Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2023 24(9):779-795
www.jzus.zju.edu.cn; www.springer.com/journal/11585
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Review

https://doi.org/10.1631/jzus.B2200605

Detection and quantitative analysis of tumor-associated tertiary lymphoid structures

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Abstract: Tumor-associated tertiary lymphoid structures (TLSs) are ectopic lymphoid formations within tumor tissue, with mainly B and T cell populations forming the organic aggregates. The presence of TLSs in tumors has been strongly associated with patient responsiveness to immunotherapy regimens and improving tumor prognosis. Researchers have been motivated to actively explore TLSs due to their bright clinical application prospects. Various studies have attempted to decipher TLSs regarding their formation mechanism, structural composition, induction generation, predictive markers, and clinical utilization. Meanwhile, the scientific approaches to qualitative and quantitative descriptions are crucial for TLS studies. In terms of detection, hematoxylin and eosin (H&E), multiplex immunohistochemistry (mIHC), multiplex immunofluorescence (mIF), and 12-chemokine gene signature have been the top approved methods. However, no standard methods exist for the quantitative analysis of TLSs, such as absolute TLS count, analysis of TLS constituent cells, structural features, TLS spatial location, density, and maturity. This study reviews the latest research progress on TLS detection and quantification, proposes new directions for TLS assessment, and addresses issues for the quantitative application of TLSs in the clinic.

Key words: Tertiary lymphoid structure; Tumor microenvironment; Chemokine signature; Spatial omics; Artificial intelligence; Radiomics

1 Introduction

The immune system is a key player in the response to tumorigenesis. Therefore, in addition to surgery, chemotherapy, radiotherapy, and targeted therapy, immunotherapy has also been a key tumor treatment method, which reduces the toxicity of other treatments. However, limitations to the use of immunotherapy include low response rate to single agents, the onset of immune-related adverse side effects, and the lack of predictive markers of treatment response (Wang et al., 2018, 2019; Ramos-Casals et al., 2020). Researchers have focused on the tumor microenvironment (TME), where multiple immune cells regulate tumor growth and progression (Galon et al., 2006; Fridman et al., 2017). Recent studies have shown that activated lymphocyte subpopulations in the TME interact through co-stimulation, co-localization, and aggregation processes under tumor antigen exposure conditions to develop advanced and specific structures of immune cells called tumor-associated tertiary lymphoid structures (TLSs). TLSs are ectopic lymphoid formations that develop in non-lymphoid tissues at chronic inflammation sites, such as tumors (Drayton et al., 2006) and autoimmune diseases (Manzo et al., 2010), reflecting the lymphoid cell accumulation after prolonged exposure to inflammatory signals from antigen stimulation, chemokines, and cytokines (Dieu-Nosjean et al., 2016). The morphological, cellular, and molecular characteristics of TLSs resemble secondary lymphoid organs (SLOs), but differ from those in lacking a capsule and the ability to form in non-lymphoid tissues (Neyt et al., 2012; Pimenta

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Received Nov. 27, 2022; Revision accepted Feb. 27, 2023; Crosschecked Aug. 29, 2023

and Barnes, 2014; Koenig and Thaunat, 2016; Rodriguez and Engelhard, 2020). TLS formations drive various immune functions through complex immune responses in various pathological settings, including autoimmune diseases (Manzo et al., 2010; Lucchesi and Bombardieri, 2013), infectious diseases (Neyt et al., 2012), organ transplant rejection (Thaunat et al., 2010), and malignancies (Dieu-Nosjean et al., 2014; Pitzalis et al., 2014; Colbeck et al., 2017). Due to its promising clinical value in tumor therapy, researchers have developed high interest in exploring TLSs. Firstly, the presence of TLSs has indicated positive clinical implications for tumors. Most tumor-associated TLS studies suggest that TLSs are related to longer patient survival time (Table 1). This has been described for oral squamous carcinoma (Li QX et al., 2020), non-small lung cancer (Dieu-Nosjean et al., 2008; Germain et al., 2014; Tang et al., 2020), breast cancer (Liu et al., 2017; Prabhakaran et al., 2017; Song et al., 2017), colorectal adenocarcinoma (Posch et al., 2018; Trajkovski et al., 2018), hepatocellular carcinoma (Calderaro et al., 2019; Li H et al., 2020, 2021), endometrial cancer (Horeweg et al., 2022; Qin et al., 2022), cervical cancer (Zhang et al., 2022), melanoma (Ladányi et al., 2007; Cabrita et al., 2020), and esophageal squamous cell carcinoma (Deguchi et al., 2022; Li RT et al., 2022). Secondly, TLSs were shown to improve the responsiveness of some tumor therapies, such as chemotherapy (Benzerdjeb et al., 2021; Delvecchio et al., 2021; Deguchi et al., 2022) and immunotherapy (Cabrita et al., 2020; Helmink et al., 2020; Petitprez et al., 2020; Zhou et al., 2021). A study on the clinical relevance of TLSs by Deguchi et al. (2022) in esophageal carcinoma indicated that III–IVa stage patients with effective neoadjuvant chemotherapy possessed more TLSs of the germinal center (GC) than those with lower treatment efficacy (P=0.0224). Thirdly, TLSs could be a candidate marker for immune checkpoint inhibitor therapy (Dieu-Nosjean et al., 2014; Sautès-Fridman et al., 2019; Rodriguez and Engelhard, 2020). This pan-cancer study analyzed 11 different tumor types to verify the robustness of the predictive value of TLSs in immune checkpoint inhibitor therapy. Despite programmed death-ligand 1 (PD-L1) expression, mature TLS status significantly enhanced immune response and survival in both PD-L1-positive and PD-L1-negative patients. Therefore, mature TLSs could objectively predict immune response efficacy (Vanhersecke et al., 2021).

The above findings are still far from the reproducibility and robustness required for tumor treatment and fail to address the accompanying problems. The induction of TLS formation in animal models (Delvecchio et al., 2021; van Hooren et al., 2021; Clubb et al., 2022), their structural composition, and the mechanism of action in the TME are being gradually explored; however, detection and quantification could be more challenging. The lack of standardized TLS quantification and convenient detection method limits a deeper understanding of these structures and hinders their clinical application. This study reviews the latest progress in TLS detection and quantification and proposes new directions for TLS assessment.

2 Formation and structural composition of TLSs

2.1 TLS formation

Because of the complexity of TLS formation, the precise mechanism behind tumor-associated TLSs still needs to be deciphered. Referring to the SLO formation process and preliminary mechanism studies in mouse models of TLSs, the formation process of TLSs could involve multiple co-existing signaling pathways that interact with each other (Rodriguez et al., 2021; Chaurio et al., 2022). TLS formation can be summarized into four main steps: (1) antigen exposure, (2) stromal cell activation, (3) lymphocyte recruitment, and (4) TLS maturation (Drayton et al., 2006; Gago da Graça et al., 2021) (Fig. 1). The presence of tumors indicates a form of chronic inflammation. The release of tumor antigens and immunogenic tumor cell death causing the emergence of inflammatory cytokines are essential factors in developing TLSs. For example, tumor necrosis factor-lymphotoxin (TNF-LT) stimulates lymphoid tissue inducer (LTi) cells with lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) (Eberl et al., 2004; Drayton et al., 2006; Rodriguez et al., 2021). For example, T helper 17 (Th17) cells, T follicular helper (Tfh) cells, B cells, group 3 innate lymphoid cells (ILC3), and M1 macrophages, although their roles have not been clarified (Bénézech et al., 2015), interact with lymphoid tissue organizer (LTo) cells being stromal cells, lymphocytes, or endothelial cells. In a mouse melanoma model, cancer-associated fibroblasts (CAFs) could act as LTo cells with lymphotoxin-β receptor (LTβR) to involve in TLS

Table 1 Initial studies on TLSs with clinical prognosis

Cancer type	Author (year)	Patients	Main findings	Outcome
Lung cancer	Germain et al. (2014)	74 (early stage); 122 (advanced stage); 61 (prospective study)	B-cell density within TLSs represents a new prognostic biomarker for NSCLC patient survival.	Positive
	Tang et al. (2020)	133	Greater area of TLSs and proportion of B cells were associated with longer survival rates.	Positive
Breast cancer	Song et al. (2017)	108	A valuable tool for predicting the pathologic complete response of triple-negative breast cancer.	Positive
	Liu et al. (2017)	248	An essential favorable prognostic indicator in $HER2^+$ breast cancer patients.	Positive
	Prabhakaran et al. (2017)	366	The presence of TLSs within the TME results in better survival outcomes.	Positive
	Sofopoulos et al. (2019)	167	Patients with peritumoral TLSs had worse disease-free survival and overall survival outcomes.	Negative
Colorectal adenocarcinma	Posch et al. (2018)	109	The TLS maturation process harbors important prognostic information on the risk of disease recurrence.	Positive
	Trajkovski et al. (2018)	103	The density of TLSs influences the control of tumor progression.	Positive
Hepatocellular carcinoma	Calderaro et al. (2019)	273	Intra-tumoral TLSs may reflect sustained anti-tumor response.	Positive
	Li H et al. (2021)	240	High TLS density improved patient survival outcomes and represented a promising prognostic biomarker for HCC.	Positive
	Finkin et al. (2015)	82	A high TLS was associated with increased recurrence risk and decreased overall survival after HCC resection.	Negative
Endometrial cancer	Qin et al. (2022)	104	TLS and B cell infiltration into tumors were associated with favorable survival outcomes in patients with EC.	Positive
	Horeweg et al. (2022)	660	Favorable solid prognostic impact of TLSs.	Positive
Melanoma	Ladányi et al. (2007)	82	TLSs were associated with a better disease outcome.	Positive
	Cabrita et al. (2020)	186	TLSs have a key role in the TME in melanoma.	Positive
Esophageal carcinoma	Deguchi et al. (2022)	236	TLS maturation may be a useful target for predicting the efficacy of immunotherapy.	Positive
	Li RT et al. (2022)	185	TLSs can be used as a new prognostic biomarker.	Positive
Cervical cancer	Zhang et al. (2022)	93	TLSs are a potentially valuable prognostic predictor for cervical cancer.	Positive
Oral squamous cell carcinoma	Li QX et al. (2020)	168	TLSs are an independent positive prognostic factor.	Positive
Clear cell renal cell carcinoma	Xu et al. (2022)	232	CXCL13 expression significantly predicted aggressive disease progression and poor prognosis.	Negative

CXCL13: CXC-chemokine ligand 13; EC: endometrial cancer; HCC: hepatocellular carcinoma; $HER2^+$: human epidermal growth factor receptor-2 positive; NSCLC: non-small-cell lung carcinoma; TLS: tertiary lymphoid structure; TME: tumor microenvironment.

formation (Rodriguez et al., 2021). $LT\alpha_1\beta_2$ from LTi cells binds to LTβR from LTo cells (Colbeck et al., 2017), which leads to the development of TLSs (Lochner et al., 2011; Peters et al., 2011; Guedj et al., 2014; Jones et al., 2016). LTi cells also secrete chemokines (e.g., CXC-chemokine ligand 13 (CXCL13)) (Meier et al., 2007) and cytokines (e.g., interleukin (IL)-17, IL-7, and IL-21) (Deteix et al., 2010; Jones et al., 2016; Chaurio et al., 2022), and selectively recruit more LTo cells and immunocytes (Rodriguez et al., 2021). This in turn leads to the secretion of vascular endothelial growth factor C (VEGFC) and adhesion molecules, such as

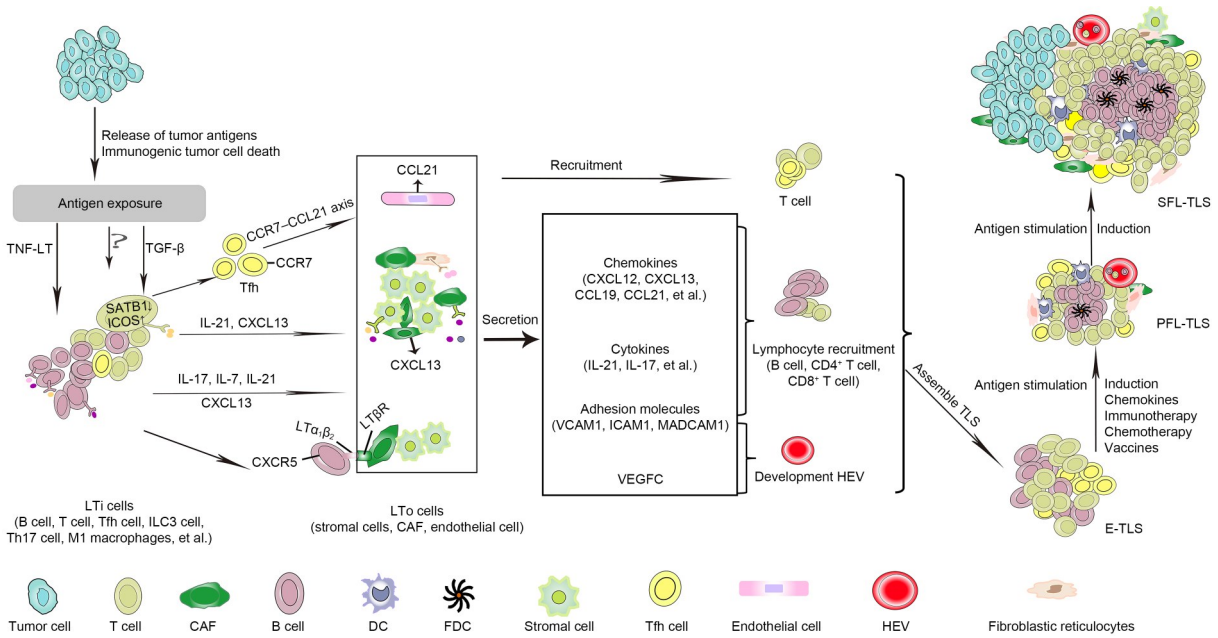

Fig. 1 General process of TLS formation and growth. The initiation mechanism of TLS formation is antigen exposure (Kang et al., 2021). Tumor-associated inflammation factors (e.g., TNF-LT and TGF-β) stimulate LTi cells, including Th17 cells, Tfh cells, T cells, B cells, ILC3, M1 macrophages, etc., which secrete chemokines CXCL13 and cytokines (e.g., IL-17, IL-7, and IL-21), as well as $LT\alpha_1\beta_2$ to recruit LTo and immunocytes to the local TME. These processes lead to the secretion of VEGFC, adhesion molecules, cytokines, and chemokines, all of which promote more lymphocyte recruitment and the development of HEVs (Schumacher and Thommen, 2022). Moreover, Tfh recruitment is mediated by TGF-β suppressing the expression of SATB1. The decreased expression of SATB1 leads to an increase in inducible T cell co-stimulator (ICOS), which promotes T cell recruitment (Chaurio et al., 2022). The aggregation of lymphocytes forms early TLSs, that is, structurally defined ectopic dense lymphoid tissue aggregates. The development of mature TLSs depends on continuous antigenic stimulation and induction (Rodriguez et al., 2021). CAF: cancer-associated fibroblast; CCL21: CC-chemokine ligand 21; CCR7: chemokine CC-motif receptor 7; $CD4^+$: cluster of differentiation 4-positive; CXCL13: CXC-chemokine ligand 13; CXCR5: chemokine C-X-C-motif receptor 5; DC: dendritic cell; FDC: follicular dendritic cell; HEV: high endothelial venule; ICAM1: intercellular adhesion molecule 1; IL: interleukin; ILC3: group 3 innate lymphoid cells; $LT\alpha_1\beta_2$: lymphotoxin-$\alpha_1\beta_2$; LTβR: lymphotoxin-β receptor; LTi: lymphoid tissue inducer; LTo: lymphoid tissue organizer; MADCAM1: mucosal addressable cell adhesion molecule 1; SATB1: special AT-rich binding protein-1; Tfh: T follicular helper; TGF-β: transforming growth factor-β; Th17: T helper 17; TLS: tertiary lymphoid structure; E-TLS: early TLS; PFL-TLS: primary follicle-like TLS; SFL-TLS: secondary follicle-like TLS; TNF-LT: tumor necrosis factor-lymphotoxin; TME: tumor microenvironment; VCAM1: vascular cell adhesion molecule 1; VEGFC: vascular endothelial growth factor C.

vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1), and mucosal addressable cell adhesion molecule 1 (MADCAM1), chemokines such as CXCL12, CXCL13, CC-chemokine ligand 19 (CCL19), and CCL21, and cytokines by LTo cells and immunocytes (Wang et al., 2002; Furtado et al., 2007; Vondenhoff et al., 2009), which promote the recruitment and aggregation of lymphocytes and the development of high endothelial venules (HEVs). HEVs are also thought to mediate lymphocyte recruitment (di Caro et al., 2014). The recruitment of B cells is more likely to occur because of the actively involved CXCL13. A recent study indicated that the promotion of T cell differentiation into Tfh is mediated by transforming growth factor-β (TGF-β) suppressing the expression of special AT-rich binding protein-1 (SATB1) (Chaurio et al., 2022). This study, while exploring the recruitment of B cells by T cells through IL-21 and CXCL13, revealed that low expression of SATB1 promotes T cell differentiation into Tfh. Furthermore, chemokine CC-motif receptor 7 (CCR7) on Tfh via the CCL21 axis recruited more T cell aggregation. These aggregated lymphocytes assemble into early TLSs without GC (Luther et al., 2002; Daum et al., 2021). Studies of lung and colorectal cancers have demonstrated that the process from the early to the mature TLS stage is sequential maturation (Posch et al., 2018; Siliņa et al., 2018a). Therefore, continuous antigenic

stimulation is important during TLS maturation. Meanwhile, relevant experimental evidence indicated that chemokines, immunotherapy, and vaccines can induce TLS maturation (Delvecchio et al., 2021; Rodriguez et al., 2021; van Hooren et al., 2021; Clubb et al., 2022) (Fig. 1). TLS formation is a complex phenomenon that different cytokines and cells can regulate under various disease conditions (Mueller et al., 2018). However, the molecular and cellular mechanisms of TLS formation are unclear, and the processes leading to TLS formation in tumors should be further explored.

2.2 Structural components of TLSs

The structural composition of TLSs in tumors was first described in non-small cell lung cancer (Dieu-Nosjean et al., 2008) and melanoma (Ladányi et al., 2007). At the histological level, immature TLSs possess the morphology of irregular, tightly packed aggregates consisting of various types of immune cells. In contrast, mature TLSs have a more apparent cellular zone and GCs with bright and dark zones (Pipi et al., 2018). TLSs comprise three main components at the cellular level: the external T cell zone, the internal B cell zone, and the surrounding HEV zone (Coppola et al., 2011). The T cell compartment contains clusters of cluster of differentiation 4-positive ($CD4^+$) T cells, $CD8^+$ T cells, and mature dendritic cells (DCs) expressing DC-lysosome-associated membrane glycoprotein-positive (DC-$LAMP^+$) or $CD83^+$ (Goc et al., 2014; Sautès-Fridman et al., 2019). These could be associated with cellular anti-tumor immunity. The GCs developed the B cell compartment containing follicular dendritic cells (FDCs) expressing CD21/CD23 and several B cells. These also include effector B cells, plasma cells, and memory B cells, to which most of the anti-tumor effects of TLSs are associated (Sautès-Fridman et al., 2019; Helmink et al., 2020; Petitprez et al., 2020; Delvecchio et al., 2021). Additionally, B cells in TLSs could be involved in an effective T-cell response after immune checkpoint inhibitor treatment (Helmink et al., 2020). Meylan et al. (2022) explored TLSs and the B-cell development thereof in renal cancer using spatial transcriptomics, bulk RNA sequencing (RNA-seq), and immunofluorescence. In the TLS-positive group, gene signatures from the B cell lineage (with genes from naive cells to plasma cells), T cells (involving different subtypes), and fibroblasts were preferentially expressed in the TLS region. Mature TLSs are characterized by $CD4^+PD1^+$ T cells, $CD4^+PD1^+CXCR5^+$ Tfh cells, and CXCL13 producing cells. GCs contain a network of $CD23^+$ FDCs in the interior, $BCL6^+CD20^+$ B cells, and HEVs with peripheral node addressin (PNAd) (Meylan et al., 2022). HEVs are developed by full cuboidal endothelial cells expressing PNAd, which could mediate the rapid recruitment of lymphocytes (Martinet et al., 2011). Moreover, controlling the type of lymphocytes in the lymphoid tissue increases the possibility of recruitment of naive and central memory T lymphocytes (Liao and Ruddle, 2006; Martinet et al., 2011, 2013; Girard et al., 2012; di Caro et al., 2014; Ager, 2017). The different states of TLSs can be identified by multiplex immunofluorescence. TLSs are classified into three stages depending on the presence of FDCs and the proliferation of the B-cell component. Early TLSs (E-TLSs) consist primarily of dense aggregates of lymphocytes without FDCs and the absence of segregated T and B cell zones. FDCs are present in the primary follicle-like TLSs (PFL-TLSs), but no GC can be observed. Secondary follicle-like TLSs (SFL-TLSs) represent an active GC reaction and resemble the SLOs (Posch et al., 2018; Siliņa et al., 2018a; Calderaro et al., 2019; van Dijk et al., 2020; Wang et al., 2021) (Fig. 2).

3 Methods for TLS detection

The current methods to detect the presence of TLSs are H&E staining, multiplex immunohistochemistry (mIHC) or multiplex immunofluorescence (mIF), and the real-time quantitative polymerase chain reaction (qPCR) of chemokines. H&E and mIHC/mIF are easy to quantify but there is a bias in determination by individual physicians. The detection of chemokines by qPCR is a more accurate approach; however, this only allows for the indirect determination of the presence of TLSs but not direct visualization. Meanwhile, the above methods rely on harvesting tumor samples from representative areas; therefore, inadequate sampling in the case of inoperable tumors hinders the precise evaluation of the TLSs (Siliņa et al., 2018b). Due to limitations commonly encountered in clinical situations, indirect methods of evaluating TLSs, such as predictive markers in the blood circulation or body fluids, as well as comparable imaging features, are promising approaches with relevant research underway.

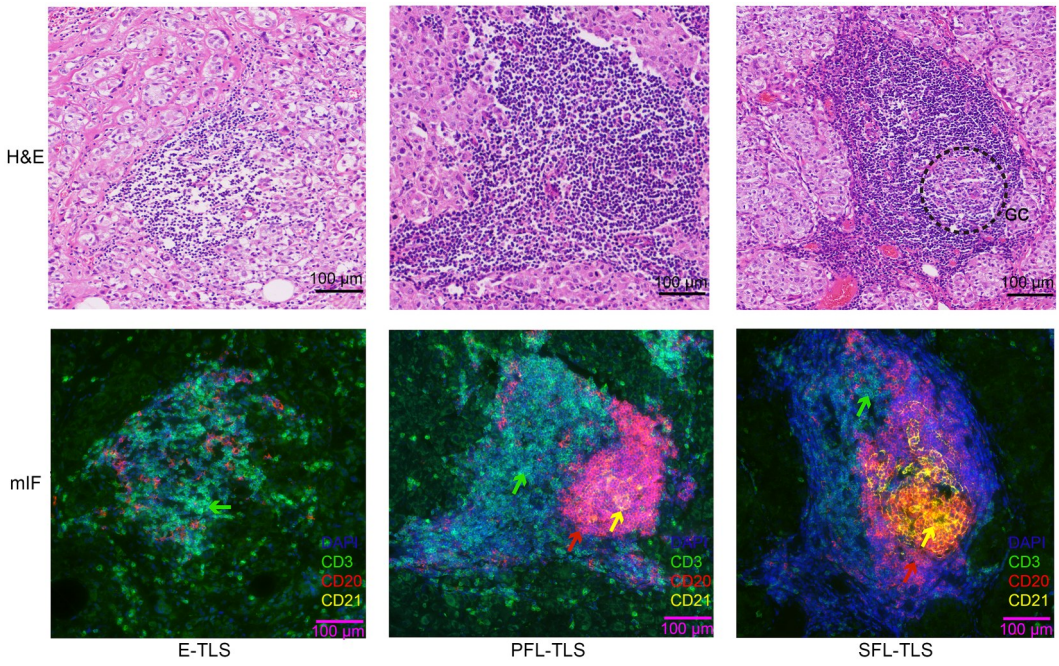

Fig. 2 Three stages of TLSs in breast cancer. E-TLS consists primarily of dense aggregates of lymphocytes (H&E and mIF), PFL-TLS is composed of B-cell clusters with FDCs (H&E and mIF), and SFL-TLS has an active GC (H&E and mIF). Colored arrows point to areas where the corresponding immunofluorescence staining marked the location of the cells. CD: cluster of differentiation; FDCs: follicular dendritic cells; GC: germinal center; H&E: hematoxylin and eosin; mIF: multiplex immunofluorescence; TLS: tertiary lymphoid structure; E-TLS: early TLS; PFL-TLS: primary follicle-like TLS; SFL-TLS: secondary follicle-like TLS.

3.1 H&E staining

H&E staining can detect TLSs based on their morphological features in formalin-fixed and paraffin-embedded tumor sections. The presence of TLSs can be initially determined by identifying dense mature lymphoid cell aggregates having curved and smooth outlines within the TME. The main advantages of H&E staining include simple execution and low cost, although it can extract limited information, not allowing to explore the full depth of cellular interactions in the TME. Using this method, the number of "TLS-like" structures can be quantitated without qualitative description (cell types, activation, etc.).

3.2 Multiplex immunofluorescence

Tissue immune staining using mIHC of consecutive sections or mIF of a single section can probe more deeply into the spatial biology of TME, such as capturing intercellular interactions to detect and determine maturity in spatial relationships (Hoyt, 2021). Especially, mIF facilitates the simultaneous analysis of multiple markers (Buisseret et al., 2017); hence, it is more accurate in evaluating the maturation status and distribution of cells (Table 2). Antigens are expressed in the B cell zone, such as CD19 and CD20 by B cells, or CD138 by plasma cells, whereas the FDC surface antigens CD21 and CD23 (in the mature TLSs harboring GCs where CD21 expression is decreased and CD23 expression is increased) and Ki67 are directly associated with cell proliferation (Sautès-Fridman et al., 2019). Regarding the T cell zone, the surface antigen CD3 is expressed in all T cells, while CD4 and CD8 in specialized T cells and DC-LAMP$^+$ mature dendritic cells (Goc et al., 2014). PNAd is expressed by HEV endothelial cells (Martinet et al., 2013). The choice of antigen marker number and fluorescence spectra is critical for high-resolution antigen staining. Advances in multispectral imaging technology have increased the number of fluorescent probes detected simultaneously by conventional fluorescence microscopy, enhancing the efficiency and quality of immunofluorescence staining (Hoyt, 2021). Siliņa et al. (2018b) systematically described the mIF staining technique and multispectral imaging procedure for TLSs. The current application of mIF staining allows the simultaneous labeling of over six TLS marker proteins, which facilitates the

Table 2 TLS maturity-related markers

Marker	Cellular localization	E-TLS	PFL-TLS	SFL-TLS	References
CD20	B cell	+	+	+	Boivin et al., 2018; Sautès-Fridman et al., 2019; Schumacher and Thommen, 2022
CD3	T cell	+	+	+	Mueller et al., 2018; Siliņa et al., 2018b; Cabrita et al., 2020
DC-LAMP/CD83	DC	−	−/+	+	Dieu-Nosjean et al., 2008; Siliņa et al., 2018b; Sautès-Fridman et al., 2019
CD21	FDC	−	+	+	Posch et al., 2018; Siliņa et al., 2018b
CD23	FDC	−	−	+	Posch et al., 2018; Siliņa et al., 2018b
PNAd	HEV	−	+	+	Song et al., 2017; Meylan et al., 2022
BCL6	GC	−	−	+	de Silva and Klein, 2015; Meylan et al., 2022

BCL6: B-cell lymphoma 6 protein; CD: cluster of differentiation; DC: dendritic cell; FDC: follicular dendritic cell; GC: germinal center; HEV: high endothelial venule; LAMP: lysosome-associated membrane glycoprotein; TLS: tertiary lymphoid structure; E-TLS: early TLS; PFL-TLS: primary follicle-like TLS; SFL-TLS: secondary follicle-like TLS; PNAd: peripheral node addressin.

characterization of TLS morphology and structure (Posch et al., 2018; Siliņa et al., 2018b; Laidlaw and Cyster, 2021).

3.3 Genomic signature of TLSs

qPCR of fresh tumor biopsy can qualitatively detect TLSs by assessing the expression of specific chemokine genes. These genes were clustered using the Pearson's correlation distance metric leading to the final 12-chemokine (CK) score, including *CCL2*, *CCL3*, *CCL4*, *CCL5*, *CCL8*, *CCL18*, *CCL19*, *CCL21*, *CXCL9*, *CXCL10*, *CXCL11*, and *CXCL13* (Coppola et al., 2011; Messina et al., 2012; Prabhakaran et al., 2017; Li R et al., 2021). Thus, TLSs could be detected and quantified at the gene level, which could prove the existence of TLSs and provide the means for quantification. The 12-CK score was independent of tumor stage, location, or treatment received. Furthermore, a strong correlation was observed between the chemokine genes and TLS-enriched tumors. More importantly, a high 12-CK score significantly improved the overall survival (Tokunaga et al., 2020; Li R et al., 2021). Zemp et al. (2021) highlighted that a group of high 12-CK patients had improved progression-free survival (P=0.004), disease-specific survival (P=0.004), and overall survival (P=0.03). The patients benefited from almost one year of the Atezolizumab treatment. In addition, high 12-CK scores had favorable oncologic outcomes across multiple tumors, including colorectal cancer, melanoma, hepatocellular carcinoma, and breast cancer (Coppola et al., 2011; de Chaisemartin et al., 2011; Messina et al., 2012; Prabhakaran et al., 2017). The 12-CK score showed excellent robustness and comparability across multiple tumors based on the pan-cancer gene expression analysis of TLS markers among 30 types from up to 9880 tumors. The expression has been quantified as the mean of $\log_2$(TPM+1) (TPM: transcripts per million) of the signature of 12 genes, indicating that the distribution was heterogeneous. For instance, this included lung tumors with high signature expression, glioblastoma of the brain, and uveal melanoma of the eye with low expression (Sautès-Fridman et al., 2019). Simultaneously, next generation sequencing (NGS) provided a comprehensive analysis of TLS-associated chemokines at the gene level. Nakamura et al. (2022) examined chemokines of the TME in Merkel cell carcinoma through NGS, and found that the expression levels of five chemokine genes, viz., *CCL5*, *CCR2*, *CCR7*, *CXCL9*, and *CXCL13*, were significantly higher in the TLS-positive group than in TLS-negative specimens. Therefore, there is significant robustness in uncovering TLSs at the gene level, and the relevant genes could be predictive and prognostic tumor markers for TLSs. However, the application of such methods in clinical practice should be combined with additional imaging methods, such as H&E and mIHC or mIF, to provide quantitative and qualitative information on the presence of TLSs.

3.4 Spatial omics of TLSs

Spatial omics is a revolutionary field of spatial tumor research, which can capture the high-throughput protein and RNA characteristics of TME at the genome level. Decoding the spatial structure of tumor-associated TLSs could be a giant leap forward to understand and quantify TLSs (Amaria et al., 2018). Spatial omics

techniques include laser capture microdissection, image-based in situ transcription, spatial barcoding-based transcriptomics, spatial proteomics, spatial metabolomics, and spatial multi-omics technologies, with a visualization platform for spatial omics developed in 2019. GeoMx Digital Spatial Profiler is a platform with high throughput, multi-omics, and a flexible selection of regions of interest, providing researchers with comprehensive and clear information. It can aid our understanding of tumorigenesis and the TME (Van and Blank, 2019; Gupta et al., 2020; Wu et al., 2022). Two high-quality studies have obtained promising results by quantification in TLS detection using the GeoMx Digital Spatial Profiler. Cabrita et al. (2020) utilized GeoMx in a study of immunotherapy response and survival in melanoma to quantify 25 immune-associated proteins of immune cells in TLSs and explored the molecular properties of T and B cells. It was revealed that highly proliferative B cells are present around the GCs. Thus, mature TLSs were linked to B cells expressing Ki67 and CD40, supporting the idea that different stages of TLSs exist within the same TME. A high proportion of $CD4^+$ and BCL2 expressing T cells were present in TLSs having a high number of Ki67 B cells. In contrast, TME without TLSs possessed fewer T cells of this molecular phenotype. Finally, the researchers identified novel molecular markers for TLSs (Cabrita et al., 2020). Helmink et al. (2020) also targeted the protein expression profiles using spatial multi-omics techniques to decipher the role of B cells in TLSs in the immune response. It was indicated that the expression of B cell markers was significantly enhanced during pre-treatment. On-treatment samples from the immune checkpoint blockade (ICB) responder group and memory B cells could act as antigen-presenting cells driving a progressive increase in memory and naive tumor-associated T cells. In addition, B cells secrete various cytokines to activate and recruit immune effector cells such as T cells (Helmink et al., 2020). Through the analysis of TLS-related proteins, the issues involving detecting and quantifying TLSs, tumor TLS heterogeneity, the characteristics across different stages of TLSs within the same tumor, and the response of TLSs to immunotherapy will be gradually resolved through future studies.

3.5 3D imaging of TLSs

Another promising approach to address the high reliance on pathological tissue for TLS detection and quantification is three-dimensional (3D) imaging. Randolph et al. (2016) provided a 3D imaging method to characterize the lymphatic vasculature in the mesenteric tissue of Crohn's patients. Therein, the surgeon intraoperatively injected a dye for lymphadenopathy locally into the ileum. Lymphatic aggregates without capsules were identified by image scanning with a light-emitting diode, 3D imaging processing and analysis with ImarisQ13 software. The structure was identified as TLS after characterization by immunofluorescence imaging in 3D, and its volume, the number of constituent cells, and the number of B cells were evaluated by computer algorithms. With the application of 3D imaging, information on the 3D structure and spatial dynamics of TLSs, and even the types, numbers, and interactions of the constituent cells in TLSs can be more visually presented. The above data suggest that there is a highly promising prospect of 3D imaging to unveil the connection among the development of TLSs, tumor status, and tumor treatment strategies (chemotherapy, immunotherapy, radiotherapy, etc.).

3.6 Potential TLS biomarkers

The identification of TLSs is still limited by several challenges. Histopathological observation requires surgical specimens and is technically demanding. Thus, readily available and reliable TLS identification markers are urgently needed in clinical practice. Recent studies exploring TLS markers yielded preliminary results in selected tumors only. Chemokines are strongly related to the presence of TLSs, particularly CXCL13, the most accepted TLS marker (Groeneveld et al., 2021). In a study on the prognostic impact of TLSs in endometrial cancer, Horeweg et al. (2022) inadvertently found that L1-cell adhesion molecule (L1CAM) expression was significantly enhanced in TLS-positive tumors. In $L1CAM^+$ TLSs, L1CAM/CD21 co-immunofluorescence analysis showed a co-localization of L1CAM with FDCs-labeled CD21, whereas gene expression analysis revealed L1CAM overexpression. This result established L1CAM as a simple biomarker for endometrial cancer-associated TLSs. Ahmed et al. (2022) observed that low levels of serum IL-2 were significantly correlated with the presence of TLSs, suggesting its potential role as TLS biomarker in pancreatic ductal adenocarcinoma TME. Nonetheless, further studies are necessary to establish the IL-2 serum threshold. Also, the reproducibility of the abovementioned markers still

needs additional validation. As a whole, more research is required to validate TLS applications in clinical settings.

4 Quantitative analysis of TLSs

The standardized quantitative assessment of tumor-associated TLSs aims to provide a reproducible quantification method in tumors. However, the scientific quantification of TLSs is currently facing many challenges. The present techniques can be broadly divided into two categories: (1) Genomic level, such as 12-CK score and spatial histology (refer to Sections 3.3 and 3.4). This quantitative approach has demonstrated advantages in pan-cancer studies and can uncover the secrets at the gene level of TLSs, but it is expensive. (2) Histological level, where histology-based quantification methods are explored for clinically-driven purposes. The quantitative studies of tumor-associated TLSs at the histological level could be roughly summarized into three aspects: (1) Absolute TLS count. The total TLSs present inside and outside the tumor area, and the density is expressed as the number of TLSs per mm^2 area. This basic quantitative approach is applied to all types of tumor research with stability and interpretability (Helmink et al., 2020). Furthermore, some studies have quantified TLS size, spatial distribution, and constituent cell counts (Barmpoutis et al., 2021; Hoyt, 2021; Werner et al., 2021). (2) Normalization of the total area occupied by TLSs based on the total area of the tumor analyzed (Helmink et al., 2020). (3) TLS grade scores. TLS scoring models were inspired by the previous "immune score" analysis, including the quantification of tumor-infiltrating T cells. The categories that can be determined range from low to high immune cell density (Pagès et al., 2018; Marliot et al., 2020b). For instance, the tumor node metastasis-immune (TNM-I) score in colorectal cancer has been accepted in the clinical setting (Marliot et al., 2020a). The determination of immune score has given some insight into the quantification of TLSs and aided the development of quantitative criteria for TLSs. TLS scoring includes two types: grade scoring for the number of TLS distributions (Rakaee et al., 2021) and grade scoring based on TLSs and distribution features under the subdivision of TLSs relative to tumor location (Ding et al., 2022). For type one, Rakaee et al. (2021) developed three strategies of TLS scoring to optimize conventional TNM staging in non-small cell lung cancer for prognostic prediction. The first strategy is a semi-quantitative scoring model that divides TLSs into four levels of score (0–3), viz., absent, mild, moderate, and severe TLS distribution (while the researcher's specific criteria are unclear). The second strategy of the TLS scoring model is a four-level score using a manual count for the total number of lymphoid follicles and lymphocyte aggregates inside and outside the tumor. The third strategy is the manual quantification of GC+TLS. In terms of applicability, the first strategy is limited by subjectivity, is prone to variability, and is therefore not recommended. For the second strategy, the number of cut-off values for each TLS grade should be explored, and its stability needs verification. Regarding the third strategy, some studies have shown a promising prognosis for mature TLSs. In general, these models could enhance the power of TNM staging in prognosis prediction among non-small cell lung cancer patients by adding a scoring of the tumor-associated immune compartment to the TNM staging system. For type two, Ding et al. (2022) constructed a four-level scoring system based on a T (intra-tumor) score within the tumor and a P (peri-tumor) score around the tumor for intrahepatic cholangiocarcinoma. The T and P scores had four levels from 0 to 3: Score 0, no TLS; Score 1, one or two TLSs; Score 2, at least three TLSs; Score 3, massive TLSs involving the T or P region and converging. This study classified intrahepatic cholangiocarcinoma into four immune subtypes and revealed that the abundance and spatial distribution of TLSs were significantly related to patient prognosis. Type two is similar to that in the second strategy with a matching problem. On a positive note, the two types of quantitative scoring provide meaningful data to establish a correlation between TLSs and tumor prognosis. TLS analysis could help distinguish between patients with histologically similar tumors but presents differences in their clinical prognosis. Thus, while it improves patient management, its performance mainly depends on the TNM staging system (Cadiz et al., 2018; Hattori et al., 2019). However, the quantitative histological level requires highly informative manual counting, whether performed under the microscope or by whole slide imaging (WSI, a scanner produces a whole slide image). Manual counting could decrease the TLS positivity

rate and inherently includes subjectivity. Quantifying the histological level is at a distinct disadvantage compared to the genomic level, and further research is required to understand whether it applies to other cancer types.

5 AI in the assessment of TLSs

Integrating artificial intelligence (AI) with clinical medical practice has allowed the mining of large volumes of medical data and the extraction of significantly valuable information. Imaging data, including radiology and pathology images, are integral to medical data. In recent years, numerous AI studies have expanded the deep learning of graphic data, applying medical imaging analysis and computer-aided diagnosis to provide decision support to clinicians (Chan et al., 2020). Moreover, deep learning has made notable achievements in oncology research. The WSI of the lung cancer cohort was classified into one of the categories, including normal, lung adenocarcinoma, and lung squamous carcinoma, and the classification and mutation prediction of non-small cell lung cancer histopathological images using deep learning could reach a prediction effectiveness of 0.97 (Coudray et al., 2018). In radiology, the classification of textural patterns (e.g., ground glass cloudiness and micronodules) in the lung using deep convolutional neural networks achieved an average accuracy of 85% (Anthimopoulos et al., 2016). Deep learning was further employed to predict tumor grade (low or high) from the magnetic resonance imaging (MRI) images of liver cancer patients, with a reported prediction effectiveness of 0.83 (Zhou et al., 2019). Deep learning based on AI has also shown promising results in early screening, diagnosis, treatment, prediction of recurrence, and survival for various types of tumors.

5.1 Advantages of deep learning in TLS identification and quantification

The digital imaging of pathology slides using panoramic section digital scanning technology integrates cellular, tissue, and structural dimensions with AI to further explore histological features and perform qualitative and quantitative analyses of digital pathology slides. Preliminary attempts have been made at the detection and quantification of TLSs. Barmpoutis et al. (2021) identified TLS regions from lung cancer tissues by the deep convolutional neural network algorithm. In this report, the authors first defined the mathematical criteria of TLSs (i.e., the number of lymphocytes within TLSs), the TLS minimum area, and the density of lymphocytes within a given region with high stability and specificity, providing a basis for the standardization of TLS definition and quantification based on H&E images, thus facilitating the integration of TLS identification into clinical workflows. However, the algorithm generated by these authors failed to distinguish between different TLS stages and correlate TLS density with prognostic outcomes. The multi-resolution convolutional neural networks of HookNet (van Rijthoven et al., 2021) can automatically identify mature TLSs with GCs at a higher accuracy based on previous TLS identification; correspondingly, it has been used for pathological detection of lung cancer, providing advantages for the prognosis and treatment efficacy. Moreover, some digital imaging analysis platforms for quantifying TLSs in lung cancer are being developed. Future studies must demonstrate that this approach can be translated to other cancer types and ensure interlaboratory reproducibility.

The presence of GCs might be a determinant factor of the prognostic value of TLSs (Posch et al., 2018). Thus, a systematic quantitative approach was developed that described in detail the detection of different stages of TLSs (Siliņa et al., 2018b). This involved using a heptachromatic immunofluorescence technique with tyramine signal amplification combined with multispectral microscopy to acquire histological images, and finally, a tissue and cell segmentation algorithm for histological images to quantitatively analyze the data and detect mature TLSs. Similar computer-based readouts have been gradually developed, with deep learning allowing quantitatively and spatially assessing TLSs in tissue sections. Therefore, we propose that identifying TLS regions, automatically detecting the cells of interest within the tumor mesenchyme, and classifying immune cell clusters according to their differentiation degree may provide a fast and reliable method for TLS quantification.

5.2 Radiomics and TLS characterization

Radiomics is an emerging non-invasive method with immense application potential in tumor diagnosis and treatment (Xu et al., 2021). It utilizes the

computerized high-throughput extraction and quantification of high-order imaging features of tumors, and then integrates the obtained data with other clinical information to find associations with tumor diagnosis, tumor efficacy evaluation, molecular markers, and prognosis among others (Tumeh et al., 2014; Braman et al., 2017; Vaidya et al., 2020; Zhang et al., 2020). For example, this approach can extract clinically significant features of the TME and construct models by AI algorithms that predict the anti-tumor response of immune cells and the efficacy of programmed cell death protein-1 (PD-1) inhibitors (Tumeh et al., 2014; Braman et al., 2017). Another methodological innovation is the correlation of microscopic pathology with macroscopic images to predict the presence of TLSs. Exploring the association between tumor images and TLS status through imaging histology to predict the presence of TLS constitutes a breakthrough in the routine pathological tissue diagnosis of TLSs, providing a new direction in the detection and quantification of TLSs. For instance, Li PX et al. (2022) found image features associated with TLSs by radiomics for preoperative non-invasive prediction of TLSs within hepatocellular carcinoma. They constructed a preoperative prediction model of TLS with an average prediction effectiveness of 0.75, improving TLS prediction in hepatocellular carcinoma. In addition, Ren et al. (2022) studied the characteristics of TLSs in lung adenocarcinoma and associated factors. They classified the computed tomography (CT) imaging features of lung adenocarcinoma into pure ground-glass nodules (pGGNs), part-solid nodules (PSNs), and solid nodules (SNs). TLS density was significantly higher in PSNs and SNs than in pGGNs, having *P*-values of 0.008 and 0.046, respectively. Their study correlated the CT imaging features with TLS density and maturation. These emerging approaches, including the indirect association of radiomics to the presence of TLSs and deep learning for the identification, detection, and quantification of TLSs, extended the scope of TLS diagnostics from qualitative to quantitative description and have attracted the interest of an increasing number of researchers. Many relevant studies are still investigating the relationship between tumor imaging features and TLS-specific imaging features to accurately predict the presence of TLSs within tumors and provide options for TLS detection and quantification.

6 Conclusions and future prospects

Recently, the discovery of TLS formation in tumor tissues opened up a window of research opportunities for developing new immunotherapeutic approaches, and the prognostic value of tumor-associated TLSs has been extensively explored. The latest research focuses on the relationship between the structure and the regulation of tumor-specific immune responses and their induction and genetic characteristics. Importantly, the standardization of TLS detection and quantification is a prerequisite for the in-depth study of TLSs. The available data suggest that mIF and mIHC are beneficial for distinguishing between different stages of TLSs with high reproducibility, even if the relevant procedures are technically challenging. As for TLS quantification, manual scoring presents advantages, whereas the reproducibility of current methods remains to be improved.

Emerging AI approaches have overcome the limitations of manual TLS identification regarding quantification variability and other shortcomings. Meanwhile, the combination of immune scoring and AI has the potential to develop standardized quantification methods applicable to multiple cancer types. AI-based computational pathology can identify otherwise unraveled substructures at the cellular, structural, and tissue levels, further allowing for the quantification of their structural features (Nawaz and Yuan, 2016; Abels et al., 2019; Cui and Zhang, 2021). This approach further integrates additional clinical information, building models for TLS identification and tumor prognosis. The organic combination of AI and genomics or space omics facilitates powerful functions: it enables investigators to explore the differences in TLSs across tumors, leading to stable quantification at the genetic level.

In view of the above, AI could be a reliable TLS detection and quantification method. TLSs can be recognized in pathology images with high detection efficacy using AI algorithms. If different stages of TLSs could be distinguished, its description could be more accurately described. The number of TLSs, their cell composition, density, and genetic characteristics have been analyzed, providing preliminary results. However, for these techniques to be applied in clinical practice, cooperation between pathologists and computer scientists is fundamental. Moreover, standards and items for detection and quantification need to be developed, scanners for scanning pathology slides should be installed

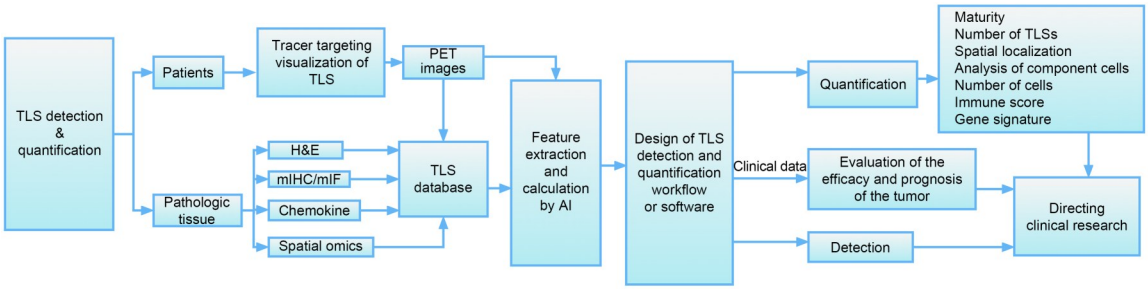

Fig. 3 Strategies for the AI detection and quantification of TLSs. If pathological tissues are available, biological information on TLSs can be obtained by H&E, mIHC/mIF, chemokine gene signature, and spatial omics. Otherwise, the images of TLSs are acquired by PET with tracer-targeted visualization. Subsequently, the TLS database is built. An AI algorithm is used to extract and calculate TLS features. The workflow or software for identifying and quantifying TLSs is constructed to serve clinical research. AI: artificial intelligence; H&E: hematoxylin and eosin; mIF: multiplex immunofluorescence; mIHC: multiplex immunohistochemistry; PET: positron emission tomography; TLS: tertiary lymphoid structure.

in every pathology center, and a sufficiently large data repository needs to be established. Designing a complete workflow for detection and quantification should be an easy task for computer scientists (Fig. 3).

We expect that future studies will report on the status of TLSs, for example, in patients with treated tumors where multiple biopsies are clinically difficult to perform. Moreover, less invasive alternative identification method is yet to be developed. Several studies have explored novel TLS biomarkers in peripheral blood and other body fluids. In addition, TLSs can be imaged by positron emission tomography (PET) and quantified by AI after patients have been injected with an appropriate targeted tracer. This non-invasive radiological approach will clearly facilitate the reproducible assessment of TLSs. The difficulty of this method, however, is finding the ideal tracer (Fig. 3).

In summary, TLS identification and quantification have multiple utilities within oncological clinical practice. The discussed studies provide preliminary results on using TLSs in the clinical setting; however, additional research is needed to fully unravel TLS biology and to develop standard criteria for TLS identification and quantification.

Acknowledgments

This work was supported by the Key Projects of Sichuan Natural Science Foundation (No. 2022NSFSC0051), the Clinical Scientist Program of Sichuan Cancer Hospital (No. YB2022003), and the Chengdu Technology Innovation R&D Project (No. 2021YF0501659SN), China.

Author contributions

Man YANG wrote and modified the manuscript. Weidong WANG supervised all aspects of the literature review design, manuscript modification, and enrichment. Yurou CHE, Kezhen LI, and Zengyi FANG carefully reviewed the manuscript's first draft and edited the Fig. 1. Simin LI, Mei WANG, and Yiyao ZHANG modified the manuscript. Zhu XU collected and edited the pathologic images containing TLSs in Fig. 2 and also drew Fig. 3. Liping LUO, Chuan WU, and Xin LAI contributed to the abstract and prospects, and edited these tables. All authors have read and approved the submitted version.

Compliance with ethics guidelines

Man YANG, Yurou CHE, Kezhen LI, Zengyi FANG, Simin LI, Mei WANG, Yiyao ZHANG, Zhu XU, Liping LUO, Chuan WU, Xin LAI, and Weidong WANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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