Tertiary lymphoid structures: Associated multiple immune cells and analysis their formation in hepatocellular carcinoma

Ye Nie | Hanlu Fan | Jianhui Li | Xinjun Lei | Tianchen Zhang | Yanfang Wang | Zhenzhen Mao | Kaishan Tao | Wenjie Song

Abstract

The prognostic value of immune cells in tertiary lymphoid structures (TLSs) remains unclear in hepatocellular carcinoma (HCC). Here, 59 of 145 patients had TLSs in training set, 48 of 120 patients had TLSs in testing set. Immunohistochemistry (IHC) were used to label CD3+ T cells, CD20+ B cells, CD8+ T cells, CD208+ dendritic cells, and CD21+ follicular dendritic cells in TLSs. High CD3+, CD20+, CD208+, and CD8+ cell densities were favorable prognostic factors for overall survival (OS). High CD3+, CD20+, CD208+, and CD8+ cell densities were significantly associated with reduced early recurrence. TLSs were divided into three grades (A, B, and C) based on immune cell density. Patients with grade C or B had significantly improved OS. Patients with grade C had the lowest recurrence rate, followed by those with grade B, while patients with grade A had the highest recurrence rate. The stromal, immune, and ESTIMATE scores derived from the ESTIMATE package were significantly higher and tumor purity was significantly lower in patients with TLSs. Patients with TLSs had significantly higher relative numbers of memory B cells, plasma cells, CD8+ T cells, NK cells, and dendritic cells and lower relative numbers of Treg cells, macrophages, and M2 macrophages according to the CIBERSORT assessment. Bioinformatics analysis and experiments confirmed that KLRK1 and GZMA expression are associated TLSs formation and can predict TLSs existence. Grade B and grade C were favorable prognostic factors for OS and recurrence and could represent immune-active tumors.

Keywords

hepatocellular carcinoma, immune cell, prognosis, tertiary lymphoid structure, tertiary lymphoid structure formation

Abbreviations: GEO, Gene Expression Omnibus; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; IF, immunofluorescence; IHC, immunohistochemistry; OS, overall survival; qRT-PCR, quantitative real-time polymerase chain reaction; ssGSEA, single-sample gene set enrichment analysis; TILs, tumor-infiltrating lymphocytes; TLSs, tertiary lymphoid structures; PPI, protein–protein interaction.
1 | INTRODUCTION

Primary liver cancer is the sixth most common tumor worldwide and the third leading cause of cancer-related death.1 The majority of cases occur in patients with chronic liver disease, and the major risk factors include hepatitis B virus and hepatitis C virus infection, fatty liver, obesity, diabetes, and exposure to aflatoxin.2 HCC accounts for approximately 90% of primary liver cancers.3 At present, the main treatment methods for HCC include surgical resection, transarterial chemotherapy and embolization, radiotherapy, chemotherapy, targeted therapy, and liver transplantation. Even so, the 5-year OS for HCC patients is less than 12.5%.4 Recently, the successful application of immune checkpoint inhibitors in clinical treatment has made it possible to overcome this low OS.5 However, the objective response rate of immunotherapy in the treatment of HCC is only 22.7%.6 Therefore, factors conducive to improving the objective response rate have recently been an active topic of research.

Many studies have pointed out that TLSs are associated with improved objective response rate and are a potential marker for immunotherapy.7–9 Its local microenvironment can induce T cell initiation, prevent depletion or exhaustion, and support efficient antigen presentation and lymphocyte activation.10 TLSs refer to the aggregation of lymphocytes (including B cells, T cells, dendritic cells, follicular dendritic cells, and macrophages) in nonlymphoid tissues, and these TLSs are similar to secondary lymphoid tissue in structure, lacking a capsule and having the ability to directly contact cancer cells. TLSs are common in tumors and autoimmune and chronic infectious diseases.11 TLSs were found to be associated with a reduced risk of recurrence and improved OS in almost all solid tumors.12 In HCC, only approximately 48% of patients have TLSs. TLSs have been shown to be a low-risk factor for early HCC recurrence but to have no effect on OS,13 though this idea is controversial. More importantly, the prognostic value of immune cells in TLSs remains unclear in HCC.

TLS formation is associated with the recruitment of lymphoid tissue inducer cells to inflammatory sites by CXCL13 and IL7.14 Lymphoid tissue inducer cell surface lymphotoxic α1β2 receptors activate peripheral stromal cells by binding LTβR on the stromal cells. Activated stromal cells can directly secrete or produce chemokines, especially CXCL11, CXCL13, CCL19, and CCL21, which can induce the migration of lymphocytes from high endothelial venules to the outer wall, in combination with the IL17 receptor signaling pathway.15 Adhesion molecules secreted by stromal cells (vascular cell adhesion molecule-1 and intercellular adhesion molecule-1) contribute to the adhesion of lymphocytes into cellular masses, leading to the formation of TLSs. Promoting the formation of TLSs is a key strategy to improve the immunotherapy response,16,17 and it is also a hotspot of current research. However, strategies to accomplish this goal are substantially challenging to develop, so more in-depth research is needed.

In this study, we aimed to determine the prognostic value of CD3+ T cells, CD8+ T cells, CD20+ B cells, and CD208+ dendritic cells in TLSs and to re-evaluate the prognostic value of TLSs categorized based on lymphocyte density. In addition, bioinformatics analysis was used to identify genes and molecular mechanisms related to TLSs formation.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Samples were collected from patients who underwent their first HCC resection in Xijing Hospital from 2015 to 2019. Exclusion criteria: preoperative radiofrequency ablation, transarterial chemotherapy and embolization, immunotherapy and other anticancer treatments; and preoperative tumor rupture or extrahepatic metastasis. A total of 265 patients met the exclusion criteria. A training set of 145 patients from 2015 to 2017, A training set of 145 patients from 2015 to 2017, a testing set of 120 patients from 2018 to 2019. Further analysis was performed using formalin-fixed and paraffin-embedded HCC tissues and 39 paired fresh frozen tumor tissue samples (stored in liquid nitrogen). The clinicopathological features are shown in Supplementary file (Table S1). The pathologic tumor type was diagnosed by three pathologists after surgical excision. Patients underwent outpatient review every 3 months in the first year, followed by outpatient review every 6 months, and all postoperative patients were contacted by telephone every 3 months. Recurrence within 2 years of surgical resection was considered early recurrence because such cases are considered metastasis of the original tumor after resection rather than a new tumor.

2.2 | Total RNA extraction and quantitative real-time polymerase chain reaction (qRT–PCR)

Total RNA was extracted using TRIzol reagent (Takara, Tokyo, Japan) and then reverse-transcribed using the PrimeScript RT–PCR Kit (Accurate Biology, China) according to the manufacturer’s instructions. A SYBR Green PCR Kit (Accurate Biology, China) was used to perform qRT–PCR. Primer sequences are shown in Supplementary file (Table S2).
2.3 | Pathological examination

Hematoxylin–eosin (HE) staining was used to identify the presence of TLSs, and detailed staining procedures have been described in previous studies. At least one TLS was confirmed positive in each section. This process

Formalin-fixed and paraffin-embedded samples were sectioned continuously at a thickness of 4 μm by Servicebio.

FIGURE 1  Histological appearance of TLSs and IHC. (A) Lymphoid aggregates (Agg, HE ×20). (B) Primary follicles (FL, HE ×20). (C–G) Representative IHC images showing CD20+, CD3+, CD8+, CD208+ and CD21+ cells (brown, ×20) cells in TLSs.
was performed by three pathologists. According to maturity, the TLSs were classified as: aggregates (tumor samples show only aggregates but no primary follicles) and primary follicles (tumor sample show at least one primary follicle or germinal center, with or without aggregates). Slides were scanned using a Nanozoomer scanner (Hamamatsu), and the surfaces of tissue areas were calculated using Nanozoomer Digital Pathology View software.

2.4 | IHC

The IHC labeling method used for formalin-fixed and paraffin-embedded sections have been described in previous studies. Sections were stained for CD3 (26582s, CST), CD8 (CL488-66868, Proteintech), CD20 (60271-1-Ig, Proteintech), CD208 (EPR24265-8, abcam), and CD21 (EP3093, abcam). IHC images were captured with an Olympus DP20 camera attached to a CX31 microscope. For IF, an Olympus FlourView FV1000 confocal microscope was used. All TLSs in the whole section viewed under a 20X microscope were recorded. ImageJ software (version 1.8.0) was used to manually count positive markers in TLSs, and the counting process was jointly completed by three pathologists as mentioned above. Cell density was recorded as cells/mm².

2.5 | Extraction of sequencing data and differential gene analysis

HCC sequencing data (GSE14520) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), and 223 tumor samples and 220 normal samples remained after samples lacking follow-up time were removed. Gene difference analysis was performed using the Limma package.

2.6 | Consistency cluster analysis and single-sample gene set enrichment analysis (ssGSEA)

To identify samples with TLSs, the following procedure was used. Based on 13 TLSs-related genes (CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, CXCL13, and IL7), the ConsensusClusterPlus, GSVA and GSEABase packages were used to perform consistent cluster analysis and ssGSEA.

![Graphs showing Kaplan–Meier survival analyses](image-url)

**FIGURE 2** Kaplan–Meier survival analyses by the log-rank (Mantel–Cox) test. In training set. (A) Patients with TLSs had better early relapse-free survival ($N_{\text{Agg}} = 29, N_{\text{FL}} = 30$). (B) TLSs were not correlated with late recurrence ($N_{\text{Agg}} = 29, N_{\text{FL}} = 30$). (C) The risk of early recurrence is different according to the maturation state of TLSs (primary follicles vs. aggregates; $N_{\text{TLS-}} = 86, N_{\text{Agg}} = 29, N_{\text{FL}} = 30$). In testing set. (D) Patients with TLSs had better early relapse-free survival ($N_{\text{Agg}} = 23, N_{\text{FL}} = 25$). (E) TLSs were not correlated with late recurrence ($N_{\text{Agg}} = 23, N_{\text{FL}} = 25$). (F) The risk of early recurrence is different according to the maturation state of TLSs (primary follicles vs. aggregates; $N_{\text{TLS-}} = 72, N_{\text{Agg}} = 23, N_{\text{FL}} = 25$).
2.7 Calculation of the immune score and construction of the protein–protein interaction (PPI) network

GEO samples were scored using the ESTIMATE package, producing a stromal score, immune score, ESTIMATE score, and tumor purity. The interactions between genes were evaluated using the STRING website (https://cn.string-db.org/), and Cytoscape software (version 3.9.0) was used to visualize the interactions between genes.

2.8 Assessment of the relative number of tumor-infiltrating lymphocytes (TILs)

To compare the differences in lymphocyte infiltration between patients with and without TLSs, CIBERSORT, an online analysis site developed by Stanford University (https://cibersortx.stanford.edu/), was used to assess the relative number of TILs in GEO samples. The TIMER website (https://cistrome.shinyapps.io/timer/) was used to analyze the correlation between genes and the number of immune cells.

2.9 Gene set enrichment analysis (GSEA)

We used GSEA software (version 4.1.0) to analyze pathways involved in the formation of TLSs. The greatest advantage of this method is that the expression data of genes with small expression differences can be included in the analysis, which greatly improves the accuracy of the results. We divided the GEO samples into TLS+ and TLS− groups and then carried out relevant molecular mechanism analysis in the GO and KEGG databases. According to the gene expression cut-off, the samples were divided into a high expression group and a low expression group to analyze the molecular mechanisms of genes related to TLS formation.

2.10 Statistical analysis

The X-tile software (version 3.6.1) was used to calculate the cut-off. Kaplan–Meier tumor recurrence and OS analyses were performed using the log-rank (Mantel–Cox) test and Cox-proportional hazards regressions. Related R packages used included ConsensusClusterPlus, Limma, GGPUBr, Reshape2, ESTIMATE, GSEABase, and GSVA.

**FIGURE 3** Kaplan–Meier survival analyses by the log-rank (Mantel–Cox) test. In training set. (A) High CD3+ cell density had no effect on OS ($N_{\text{Low-CD3}} = 22, N_{\text{High-CD3}} = 37$). (B–D) High CD8+ ($N_{\text{Low-CD8}} = 25, N_{\text{High-CD8}} = 34$), high CD20+ ($N_{\text{Low-CD20}} = 30, N_{\text{High-CD20}} = 29$), and high CD208+ ($N_{\text{Low-CD208}} = 33, N_{\text{High-CD208}} = 26$) cell densities were significantly correlated with improved OS. (E–H) High CD3+ ($N_{\text{Low-CD3}} = 17, N_{\text{High-CD3}} = 42$), high CD8+ ($N_{\text{Low-CD8}} = 28, N_{\text{High-CD8}} = 31$), high CD20+ ($N_{\text{Low-CD20}} = 28, N_{\text{High-CD20}} = 31$), and high CD208+ ($N_{\text{Low-CD208}} = 20, N_{\text{High-CD208}} = 39$) cell densities were significantly correlated with reduced early recurrence. (I–L) High CD3+ ($N_{\text{Low-CD3}} = 17, N_{\text{High-CD3}} = 42$), high CD8+ ($N_{\text{Low-CD8}} = 28, N_{\text{High-CD8}} = 31$), high CD20+ ($N_{\text{Low-CD20}} = 28, N_{\text{High-CD20}} = 31$), and high CD208+ ($N_{\text{Low-CD208}} = 20, N_{\text{High-CD208}} = 39$) cell densities were not correlated with late recurrence.
Differences between the two groups were analyzed using the Wilcoxon test. The Spearman correlation test was used for correlation analysis. *p* < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Intra-tumoral TLS

The HE staining results showed that 59 of 145 patients had TLSs in the training set, including 29 patients with aggregates (Figure 1A) and 30 patients with primary follicles (Figure 1B). 48 of 120 patients had TLSs in testing set, including 23 patients with aggregates and 25 patients with primary follicles.

3.2 | IHC was used to label immune cells in TLS

We used IHC to label CD20+ B cells (Figure 1C), CD3+ T cells (Figure 1D), CD8+ T cells (Figure 1E), CD208+ dendritic cells (Figure 1F), and CD21+ follicular dendritic cells (Figure 1G) in TLSs. The tissue area of the sections was 25.71–138.71 mm². The densities of CD3+, CD8+, and CD208+ cells were calculated to be 1.25–47.70 cells/mm², 1.19–50.1 cells/mm², 0–14.20 cells/mm², and 0–5.46 cells/mm², respectively.

3.3 | Survival analysis

Patients with TLSs had significantly lower rates of early recurrence (Figure 2A), but to have no effect on late recurrence (Figure 2B). In addition, the recurrence rate of patients with primary follicles was significantly lower than that of patients with aggregates, while the recurrence rate of patients with aggregates was lower than that of patients without TLSs, but there was no statistical significance (Figure 2C). The same conclusion can be reached in the testing set (Figure 2D–F). Kaplan–Meier survival analysis was used to reveal the prognostic value of CD3+, CD8+, and CD208+ cell densities. CD3+ cell density did not affect OS (*p* = 0.211) (Figure 3A). Samples high CD8+ (Figure 3B), high CD20+ (Figure 3C)
and high CD208+ cell (Figure 3D) densities had significantly improved OS compared with those with low CD20+, low CD208+, and low CD8+ cell densities, respectively (p = .007, p = .024, and p = .007, respectively). Samples with high CD3+ (Figure 3E), high CD8+ cell (Figure 3F), high CD20+ (Figure 3G), and high CD208+ (Figure 3H) densities had a significantly reduced rate of early recurrence compared with those with low CD3+, low CD8+, low CD20+, and low CD208+ cell densities, respectively (p = .001, p = .008, p = .002, and p = .015, respectively). The density of CD3+ (Figure 3I), CD8+ (Figure 3J), CD20+ (Figure 3K) and CD208+ (Figure 3L) cells did not affect the late recurrence (p = .132, p = .740, p = .422, and p = .078, respectively). There were only 9 CD21+ samples, so more samples are needed to prove its prognostic value. The above conclusion is verified in the testing set (Figure 4A–L). In univariate analysis, the variables associated with better OS were high CD8+ cell density (HR 0.18; p = .02), high CD20+ cell density (HR 0.30; p = .03) and high CD208+ cell density (HR 0.27; p = .01) (Table 1). In multivariate analysis, the variables associated with better OS were high CD8, CD3+, CD20+, and CD208+ cell density (HR 0.23; p = .01) were associated with reduced early recurrence; CD8+, CD3+, CD20+, and CD208+ cell density were not associated with late recurrence (Table 2). In multivariate analysis, the variables associated with reduced early recurrence were high CD8+ (HR 0.18; p = .04), CD3+ (HR 0.19; p = .01), CD20+ (HR 0.07; p = .01) and CD208+ (HR 0.17; p = .03) cell density (Table 2). These conclusions were reconfirmed by performing univariate and multivariate analyses in the testing set (Tables 3 and 4).

### 3.4 TLSs affects both OS and recurrence-free survival

Spearman correlation analysis showed that the densities of CD3+, CD20+, CD208+, and CD8+ cells were moderately to strongly correlated (Figure 5A), indicating that the proportions of immune cells in TLSs were different. TLSs with low densities of CD3+, CD20+, CD8+, and CD208+ cells were defined as grade A (low-CD3+ CD20+ CD8+ CD208, 43 cases), and TLSs with a high density of any one to three of the cells were defined as grade B (low-any one to three cells, 43 cases), and TLSs with CD21+ follicular dendritic cells or high densities of CD3+, CD20+, CD208+, and CD8+ cells were defined as grade C (CD21+ or high-CD3+ CD20+ CD8+ CD208, 21
cases) (Figure 5B). Grade A is composed of Aggs; Grade C includes only mature TLSs and a few TLSs with germinal centers, the density of TLSs is high; Grade B includes Aggs and mature TLSs, the TLSs density is between Grade A and Grade B. Grade C were significantly associated with improved OS, while grade A were not significantly associated with improved OS compared with a lack of TLSs (TLS−) (Figure 5C). Regarding recurrence, patients with grade C had the lowest recurrence rate, followed by patients with grade B, while patients with grade A had the highest recurrence rate, which was almost the same as that of TLS− patients (Figure 5D). The GradeB+C (Grade B and Grade C) was linked with a favorable OS (HR 0.26; \( p = 0.01 \) and HR 0.50; \( p = 0.006 \) in univariate and multivariate analysis, respectively) and a lower risk of recurrence (HR 0.23; \( p < 0.001 \) and HR 0.71; \( p < 0.001 \) in univariate and multivariate analysis, respectively) (Table 5).

3.5 | GEO sample classification based on TLS presence

By consistent cluster analysis, 223 GEO samples were divided into two categories: TLS+ (70 samples) and TLS− (153 samples) (Figure 6A). In addition, ssGSEA was used to score the 223 GEO samples, and the samples with scores in the top 48% were regarded as TLS+ (108 samples) and the rest were TLS− (115 samples) (Figure 6B). As the classification results of the two methods were different, we used those samples that were identified consistently by both methods, resulting in 98 TLS− samples (Figure 6C) and 53 TLS+ samples (Figure 6D). Finally, we used Kaplan–Meier survival curves to verify the accuracy of the classification results. The results showed that the existence of TLSs was a factor protecting against early recurrence and had no effect on late recurrence (Figure 6E,F), implying that the classification results were relatively accurate.

3.6 | Differences in immune score and immune infiltration

To compare the immune differences between TLS+ and TLS− samples, we determined the immune scores of GEO samples. Kaplan–Meier survival analysis showed that patients with higher stromal scores, immune scores, and ESTIMATE scores had higher OS (Figure 7A–C),
while patients with higher tumor purity had lower OS (Figure 7D). Patients with TLSs had significantly higher stromal scores, immune scores, and ESTIMATE scores and lower tumor purity (Figure 7E–H). The relative numbers of memory B cells, plasma cells, CD8+ T cells, NK cells, and dendritic cells were significantly higher in TLS+ patients than in TLS− patients, while the opposite was true for Treg cells, macrophages, and M2 macrophages (Figure 7I).

### 3.7 Molecular mechanisms related to TLS formation

We selected the top 5 pathways identified in the GO and KEGG database enrichment analyses, and the results showed that the formation of TLSs was related to the antigen receptor-mediated signaling pathway, immune response regulating signaling pathway, T cell receptor signaling pathway, chemokine signaling pathway, and toll-like receptor signaling pathway (Figure 8A).

### 3.8 Identification of TLS-related genes and generation of a PPI network

There were 9759 differentially expressed genes in the GEO tumor compared with normal samples (p < .01) and 1285 differentially expressed genes in the GEO samples with and without TLSs (p < .01). Among them, 275 genes were associated with OS (p < .05), and 168 genes were associated with recurrence (p < .05). A total of 135 genes affected both OS and recurrence (Figure 8B). We used the STRING website to evaluate the interaction between these 135 genes and selected molecules with interaction scores >0.5 for visualization (Figure 8C). The core molecules of the PPI network were KLRK1, GZMA, KLRB1, and CXCL13. GZMA and KLRK1 were expressed at low levels in HCC, and their expression can predict the presence of TLSs (Figure 8D).

### 3.9 KLRK1 and GZMA are associated with TLS formation

GZMA and KLRK1 expression levels were significantly higher in samples with TLSs, (Figure 9A,B) which are good prognostic factors for OS and recurrence (Figure 9C–F). GZMA expression was moderately to strongly correlated with the relative number of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells (Figure 9G). Similarly, KLRK1 expression was moderately to strongly correlated with the relative number of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells (Figure 9H).
There was a moderate to strong correlation between the expression of multiple chemokines and the expression of GZMA and KLRK1, especially CCL5 (Figure 9I). We chose to top 5 significantly enriched the signal pathway, the results are as follows GZMA may regulate the antigen receptor-mediated signaling pathway, immune response regulating signaling pathway, T cell receptor signaling pathway, chemokine signaling pathway (Figure 9J); KLRK1 may regulate the antigen receptor-mediated signaling pathway, immune response regulating signaling pathway, T cell receptor signaling pathway, B cell receptor signaling pathway, chemokine signaling pathway (Figure 9K).

We verified that the GZMA and KLRK1 expression levels were significantly higher in tissues with TLSs by qRT–PCR (Figure 10A,B), and high GZMA and KLRK1 expression was a favorable prognostic factor for OS and recurrence (Figure 10C–F). In addition, we verified that there was a moderate to strong correlation between the expression of multiple chemokines and the expression of GZMA and KLRK1 (Figure 10G). GZMA and KLRK1 expression can predict the presence of TLSs (Figure 10H).

### DISCUSSION

In this study, we first verified that TLSs were a low-risk factor for early recurrence in HCC and had no effect on late recurrence. High CD20+ , high CD208+, and high CD8+ cell densities were associated with significantly increased prognosis and were independent prognostic factor. The number of T cells is positively correlated with the prognosis of tumor patients. 24 In our results, CD3+ T cell located in TLSs had no effect on OS, but high CD3+ cell density showed a favorable prognostic trend, this phenomenon may be related to the insufficient sample size or local microenvironment of TLSs. CD8+ T cells are positively correlated not only with good prognosis but also with the immunotherapy response. 25, 26 Cytotoxic CD8+ T cells specifically recognize the endogenous peptide MHC I complexes and kill tumor cells by expressing FasL or secreting TNF-α. 27 The exhausted CD8+ T cells have a positive impact on the poor prognosis, and the completely exhausted CD8+ T cells do not respond to immunotherapy. 28, 29 Dendritic cells are one of the important sources of PD-L1, and inhibition of PD-L1 expression in

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**TABLE 4** Analysis of early and late HCC recurrence in a testing set

| Variables         | Early recurrence | Late recurrence |
|-------------------|------------------|----------------|
|                   | Univariate analysis |               |
|                   | HR (CI 95%)      | p Value      |
|                   |                   | HR (CI 95%) | p Value |
| BCLC (0-A/B-C)    | 1.09 (0.24–4.87) | .91 | 1.21 (0.12–3.63) | .86 |
| HBsAg (+/−)       | 2.03 (0.39–4.49) | .40 | 2.98 (0.25–5.90) | .38 |
| Cirrhosis (+/−)   | 1.51 (0.34–6.77) | .59 | 0.67 (0.07–6.46) | .73 |
| AFP (≥400/<400)   | 1.29 (0.25–6.66) | .76 | 0.24 (0.03–2.32) | .22 |
| CD8 (High/Low)    | 0.03 (0.01–0.43) | .02 | 0.02 (0.01–2.91) | .35 |
| CD3 (High/Low)    | 0.15 (0.02–0.54) | .01 | 0.31 (0.24–3.60) | .51 |
| CD20 (High/Low)   | 0.12 (0.01–0.95) | .005 | 0.45 (0.05–4.43) | .49 |
| CD208 (High/Low)  | 0.33 (0.14–0.67) | .001 | 0.03 (0.02–2.37) | .45 |

| Variables         | Early recurrence | Late recurrence |
|-------------------|------------------|----------------|
|                   | Multivariate analysis |               |
|                   | HR (CI 95%)      | p Value      |
|                   |                   | HR (CI 95%) | p Value |
| BCLC (0-A/B-C)    | 1.42 (0.26–7.68) | .68 | 0.83 (0.16–4.38) | .83 |
| HBsAg (+/−)       | 3.74 (0.28–5.76) | .32 | 1.39 (0.23–8.21) | .72 |
| Cirrhosis (+/−)   | 0.75 (0.28–2.91) | .80 | 1.10 (0.21–5.80) | .92 |
| AFP (≥400/<400)   | 0.90 (0.12–6.81) | .92 | 1.02 (0.15–6.77) | .86 |
| CD8 (High/Low)    | 0.26 (0.14–0.75) | .03 | 0.15 (0.02–1.35) | .02 |
| CD20 (High/Low)   | 0.12 (0.01–0.78) | .04 | 0.51 (0.33–0.840 | .01 |
| CD208 (High/Low)  |                   |               | 0.51 (0.33–0.840 | .01 |
dendritic cells can greatly promote CD8+ T cell response and inhibit tumor cell growth. Follicular dendritic cells and follicular helper T cells work together on B cells to further differentiate into plasma cells, which in turn produce antibodies to kill tumor cells. Moreover, follicular dendritic cells can concentrate antigens and retain them on the cell surface for several weeks to several years. After B cells recognize antigens retained on the surface of follicular dendritic cells, somatic high-frequency mutations can occur, resulting in the diversity of antibodies.

Recently, Hui Li et al. reported that TLSs have no effect on OS in HCC. It is well known that OS in patients with recurrent cancer is significantly lower than that in patients without recurrence. Clearly, it is not plausible that TLSs have not an effect on OS in HCC patients. It has been previously reported that different proportions of TILs have...
FIGURE 6  Clustering of GEO samples based on TLS presence. (A) Consistent cluster analysis was used to divide GEO samples into two categories: cluster 1 represents TLS− samples, and cluster 2 represents TLS+ samples. (B) Single-sample gene set enrichment analysis was also used to categorize samples based on the presence of TLSs: the samples with scores in the top 48% were classified as TLS+ samples, and the remaining were classified as TLS− samples (differences between the two groups were analyzed using the Wilcoxon test). (C, D) The results of the above two methods were overlapped, producing 98 common TLS− samples and 53 common TLS+ samples. (E, F) The presence of TLSs was a protective factor for early recurrence but not for late recurrence ($N_{TLS−} = 98$, $N_{TLS+} = 53$; Kaplan–Meier survival analyses by the log-rank (Mantel–Cox) test).

FIGURE 7  Immune differences. (A) The stromal score had no effect on OS by the log-rank (Mantel–Cox) test ($N_{Low-stromal score} = 140$, $N_{High-stromal score} = 83$). (B–D) Patients with higher immune scores ($N_{Low-immune score} = 90$, $N_{High-immune score} = 133$) and ESTIMATE scores ($N_{Low-ESTIMATE score} = 90$, $N_{High-ESTIMATE score} = 133$) had higher OS by the log-rank (Mantel–Cox) test. Patients with higher tumor purity ($N_{Low-tumor purity} = 131$, $N_{High-tumor purity} = 92$) had lower OS by the log-rank (Mantel–Cox) test. (E–H) TLS+ patients had significantly higher stromal scores, immune scores, and ESTIMATE scores and lower tumor purity than TLS− patients by the Wilcoxon test. (I) The relative numbers of memory B cells, plasma cells, CD8+ T cells, NK cells, and dendritic cells were significantly higher in TLS+ patients than in TLS− patients, while the opposite was true for Treg cells, macrophages, and M2 macrophages.
different effects on the prognosis of tumor patients.\textsuperscript{33,34} In the correlation analysis results of immune cell density, the immune cell density showed moderate to strong correlation, indicating that the proportions of immune cells in the TLSs were not exactly the same. Most of the TILs come from TLSs,\textsuperscript{35} CD3+ T cells, CD20+ B cells, CD8+ T cells, and CD208+ dendritic cells all have favorable prognostic value. Therefore, we believed that the different proportions of immune cells in TLSs had different effect on prognosis. Then, we proposed a hypothesis to classify TLSs based on the density of immune cells within TLSs to indicate the prognostic value of TLSs. Survival analysis was used to verify the validity of the TLS classification results. Clearly, grade B and grade C were significantly associated with longer OS and lower relapse-free survival. Grade A were not significantly associated with OS or relapse-free survival compared with a lack of TLSs. These results indicate that not all TLSs are favorable prognostic factors, and these differences in prognostic ability may be caused by the different proportions of immune cells in TLSs and the heterogeneity of the tumor microenvironment.

The ESTIMATE score is calculated based on transcriptome data from a sample, and its accuracy has been demonstrated in previous reports.\textsuperscript{36} The higher the stromal score, immune score, and ESTIMATE score are, the stronger the immune status is. Tumor purity represents the ratio of tumor cell volume to tumor volume.\textsuperscript{37} In our results, patients with TLSs not only had significantly higher stromal scores, immune scores, and ESTIMATE scores but also had more CD8+ T cells, indicating stronger immunity in patients with TLSs. Patients without TLSs had more Treg cells and M2 macrophages. Sia et al. recently classified...
HCC into “immune-active” and “immune-exhausted” types based on gene expression profiles; in that study, the active type was characterized by more CD8+ T cells and TLSs, while the immune-exhausted type showed enrichment of M2 macrophages. These results are consistent with our results. As such, we believe that grade B and grade C could represent “immune-active” tumors.

TLSs promote lymphocyte infiltration and activation to increase the antitumor immune response and play an important role in enhancing the immunotherapy response. Promoting the formation of TLSs is an important strategy for successfully treating immune-exhausted tumors. Previous studies have shown that CCL21 expression and CD3+ CD4+ T cell recruitment contribute to TLS formation. Type I interferon (IFN) induces the expression of chemokine C-X-C matrix ligand 13 (CXCL13) in lung fibroblasts, which further promotes the expression of C-X-C motif receptor 5 (CXCR5), thereby promoting TLS formation.
formation. GZMA can activate a variety of immune cells and induce an inflammatory environment and regulate the TLR9-MyD88 pathway to produce IFN. The expression of KLRK1 in NK cells, CD8+ T cells, and other immune cells is significantly reduced, thus promoting tumor immune escape. KLRK1 expression is involved in the interaction between autoantigen-presenting cells and stromal cells. It is worth mentioning here that stromal cells are key in the formation of TLSs. In the GO and KEGG enrichment analysis results, TLS formation was related to the T cell receptor pathway and the high expression of KLRK1 and GZMA also regulated the T cell receptor pathway. The activation of T cell receptor signal not only causes T cell proliferation and cytokine production but also promotes T cell differentiation to effector T cells and perform functions. Induction of TLSs by cytokines is one of the most commonly used methods. The expression of GZMA and KLRK1 was strongly correlated with the expression of multiple chemokines by Spearman test. The combination of GZMA and KLRK1 can predict the presence of TLSs. GZMA and KLRK1 have the potential to promote the formation of TLSs. A limitation of this study is that more subtypes of T and B cells were not included in the TLS classification.

**AUTHOR CONTRIBUTIONS**

Ye Nie, Hanlu Fan, and Jianhui Li contributed equally to this work. Ye Nie, Kaishan Tao, and Wenjie Song designed the whole study. Ye Nie and Jianhui Li conducted the statistical analysis. Jianhui Li, Xinjun Lei, and Zhenzhen Mao did the qRT-PCR analysis. Ye Nie and Hanlu Fan drafted the manuscript. Tianchen Zhang and Yanfang Wang made the relevant edits to the manuscript. Zhenzhen Mao and Wenjie Song revised the manuscript. All authors read and approved the final manuscript.

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**DISCLOSURES**

All authors declare that they have no conflicts of interest.
DATA AVAILABILITY STATEMENT
The data presented in the study are deposited in the FigShare repository, accession link: https://figshare.com/s/93afe076e2ed13d32f4d.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All cases were collected from Xijing Hospital, with patients’ informed consent and the approval of the Medical Ethics Committee of First Affiliated of Fourth Military Medical University (approval number: KY20172013-1).

CONSENT FOR PUBLICATION
The manuscript has never been published, neither is being considered for publication elsewhere in whole or in part in any language.

ORCID
Ye Nie @ https://orcid.org/0000-0002-2887-707X
Jianhui Li @ https://orcid.org/0000-0002-7946-0768
Wenjie Song @ https://orcid.org/0000-0002-8916-8859

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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