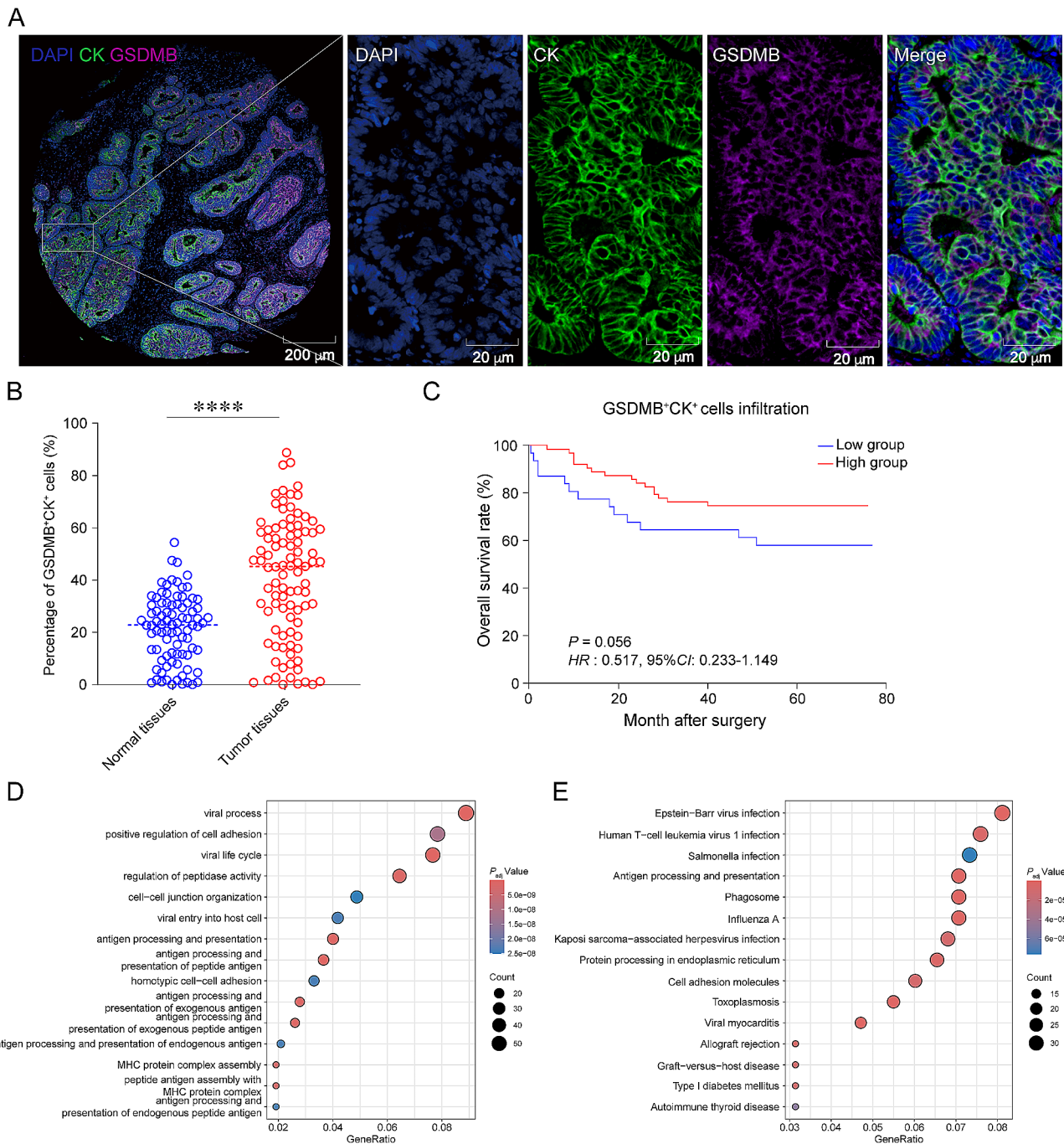


Fig. 6 Prognostic value of GSDMB⁺CK⁺ cells in human colon cancer. **(A)** mIHC and single-color images were stained in the colon cancer TMA, and the arrows correspond to the GSDMB⁺CK⁺ cells. CK: green, GSDMB: purple. **(B)** Comparison of the proportions of GSDMB⁺CK⁺ cells between normal colon tissues and colon cancer tissues. **(C)** Kaplan-Meier test analyzed the proportions of GSDMB⁺CK⁺ cells in colon cancer patients. **(D)** and **(E)** Dot plots showing the top 15 pathways of GO and KEGG results. The GO results in **(D)**, and KEGG results in **(E)**. The P value in **(C)** was calculated by using the Kaplan-Meier test, whereas HR and 95%CI using univariate Cox's regression. Statistical analysis in **(B)** was performed using an unpaired, two-tailed Student's t -test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$

CD8⁺T cell subsets, facilitated by IFN- γ and TGF- β , the co-stimulatory molecule ICOS, and the immune checkpoint molecules TIGIT and TIM-3. These interactions are further enhanced by the engagement of FasL on GZMA⁺IFN- γ ⁺CD8⁺TILs with the Fas receptor

on GSDMB⁺CK⁺ cells. Notably, the immunosuppressive impact of GSDMB⁺CK⁺ cells on GZMA⁺IFN- γ ⁺CD8⁺TILs can be augmented through their interactions with TIGIT and TIM-3 (*HAVCR2*),

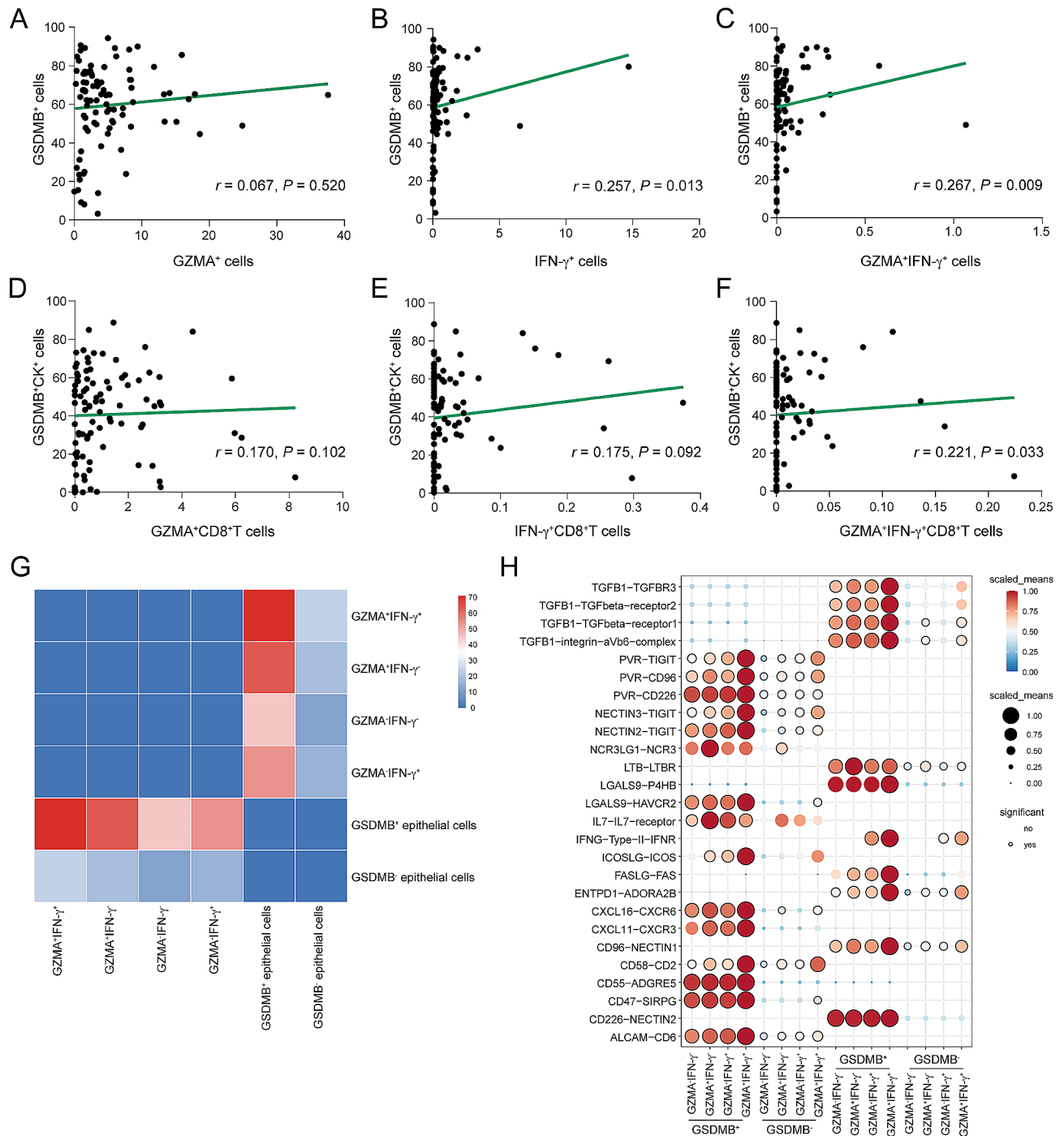


Fig. 7 Correlation among IFN- γ ⁺CD8⁺TILs, GZMA⁺CD8⁺TILs and GSDMB⁺CK⁺ cells in the TME of colon cancer. **(A-C)** The Spearman correlations for GZMA⁺ cells and GSDMB⁺ cells in **(A)**, IFN- γ ⁺ cells and GSDMB⁺ cells in **(B)**, GZMA⁺IFN- γ ⁺ cells and GSDMB⁺ cells in **(C)**. **(D-F)** The Spearman correlations for GZMA⁺CD8⁺T cells and GSDMB⁺CK⁺ cells in **(D)**, IFN- γ ⁺CD8⁺T cells and GSDMB⁺CK⁺ cells in **(E)**, GZMA⁺IFN- γ ⁺CD8⁺T cells and GSDMB⁺CK⁺ cells in **(F)**. **(G)** Heat map displaying the interactions among epithelial subsets and CD8⁺TIL subsets. **(H)** Dot plot showing the strength of ligand-receptor pairs among epithelial subsets and CD8⁺TIL subsets

highlighting the critical roles of these pathways in immune evasion mechanisms [31–33] (Fig. 8).

Notably, GZMA⁺IFN- γ ⁺CD8⁺TILs subset demonstrated enhanced activation and effector functions compared to other CD8⁺TIL populations. However,

the proportion of GZMA⁺IFN- γ ⁺CD8⁺TILs was significantly reduced in colon cancer patients, likely due to immune escape [32]. Additionally, inhibitory cytokines secreted by tumor cells, regulatory T cells (Tregs), or myeloid-derived suppressor cells (MDSCs) within the

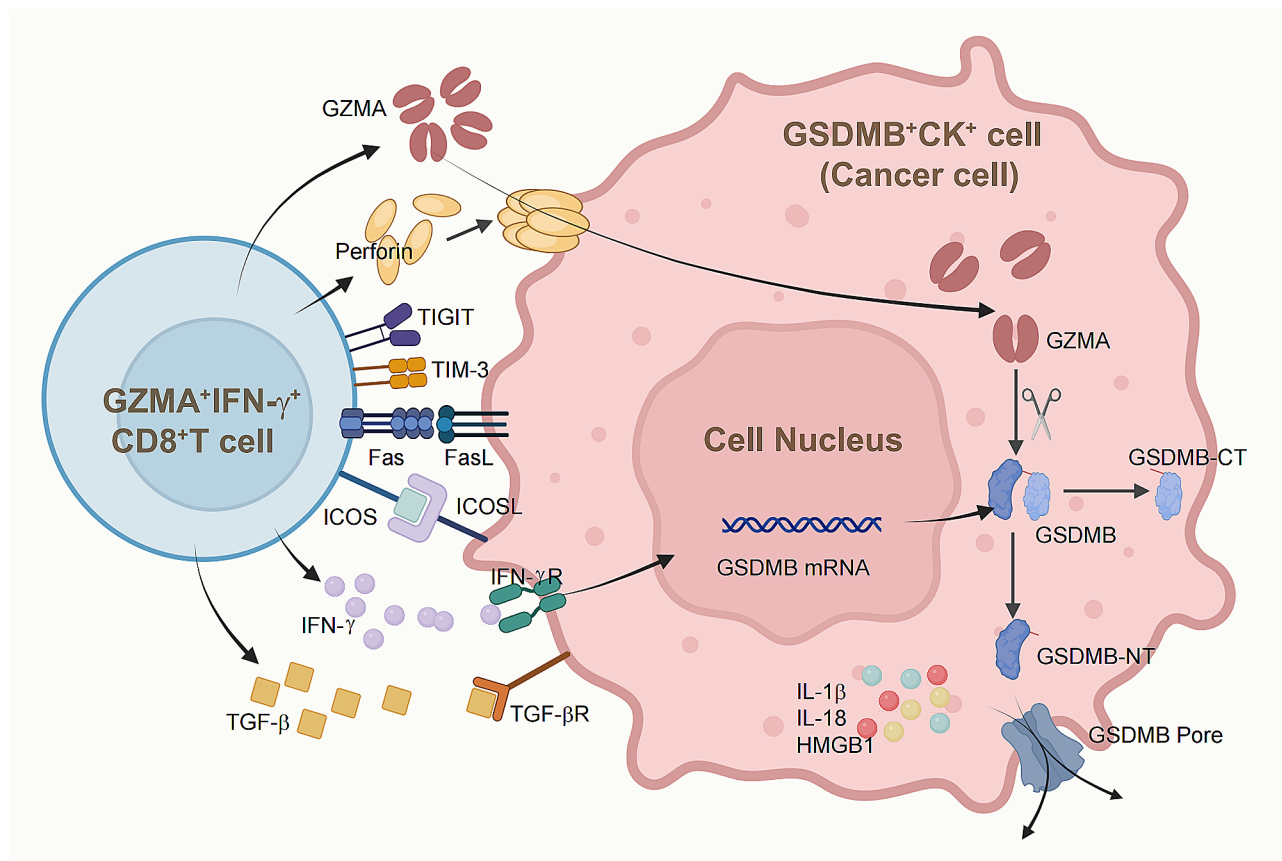


Fig. 8 Diagram of the interactions between GZMA⁺IFN- γ ⁺CD8⁺TILs and GSDMB⁺CK⁺ cells in the TME of colon cancer. Created with BioRender.com

TME further suppress the proliferation and functionality of GZMA⁺IFN- γ ⁺CD8⁺TILs [14]. Persistent tumor antigens in the TME can also lead to T cell exhaustion, characterized by diminished functionality, reduced proliferative capacity, and high inhibitory receptor expression [34].

The IFN- γ signaling pathway was significantly enhanced in GZMA⁺IFN- γ ⁺CD8⁺TILs, highlighting their crucial function in mediating IFN- γ -dependent immune responses. Additionally, the elevation of molecules associated with the MHC-II suggested that GZMA⁺IFN- γ ⁺CD8⁺TILs enhance antigen presentation, thereby strengthening immune surveillance capabilities. Upon stimulation by antigenic peptide-loaded MHC-II molecules, activated CD4⁺TILs secrete cytokines such as IL-2, which promote the proliferation and differentiation of CD8⁺TILs [35]. Additionally, activated CD8⁺TILs can also express MHC-II molecules, allowing them to present antigens [36]. By regulating the activation, proliferation, and apoptosis of CD8⁺TILs, MHC-II plays a critical role in maintaining immune homeostasis and preventing autoimmune responses [35].

Metabolic pathways such as glycolysis, oxidative phosphorylation, and fatty acid metabolism were notably more active in GZMA⁺IFN- γ ⁺CD8⁺TILs. This activity

underscores the cells' heightened adaptability to the metabolic and energetic demands of the TME. The functionality of GZMA⁺IFN- γ ⁺CD8⁺TILs was significantly influenced by the IL-12 signaling pathway, TCR signaling pathway, and PD-1 signaling pathway. The IL-12 and PD-1 pathways, in particular, are thought to play key roles in modulating immune responses and evasion from the immunosurveillance [23, 24]. Consequently, we suggest boosting IL-12 expression as a strategy to amplify the infiltration ratio of GZMA⁺IFN- γ ⁺CD8⁺TILs, potentially elevating the sensitivity of colon cancer cells to both immunotherapy and chemotherapy, thus intensifying antitumor efficacy. These insights furnish novel perspectives on the mechanisms by which GZMA⁺IFN- γ ⁺CD8⁺TILs modulate the TME of colon cancer, providing innovative angles for therapeutic intervention.

Although no statistical correlation was observed between the infiltration ratio of GSDMB⁺CK⁺ cells and patients' prognosis in colon cancer, these cells exhibit enhanced immunogenic properties, contributing to antigen processing, presentation, and intercellular adhesion. This underscores the multifaceted role of GSDMB⁺CK⁺ cells in the pathogenesis of colon cancer. Correlation analyses identified a positive association with GZMA⁺IFN- γ ⁺CD8⁺TILs, suggesting intricate

cellular interactions. GSDMB-mediated pyroptosis releases various intracellular inflammatory factors, such as IL-1 β and IL-18, thereby enhancing the local inflammatory response and activating immune cells, including GZMA⁺IFN- γ ⁺CD8⁺TILs [14]. This activation further stimulates these CD8⁺TILs to secrete increased amounts of GZMA and IFN- γ , thereby enhancing their cytotoxic effects on tumor cells and forming a positive feedback loop [14]. Tumor cells express inhibitory molecules which interact with receptors on these TILs, like TIM-3, and TIGIT, inhibiting their activation and function. Therefore, antagonizing the synergistic effect of TIGIT and TIM-3 offers a potent strategy to enhance the activity and infiltration of GZMA⁺IFN- γ ⁺CD8⁺TILs. This approach can significantly boost their anti-tumor immune response, potentially surpassing the efficacy of PD-1 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) monoclonal antibody therapies. These findings demonstrate the significant influence of GZMA⁺IFN- γ ⁺CD8⁺TILs on the immune microenvironment in colon cancer, which in turn affects patient prognosis by modulating tumor cells expressing GSDMB.

Our research sheds light on the clinical implications and functional roles of CD8⁺T cells expressing GZMA and IFN- γ , as well as colon cancer cells expressing GSDMB. It delved into their intricate mechanisms of interaction, laying the groundwork for novel pathways in prognostic evaluation, therapeutic efficacy monitoring, overcoming resistance to immunotherapy, and crafting immunotherapeutic strategies for colon cancer patients. However, despite selecting an effective IFN- γ antibody for staining, we noted suboptimal immunohistochemical staining of IFN- γ , resulting in an undetectable level of GZMA⁺IFN- γ ⁺CD8⁺TILs in many colon cancer samples. This observation introduces a potential statistical bias in assessing the impact of these TILs on patient prognosis. Consequently, we refrained from conducting survival analysis based on this parameter. Future studies should increase the sample size to investigate the correlation between the infiltration of GZMA⁺IFN- γ ⁺CD8⁺TILs and the prognosis of colon cancer patients, to determine whether this ratio can serve as a reliable biomarker for prognosis assessment and treatment efficacy monitoring in colon cancer. Moreover, it is imperative for future studies to unravel the precise mechanisms by which GZMA⁺IFN- γ ⁺CD8⁺TILs target GSDMB-expressing colon cancer cells and facilitating pyroptosis. Additionally, it's crucial to examine the effects of pyroptosis on the diverse immune cells, inflammatory mediators, and signaling cascades within the TME of colon cancer. Future endeavors should also emphasize the integration of GSDMB-targeted therapies with chemotherapy or other immunotherapeutic modalities, striving to augment treatment efficacy.

Abbreviations

CK	Cytokeratin
CRC	Colorectal cancer
CRS	Cytokine release syndrome
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
DEGs	Differentially expressed genes
dMMR	Mismatch-repair deficiency
GEO	Gene Expression Omnibus
GSDMB	Gasdermin B
GSEA	Gene Set Enrichment Analysis
GZMA	Granzyme A
GZMs	Granzymes
HRP	Horseradish peroxidase
ICIs	Immune checkpoint inhibitors
IFN- γ	Interferon- γ
MDSCs	Myeloid-derived suppressor cells
MHC-II	Major histocompatibility complex class II
mIHC	Multi-color immunohistochemistry
MSI-H	High microsatellite instability
MSI-L	Low microsatellite instability
MSS	Microsatellites stable
NK cells	Natural killer cells
OS	Overall survival
PCA	Principal component analysis
PCs	Principal components
PD-1	Programmed cell death-1
pMMR	Proficient mismatch-repair
scRNA-seq	Single-cell RNA sequencing
SNN	Shared nearest neighbor
ST	Spatial transcriptome
TCR	T-cell receptors
TGF- β	Transforming growth factor- β
TILs	Tumor-infiltrating lymphocytes
TMA	Tissue microarray
TME	Tumor microenvironment
TNF- α	Tumor necrosis factor- α
Tregs	Regulatory T cells
UMAP	Uniform manifold approximation and projection
VEGFA	Vascular endothelial growth factor A

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Author contributions

(I) Conception and design: L Chen, X Zheng and J Jiang. (II) Administrative support: J Jiang. (III) Provision of study materials or patients: J Yang, X Ding, Z Fang, S Wu, Q Xu, M Yuan, H Wu; (IV) Collection and assembly of data: J Yang, X Ding, Z Fang, R Chen and X Gao; (V) Data analysis and interpretation: X Ding, J Yang, Z Fang, S Wu, Q Xu, R Chen and X Gao; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The studies involving humans were approved by the Clinical Research Ethics Committee at Outdo Biotech (Shanghai, China, SHYJS-CP-1910009). The studies were conducted in accordance with the local legislation and

institutional requirements. The participants provided their written informed consent to participate in this study.

Consent for publication

All authors consent to publication.

Competing interests

The authors declare no competing interests.

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