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REVIEW

Tertiary lymphoid structures and their therapeutic implications in cancer

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Abstract

Tertiary lymphoid structures (TLSs) are ectopic lymphoid aggregates formed by the structured accumulation of immune cells such as B cells and T cells in non-lymphoid tissues induced by infection, inflammation, and tumors. They play a crucial role in the immune response, particularly in association with tumor development, where they primarily exert anti-tumor immune functions during tumorigenesis. Current research suggests that TLSs inhibit tumor growth by facilitating immune cell infiltration and are correlated with favorable prognosis in various solid tumors, serving as an indicator of immunotherapy effectiveness to some extent. Therefore, TLSs hold great promise as a valuable biomarker. Most importantly, immunotherapies aimed to prompting TLSs formation are anticipated to be potent adjuncts to current cancer treatment. This review focuses on the formation process of TLSs and their potential applications in cancer therapy.

Keywords Tertiary lymphoid structures · Immune response · Prognosis · Biomarker · Cancer therapy

Abbreviations

TLSs	Tertiary lymphoid structures
TSA	Tumor-specific antigen
TAA	Tumor-associated antigen
TILs	Tumor-infiltrating lymphocytes
PD-L1	Anti-programmed death ligand 1
TME	Tumor microenvironment
TLOs	Tertiary lymphoid organs
ELSs	Ectopic lymphoid-like structures
GC	Germinal center
HEV	High endothelial venules
LVs	Lymphatic vessels
SLOs	Secondary lymphoid organs
PC	Plasma cells
LTi	Lymphoid tissue-inducing
LT	Lymphotoxin
PDPN	Podoplanin
FAP	Fibroblast activating protein
LTβR	Lymphotoxin β receptor
PNAd	Peripheral node addressin
MAdCAM	Mucosal addressin cell adhesion molecule
FRCs	Fibroblastic reticular cells
FDCs	Follicular dendritic cells
E-TLSs	Early tertiary lymphoid structures
PFL-TLSs	Primary follicle-like tertiary lymphoid structures
SFL-TLSs	Secondary follicle-like tertiary lymphoid structures
ILC	Innate lymphocyte
NSCLC	Non-small cell lung cancer
MCPyV	Merkel cell polyomavirus
EBVaGC	EBV-associated gastric cancer
EBVnGC	EBV-negative gastric cancer
HNSCC	Neck squamous cell carcinoma
ccRCC	Clear cell renal cell carcinoma
ST	Spatial transcriptome

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CRC	Colorectal cancer
APCs	Antigen-presenting cells
PDCs	Plasmacytoid dendritic cells
iTLSs	Intra-tumor tertiary lymphoid structures
pTLSs	Peritumor tertiary lymphoid structures
HCC	Hepatocellular carcinoma
nmCRC	Non-metastatic colorectal cancer
gADC	Gastric adenocarcinoma
DFS	Disease-free survival
OS	Overall survival
H&E	Hematoxylin-eosin staining
IHC	Immunohistochemical staining
IF	Immunofluorescent staining
scRNA-seq	Single-cell sequence
PDA	Pancreatic ductal adenocarcinoma
IL2	Interleukin 2
NLR	Neutrophil-to-lymphocyte ratio
PBMCs	Peripheral blood mononuclear cells
STS	Soft tissue sarcoma
ICB	Checkpoint blockade
RT	Radiotherapy
STING	Interferon gene
APC-HB	Hepatoblastoma related to Adenomatous Polyposis Coli
CAR-T	Chimeric antigen receptor T-cell therapy
ORR	Objective remission rates
sLN	Lymph node stroma
artTLSs	Artificial tertiary lymphoid structures
mIFH	Multiplex immunofluorescence histology
ICI	Immune checkpoint inhibitors

1 Introduction

Tumor immunotherapy is emerging as the future direction for cancer treatments due to its minimal side effects and pronounced therapeutic benefits. However, the efficacy of immunotherapy varies significantly between "hot tumor" patients, usually characterized by increased immune cell infiltration and immune activation, and "cold tumor" patients, often marked by limited immune cell presence and immune suppression [1]. The concept of "hot" or "cold" is used to refer to T-cell infiltrated, inflamed or not infiltrated, non-inflamed tumors, respectively [2]. In addition to the presence of tumor-infiltrating lymphocytes (TILs), other features such as the expression of anti-programmed death ligand 1 (PD-L1) on tumor-associated immune cells are also considered. The presence of pre-existing anti-tumor immune responses (e.g., accumulation of adaptive immune components that actively recognizes the tumor-specific antigen [TSA] or the tumor-associated antigen [TAA]) has been described as characteristic of "hot tumor". In contrast, "cold" tumors are not only poorly infiltrated but also immunologically naive with almost no expression of PD-L1. They are characterized by high proliferation, low expression of neoantigens, and low expression of markers of antigen presentation mechanisms such as major histocompatibility complex class I (MHC I), which leads to reduced recognition and clearance of the tumor. This discrepancy underscores the pivotal role of TILs in anti-tumor immunity, directly influencing the prognosis and therapeutic efficacy in patients with tumors [3]. Recent studies of the tumor microenvironment (TME) have revealed that immune cells within tertiary lymphoid structures (TLSs) play a crucial role in exerting anti-tumor effects [4].

TLSs, alternatively referred to as tertiary lymphoid organs (TLOs) or ectopic lymphoid-like structures (ELSs) [5], are induced in non-lymphoid tissues under non-physiological conditions such as chronic infections, transplant rejection, autoimmune diseases, and tumors. These formations are primarily result from organized aggregation of immune cells such as B cells and T cells [6]. The formation, structure, and function of TLSs can be influenced by their location and specific inflammatory stimuli, potentially resulting in variations in TLSs characteristics across different disease contexts or tissues [7]. The role of TLSs in diseases often hinges on the specific disease background [8]. For instance, in autoimmune diseases [9] and organ transplantation [10], TLSs are frequently regarded as detrimental to patient health and survival. Conversely, in tumors, a predominantly positive impact has been reported [4], with associations to a favorable prognosis reported in various solid tumors [6, 11]. This review primarily explores the formation process of TLSs and the research progress in the context of tumors, emphasizing the significance and potential applications of TLSs in cancer therapy.

2 Formation and structure of TLSs

TLSs are ectopic lymphoid aggregates characterized by a structural composition that includes germinal centers (GC), B-cell regions, T cell regions, high endothelial venules (HEV), and lymphatic vessels (LVs) [12]. Due to their lack of stable structural organization, including capsules, they cannot be strictly defined as organs and are referred to as tertiary lymphoid organs/structures [13].

The process of initiating TLSs formation at sites of inflammation or tissue damage is known as lymphoid neogenesis [7, 14]. While the precise mechanisms underlying lymphoid neogenesis remain incompletely elucidated, it is known to involve signaling pathways akin to those observed in lymphoid organogenesis (Fig. 1) [14]. Upon prolonged exposure to inflammation or chronic infection, lymphocytes

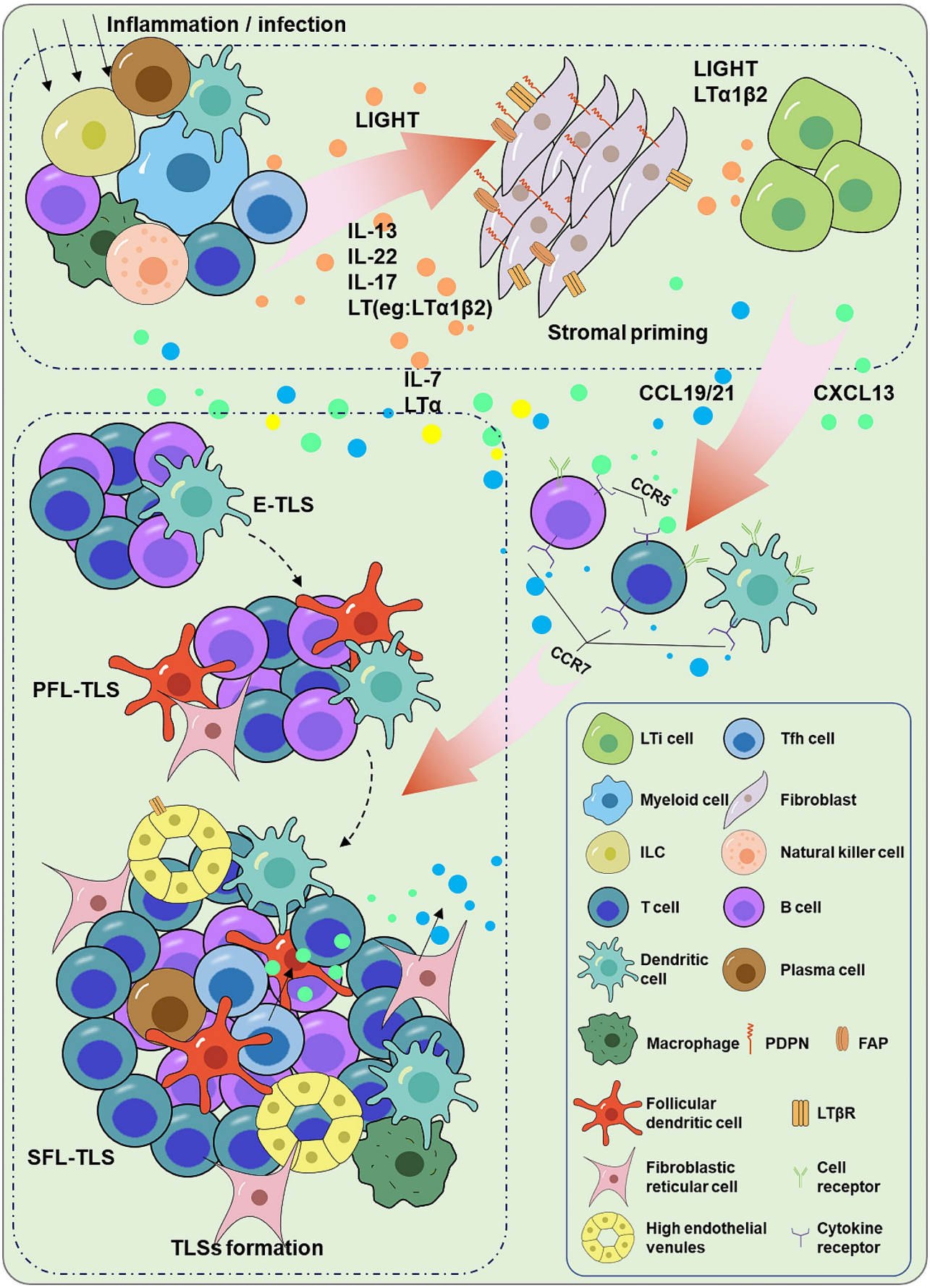
Inflammation / infection
LIGHT
LIGHT
LTα1β2
IL-13
IL-22
IL-17
LT(eg:LTα1β2)
Stromal priming
IL-7
LTα
CCL19/21
CXCL13
CCR5
CCR7
E-TLS
PFL-TLS
SFL-TLS
TLSs formation
LTi cell
Tfh cell
Myeloid cell
Fibroblast
ILC
Natural killer cell
T cell
B cell
Dendritic cell
Plasma cell
Macrophage
PDPN
FAP
Follicular dendritic cell
LTβR
Fibroblastic reticular cell
Cell receptor
High endothelial venules
Cytokine receptor

Fig. 1 Lymphoid neogenesis. Under prolonged stimulation from inflammation or chronic infection, immune cells and stromal cells orchestrate the recruitment of B cells, T cells, and other lymphocytes by expressing specific chemokines and cytokines. This gradual process leads to the formation of lymphoid aggregates with distinct structures, such as early tertiary lymphoid structures (E-TLSs), primary follicle-like TLSs (PFL-TLS), and secondary follicle-like TLSs (SFL-TLSs). Among these, E-TLSs is a dense cluster of lymphocytes comprising B cells and T cells, lacking differentiation into a FDC network and germinal center (GC). PFL-TLSs is a B cell cluster containing a mature FDC network but devoid of a GC. SFL-TLSs closely resembles secondary follicles in secondary lymphoid organs (SLO) and includes a GC containing Tfh cells. *LTi* lymphoid tissue-inducing cells, *ILC* innate lymphoid cells, *Tfh cells* T follicle helper cells, *PDPN* podoplanin, *FAP* fibroblast activating protein, *LTβR* lymphotoxin β receptor

and immune cells release cytokines, including IL-13, IL-17, IL-22, and lymphotoxin (LT). These cytokines stimulate stromal cells to differentiate into fibroblasts expressing podoplanin (PDPN) and fibroblast activation protein (FAP) [15], forming an intricate network of immune fibroblasts that recruit LTi cells to the inflammatory site [16]. Notably, IL-17 and IL-22 are produced not only by immune cells but also by LTi cells. IL-22 and LT play pivotal roles in maintaining and enhancing the expression of chemokines CXCL12 and CXCL13 [17]. Stromal cells orchestrate the recruitment of B cells, T cells, dendritic cells (DCs), and other lymphocytes through the expression of specific chemokines and cytokines, including CCL19, CCL21, CXCL13, LTβ, and IL-7 [18–20]. For instance, IL-7 is vital in the development of various immune cells and the preservation of immune homeostasis, promoting inflammatory responses mediated by T cells and macrophages [17, 18]. The expression of CXCL13 and CCL19/CCL21 further attracts cells positive for the CXCL13 receptor (CXCR5, found on B cells and T cells) and cells positive for the CCL19 and CCL21 receptor (CCR7, present on B cells, T cells, DCs, etc.) to the site [21]. This facilitates the aggregation of lymphocytes in TLSs, leading to the formation of structures such as germinal centers (GCs), B cell areas, and T cell areas. The primary function of GC is to generate plasma cells (PCs) and memory B cells, which produce high-affinity antibodies [22]. T follicle helper (Tfh) cells are the main origin of CXCL13 in GC and play an instrumental role in the formation and maintenance of TLSs [23]. Meanwhile, LTi cells and immune cells activate the lymphotoxin β receptor (LTβR) on stromal cells by expressing LTα1β2 and LIGHT (also known as TNFSF14) [24, 25], mediating normal expression of PNAd and MAdCAM to maintain HEV homeostasis and allow more lymphocytes enter into TLSs [26]. In addition, LTβR signaling is implicated in the formation of structures such as T cell zones, B cell zones, GCs, fibroblastic reticular cells (FRCs), and follicular dendritic cells (FDCs) [27, 28]. It may also induce or maintain the expression of CXCL13 and CCL21 in TLSs [29]. The establishment of these structures positively recruits immune cells and stimulates the production of chemokines and cytokines, thereby fostering the continued development of TLSs. For example, FRCs, a major source of the T cell zone regional chemokines CCL19 and CCL21, recruit T cells and DC expressing the CCR7 ligand [28]. The resulting TLSs structures vary widely depending on the nature of inflammation and the tissue involved, ranging from early TLSs (E-TLSs) with only B and T cell aggregates to primary follicle-like TLSs (PFL-TLSs) characterized by the differentiation of a mature FDC network, as well as secondary follicle-like TLSs (SFL-TLSs) featuring Tfh cell-containing GCs [30, 31].

3 Composition and interaction of cells in tumor-associated TLSs

Similar to other diseases, the cellular composition of tumor-associated TLSs mainly includes germinal center B cells, cytotoxic T cells, plasma cells, T helper cells (Th), DCs, HEV-associated cells, macrophages, and neutrophils (Table 1) [32]. While TLSs has been identified in a variety of solid tumors such as lung cancer [33], melanoma [34], and soft tissue sarcoma [35], their presence is remarkably heterogeneous and not universal across all tumor types or patients.

The specific process of TLSs formation in tumors may vary somewhat. Recent studies have unveiled some key factors influencing the formation of TLSs in tumors, notably highlighting the substitutability of LTi cells. For example, in non-small cell lung cancer (NSCLC), NCR^+ innate lymphocyte (ILC)-3 exhibits LTi properties and produces IL-22, TNF-α, IL-8, and IL-2 upon activation through interaction with tumor cells or tumor-associated fibroblasts. This activation extends to endothelial cells, promoting the formation and/or maintenance of TLSs within the tumor [50]. Additionally, activated $CD4^+$ T cells can replace LTi cells in anti-tumor immunity by downregulating the expression of genomic organizer SATB1 and secreting LIGHT in the tumor microenvironment in response to TGF-β signaling, which in turn activates endothelial cells and associated fibroblasts to generate CCL21 and CXCL13, promoting the formation of TLSs [51]. Contrary to previous perspectives suggesting LTβR signaling stimulation as a necessary factor in tumor lymphoid neogenesis [7], in some tumor types, HEV formation is not linked to LTβR signaling but is mediated by cytokines such as LTα3 [52] and IL-36γ [53]. Analysis of the localization of immune cells in TLSs and understanding tumor-immune cell interactions will provide novel avenues for subsequent precision immunotherapy.

As an important factor influencing tumor progression, the specific mechanisms through which viral infections

Table 1 Major cell types in TLSs and their markers

Structure	Cell type	Molecular markers	References
B-cell zone	B cell	CD19, CD20, CD21, CD23, CD35	[16, 36, 37]
	GC B cell	AID, Ki67, CD269	[38, 39]
	FDC	CD21, CD23, CD35	[40]
	PC	CD138, CD269	[16]
T-cell zone	T cell	CD3, CD4, CD8	[41, 42]
	Treg cell	FOXP3	[43, 44]
	TH1 cell	T-bet	[45]
	Tfh cell	PD1, CXCR5	[34]
	FRC	CCL19, CCL21	[46]
	DC	DC-LAMP, CD83, CD86	[38, 47]
Others	Neutrophil	CD66b	[48]
	Macrophage	CD68	[49]
	HEV	PNAd, MECA79	[47]

GC germinal center, *FDC* follicular dendritic cell, *Treg cell* regulatory T cell, *PC* plasma cell, *TH1 cell* T helper 1 cell, *Tfh cell* follicular helper T cell, *FRC* fibroblastic reticular cell, *DC* dendritic cell, *HEV* High endothelial venule

impact the development of TLSs in tumor patients remain undetermined. Different oncogenic viruses exert varying effects on TLSs in tumor patients. For example, a study on Merkel cell carcinoma found no correlation between Merkel cell polyomavirus (MCPyV) infection and the presence of TLSs [54]. In contrast, a study on EBV-associated gastric cancer (EBVaGC) and EBV-negative gastric cancer (EBVnGC) identified a prognostic role for TLSs exclusively in patients with EBVnGC, with no association with prognosis in patients with EBVaGC [37]. Furthermore, other environmental factors have a significant impact on TLSs. For instance, in patients with HPV^+ HNSCC, those with a history of smoking produced more TLSs compared to non-smokers [43]. Further studies are needed to establish whether viral infection is involved in the process of TLSs neogenesis in tumor patients.

Interactions between the cells in TLSs collectively promote anti-tumor immunity. T cells can drive B cells into TLSs, facilitating their differentiation. A study [55] in nasopharyngeal carcinoma revealed a subset of Th cells expressing PD1 and the B lymphocyte chemokine CXCL13 in TLSs drove B cells into TLSs and that Th-CXCL13 cells promoted PC differentiation and immunoglobulin production within nasopharyngeal carcinoma TLSs through IL-21 and CD84 interactions. In turn, differentiated B-cell-derived PCs exert anti-tumor effects along with fibroblasts, as evidenced by a study on clear cell renal cell carcinoma (ccRCC) that identified B cells in various maturation states by analyzing differential gene expression from naive cells to PCs within TLSs using spatial transcriptome (ST) technology. The study found PCs secreting immunoglobulins IgA and IgG in TLSs spreaded toward tumors alongfibroblast tracks expressing CXCL12, which was chemotactic for lymphocytes, indicating their association with anti-tumor responses [22]. Similarly, another study also revealed the close relationship between B cell differentiation and TLSs by ST. In that study, different subtypes of B cells including naïve, anterior germinal centers, germinal centers, anterior plasma cells, plasma cells, and memory B cells were detected in tumors enriched with TLSs, where naïve and anterior germinal centers were predominantly associated with early stage TLSs, whereas GCs and plasma cells were predominantly associated with mature TLSs [56]. These findings suggest that TLSs support B cell maturation and differentiation in situ, supporting a role of plasma cells in the promotion of tumor immunity in patients. There is a lack of relevant studies on immature B cells, transitional-B cells, and regulatory B cells. Of note, a recent study reported that a subpopulation of atypical B cells ($CD21^{-/low}CD11c^+$) in TLSs promoted cancer progression in non-muscle invasive bladder cancer and showed immunosuppressive effects with poor response to BCG vaccine, suggesting that different subpopulations of B cells in tumor-associated TLSs may play different roles [57]. In addition to B cells and T cells, DCs play a crucial role in TLSs as well. DCs are necessary for early Tfh differentiation. FDCs located in the GC are important for memory B cell selection, and DC-LAMP (also known as CD208 or LAMP3, is a molecular marker of dendritic cell maturation) positive mature DCs in the T cell zone serve as the major type of antigen-presenting cells (APCs) promoting cytotoxic T cell production [58]. As research progresses, new cellular components of TLSs are emerging. For instance, in a study on human CRC, plasmacytoid dendritic cells (pDCs) were identified as a new component of the T cell zone in CRC-associated TLSs. These pDCs exhibited nuclear IRF7 expression and were preferentially located near $CD4^+$ T cells, suggesting a potential role in inducing or maintaining T cell-mediated antitumor responses [59].

4 Role of TLSs on tumorigenesis

Recent studies highlight the close association between TLSs and the grading and staging of tumor development. An analysis of 176 gastric cancer patients from The Cancer Genome Atlas (TCGA) demonstrated an elevated abundance of TLSs in patients with advanced (T2–T4) gastric cancer compared to those in the early (T1) stage. Consistent findings were observed in mouse models as well [60]. Conversely, a study involving 28 colorectal cancer (CRC) patients revealed a decreased density of TLSs in T3/T4 stage tumor patients compared to both normal tissue and T1/T2 stage tumor patients [61]. These findings suggest

that TLSs may fulfill distinct functions in different tumors. Moreover, the distribution of TLSs varies across different tumor grades and subtypes. In a preliminary study of bladder cancer patients, TLSs were more prevalent in high-grade muscle-invasive bladder cancer than in non-muscle-invasive bladder cancer [62]. Similarly, an investigation of sarcoma patients revealed differing immune cell distributions among soft tissue sarcoma subtypes. Undifferentiated pleomorphic sarcoma specimens displayed diffuse intertumoral infiltration of T cells, whereas rhabdomyosarcoma specimens showed intratumoral T cells aggregating with B cells near the perivascular bed to form TLSs [63]. These variations underscore the nuanced role of TLSs in different tumor contexts and highlight their potential significance in tumor progression and immune response modulation.

TLSs has been observed in metastatic sites of various cancer types. The density of TLSs appears to be influenced by the primary tumor type and the organ of metastasis. For example, the density of TLSs in lung metastases showed significant differences among tumor types, with high densities in colon and prostate cancers, moderate densities in thyroid, kidney, liver, and breast cancers, and notably low densities in smooth muscle sarcomas and osteosarcomas [11, 16]. However, the location of TLSs was reported to be independent of the metastatic site [34]. There exists some disparity in the density of TLSs in metastases compared to the primary site, which to some extent reflects the disease progression. In a study of human melanoma, the density of TLSs was higher in metastatic melanoma than in primary melanoma, with primary melanoma predominantly being E-TLSs and a significant increase in SFL-TLSs in metastatic melanoma [64].

In addition to the site of metastasis, the relative location of TLSs to the tumor also has an impact on their function. The role of intra-tumor TLSs (iTLSs) and peritumor TLSs (pTLSs) in different tumor types varies across existing studies. A higher density of pTLSs was reported in the presence of iTLSs, suggesting a potential synergistic effect of iTLSs and pTLSs in predicting the survival of hepatocellular carcinoma (HCC) patients [41]. Conversely, in a study of oral cancer patients, the presence of iTLSs significantly reduced P53 and Ki67 scores, while pTLSs showed no correlation [65]. Currently, there is a scarcity of studies on peritumor and invasive edge TLSs. More refined investigations into iTLSs and pTLSs can contribute to a more comprehensive understanding of the spatial role of TLSs. For instance, pTLSs have been associated with poor prognosis in breast cancer patients [44]. But, a study in patients with non-metastatic colorectal cancer (nmCRC) found that high-density pTLSs and low tumor stroma percentage were independent and favorable prognostic factors in nmCRC patients, whereas iTLSs were not associated with clinical outcomes [40].

5 Impact of TLSs on other cells in TME

TLSs formed within the bodies of tumor patients act as a hub for local antitumor immune responses, possibly by generating or recruiting more immune cells in the TME to promote antitumor immunity. In HCC patients with TLSs, there was a significant increase in the relative numbers of memory B cells, plasma cells, $CD8^+$ T cells, NK cells and dendritic cells whereas the relative numbers of Treg cells, macrophages, and M2 macrophages were substantially decreased [66]. Similarly, a study in gastric adenocarcinoma (gADC) found that mature TLSs can enrich a variety of immune cells in tissues compared to normal TLSs. Tissues with mature TLSs exhibited high expression of IgA and complement factors, indicating that IgA and complement activation pathways may play a crucial role in TME of gADC [67]. Moreover, recent studies have shown that differences in the composition of TLSs may not solely depend on maturation status but are influenced by TME [68]. However, the specific mechanisms and molecules through which TME affects the composition and function of TLSs necessitate further research for clarification.

In addition to their role in promoting anti-tumor immunity, TLSs have been found in some studies to have a function in supporting tumor growth, with different cell compositions within tumor-associated TLSs potentially yielding distinct effects. For example, in a study utilizing genetically engineered mouse model of lung adenocarcinoma, it was observed that Treg cells in the lung were predominantly located in tumor-associated TLSs, where they suppressed anti-tumor immunity. Notably, after depleting Treg cells, there was an increase in the expression of DC co-stimulatory molecules and T cell proliferation rates within TLSs, resulting in enhanced anti-tumor capacity [69]. Similarly, a study in breast cancer patients revealed the presence of large numbers of Treg cells within TLSs rather than in the tumor stroma, which was associated with poor patient survival [70].

TLSs may also play a pivotal role in promoting the infiltration of lymphocytes into tumors (Fig. 2). For instance, a study in melanoma demonstrated that the regulation of lymphocytes within tumors and those within TLSs was independent. The lymphoid neogenesis and maturation of TLSs were correlated with a high density of intra-tumoral lymphocytes, suggesting that factors promoting TLSs formation may also contribute to lymphocyte infiltration within tumors [71]. Similarly, a study of gastric cancer patients found that 70% of TILs in gastric cancer tissues were CD103 $CD8^+$ T

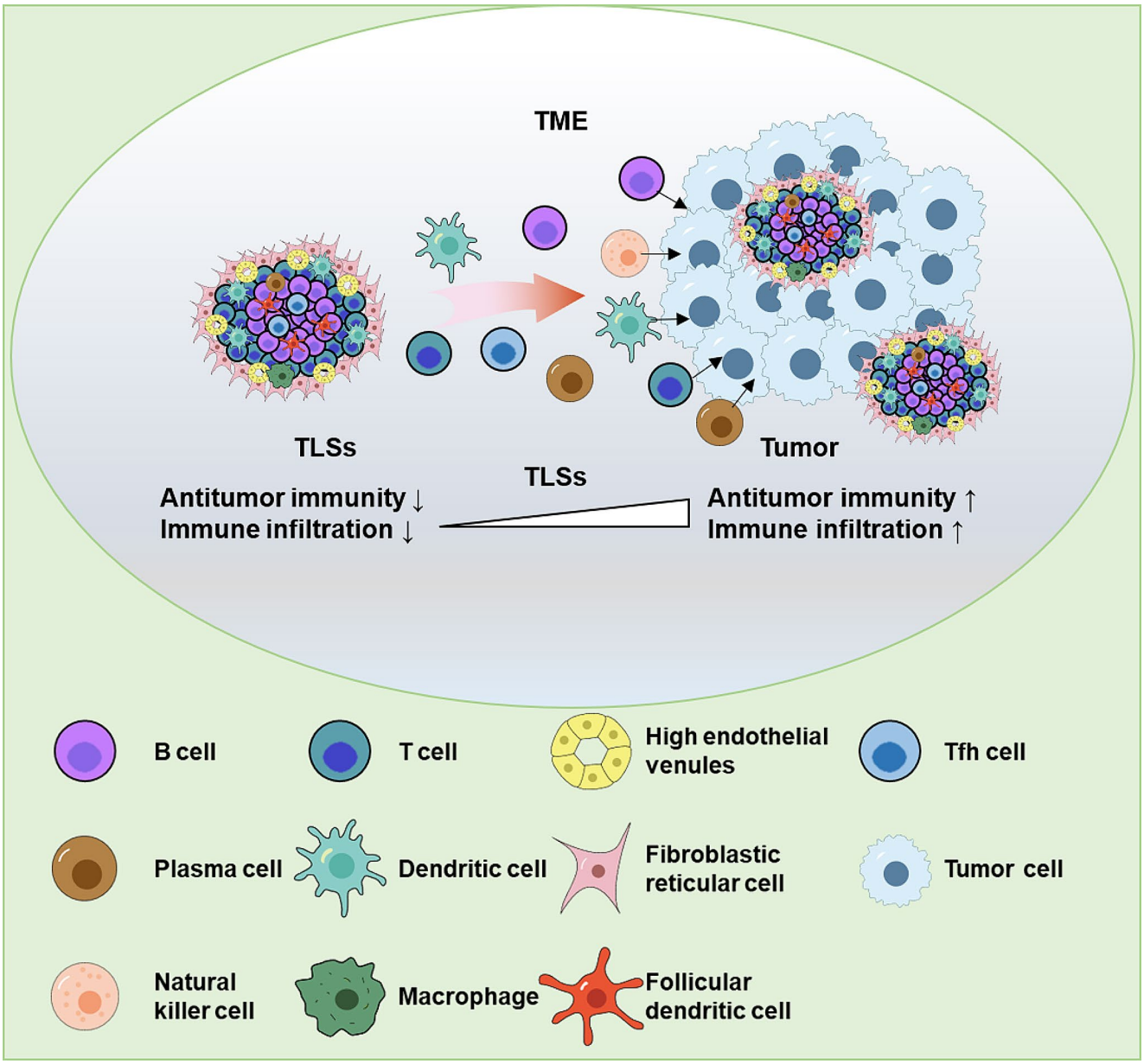

Fig. 2 Role of tumor-associated TLSs in TME. TLSs are distributed in tumor stroma, invasive margins, and/or tumor core to promote immune cell infiltration into the tumor, enhancing anti-tumor immunity. *TME* tumor microenvironment

cells. CD103 T cells, identified as tissue-resident memory T cells, were distributed around TLSs, which are activated effector T cells. TLSs likely facilitated infiltration of CD103 T cells into tumor tissue [72]. Co-infiltration of TLSs-containing intra-tumor B cells with $CD8^+$ T cells have also been observed across various tumor types [68, [73]. Beyond interactions with immune and tumor cells, TLSs also engage with other cells in the TME. In a recent melanoma study, it was found that tumor-associated sensory neurons negatively modulate antitumor immunity in TME, which limit the formation of mature TLSs by impeding the maturation of intratumoral HEVs [74].

6 TLSs as a diagnostic marker for cancer

Histologically, TLSs are typically considered to be discrete entities with smooth rounded contours composed of mature, tightly aggregated lymphocytes [75]. TLSs tend to be found in the tumor stroma, invasive margins, and/or tumor core, with higher abundance in the stroma and invasive margins [16]. The specific composition of TLSs varies depending on their proximity from the tumor cells [76]. As such, the composition and functional status of TLSs play a vital role in determining the prognosis and therapeutic efficiency in patients with cancer, among other factors.

TLSs have emerged as valuable biomarkers [3, 77], and their prognostic significance has been investigated across various tumor types. While different methods exist for identifying TLSs in tumor sections, a growing body of research highlights their association with extended disease-free survival (DFS) and overall survival (OS) in diverse malignancies (Table 2). Studies indicate a positive prognostic correlation between TLSs and various cancers, including melanoma [34, 71], colorectal cancer [40], lung cancer [78, 79], liver cancer [80], breast cancer [81, 82], gastric cancer [37, 83], pancreatic cancer [49, 84], and oral cancer [36, 38, 47, 65]. Nevertheless, conflicting findings suggest that TLSs can also been linked to poor prognosis in certain tumor types [44, 85, 86]. In a comparative study, TLSs were associated with unfavorable outcomes in diverse genitourinary cancers such as human ccRCC, while demonstrating the opposite trend in bladder cancer, with significant differences in maturation and spatial distribution of TLSs in both cancers, with bladder cancer exhibiting more mature TLSs containing easily discernible GC [85].

The role of TLSs in HCC remains controversial. Studies suggest a dual role for TLSs in HCC, with some indicating an association with better prognosis in HCC patients [41, 80, 87], while others report a connection to poor prognosis [86]. Further exploration is warranted to elucidate the specific role of TLSs in different tumor types. The prognostic significance of TLSs varies across tumors, where their mere presence is linked to a favorable prognosis in certain cases [49, 71], irrespective of quantity. In contrast, in other tumors, the density or number of TLSs is associated with prognosis [87, 88]. Moreover, the maximum diameter of TLSs in tumor sections has been identified as a factor correlating with prognosis [83]. A comprehensive assessment of the presence, density, location relative to the tumor, immune cell composition, and maturation of TLSs is essential for effectively exploiting their prognostic value in tumor patients.

Currently, the detection of TLSs in tumors relies on the examination of whole sections of tumor masses, particularly those displaying the most stromal infiltration, which has proven to be a reliable method [16]. Various evaluation methods for TLSs in tumors include histochemical staining, predominantly hematoxylin-eosin staining (H&E staining), as well as immunohistochemical staining (IHC) and immunofluorescent staining (IF). The identification of TLSs markers is achieved through dual or multiple labeling techniques. However, the accuracy of these assessment methods for TLSs in tumors can vary. For example, a breast cancer study comparing the concordance of H&E staining with IHC staining, assessed by experienced pathologists, revealed that IHC staining exhibited greater accuracy and consistency in TLSs assessment than H&E staining [89]. In the meantime, digital methods are gaining traction for TLSs evaluation, allowing for quantitative assessments of TLSs density, size, and cell content in scanned images through experimental quantitative digital pathology software. In a study focused on TLSs associated with lung cancer tissues, an automated identification and quantification method for TLSs in H&E-stained tissue images was developed [75], and similar methodologies have been applied in tumor types such as esophageal squamous cell carcinoma [38].

In addition to morphological assessments, advancements in technologies like single-cell sequencing (scRNA-seq) and spatial transcriptomics (ST) have enabled the confirmation of TLSs and provided gene expression-related information in transcriptomics. This offers a more comprehensive understanding of the interaction between TLSs and tumors. For instance, a recent study in breast cancer integrated scRNA-seq data with ST data to construct a predictive model for TLSs presence. That study successfully constructed a prediction model for TLSs and experimentally verified its strong predictive potential, thereby advancing research on tumor heterogeneity [90]. While molecular markers have been pivotal in evaluating tumor-associated TLSs, the lack of standardized assessment methods hinders direct comparative studies across different tumor types. Therefore, establishing standardized quantitative assessment methods for TLSs in tumors and defining recognized criteria for tumor-related TLSs will facilitate the detection of differences in the phenotypic and spatial distributions of TLSs, aiding in disease staging in large tumor samples.

Moreover, identifying potential blood biomarkers for predicting the presence of TLSs can offer a more convenient means of detecting TLSs in patients. For example, a study on pancreatic ductal adenocarcinoma (PDA) revealed that patients with TLSs exhibited notably higher interleukin 2 (IL2) levels in the tumor stroma compared to those without TLSs. Furthermore, only patients lacking TLSs displayed significantly higher serum IL2 levels than in the tumor stroma. Consequently, serum IL2 levels may serve as a potential marker for predicting TLSs [84]. Another study in NSCLC patients demonstrated an association between high TLSs expression and a low neutrophil-to-lymphocyte ratio (NLR) in peripheral blood mononuclear cells (PBMCs). Additionally, patients with high TLSs expression had lower counts of cells expressing HLA-DR and CD9 in their PBMCs [79]. Combining NLR and the number of cells expressing HLA-DR and CD9 in peripheral blood could potentially predict the presence of TLSs. To facilitate the clinical application of TLSs as a biomarker, it is crucial to develop non-invasive methods for predicting TLSs and standardize the characterization techniques for TLSs.

As a biomarker, the amalgamation of TLSs with other biomarkers holds promise for achieving enhanced

Table 2 Prognostic impact of TLSs in human cancers

Cancer	Tumor location	Treatment	Patients numbers	Methods	Prognostic value	References
Breast cancer	Primary tumor	NA	167	H&E and IHC (CD8, CD4, CD163, FOXP3, CD20, CD3, CD23, CD31)	Adverse	[44]
Breast cancer (Invasive breast cancer)	Primary tumor	HR^+: adjuvant endocrine therapy with adjuvant chemotherapy; HR^-: adjuvant chemotherapy.	248	H&E and IHC (CD23, CD20, CD3)	Favourable in $HER2^+$ breast cancer	[81]
Colorectal cancer	Primary tumor	Elective laparoscopic surgery	174	H&E and IHC (CD20, CD3, CD21, CD23, CXCL13, PNAd)	Favourable	[40]
Gastric cancer	NA	NA	721	H&E	Favourable	[83]
Gastric carcinoma	NA	NA	932	H&E and IHC (CD3, CD20, CD21)	Favourable in EBVnGC	[37]
Hepatocellular carcinoma	NA	NA	365	H&E and gene signature	Favourable	[80]
Hepatocellular carcinoma	Primary tumor	Surgical resection	360	H&E and IHC (CD3, CD8, FOXP3, CD20, CD68, CD57) and gene signature	Favourable	[41]
Hepatocellular carcinoma	Primary tumor	Radical surgery	126	H&E, IHC (CD20, CD21, CD23) and IF(CD20, CD21, CD23)	Favourable; NLR add better prognostic value to TLSs	[87]
Head and neck squamous cell carcinoma	NA	Surgical resection	124	IHC (CD8, CD20, CD4, CD68, FOXP3) and gene signature	Favourable	[43]
Human clear cell renal cell carcinoma	NA	Surgery	105	H&E and IHC (CD20, CD3, Bcl6, CD10, CD21) and gene signature	Adverse	[85]
Lung adenocarcinoma	NA	NA	515	Gene signature	Favourable	[78]
Melanoma	Metastatic tumor	ICB (CTLA4 blockade, 37/119)	296	IF (CXCR5, CXCL13, CD20) and gene signature	Favourable	[34]
Melanoma	Metastatic tumor	metastasectomy	64	mIFH (CD8, CD20, CD21, CD23, PNAd)	Favourable	[71]
Merkel cell carcinoma	Primary tumor and metastatic tumor	Surgery, radiation therapy or both and Chemotherapy (2/61), ICI(2/61)	61	IHC (CD3, CD20, CD21) and gene signature	Favourable	[54]
Non-small cell lung cancer	Primary tumor	NA	147	IHC (PNAd)	Favourable in advanced group patients	[79]
Oral cancer	Primary tumor	Curative surgery	65	H&E and IHC(CD21) and gene signature	Favourable	[65]
Oesophageal squamous cell carcinoma	Primary tumor	Complete resection	650	H&E and IHC (CD20, Ki67, CD21, CD4, LAMP3, CD8) and spatial transcriptomic characteristics	Favourable	[38]
Oral squamous cell carcinoma	NA	NA	106	IHC (CD3, CD20, CD21) and gene signature	Favourable	[36]
Oral squamous cell carcinoma	Primary tumor	NA	168	IHC (PNAd, CD20, CD3, LAMP3)	Favourable	[47]
Pancreatic ductal adenocarcinoma	NA	NA	55	IHC (CD3, CD4, CD8, CD20, CD21, CD163, DC-LAMP)	Favourable	[84]
Pancreatic neuroendocrine tumor	NA	Pancreatectomy	307	H&E and IHC (CD4, CD8, CD20, CD11c, CD25RO, anti-NCR1, FOXP3, CD68)	Favourable	[49]
Urachal carcinoma	NA	adjuvant chemotherapy (18/37)	37	IHC (CD4, CD8, CD20, CD3, FOXP3, HLA-DR, CD68)	Favourable	[88]

ICB checkpoint blockade, *IF* immunofluorescent staining, *mIFH* multiplex immunofluorescence histology, *H&E* hematoxylin-eosin staining, *NA* not available, *IHC* immunohistochemical staining, *NLR* neutrophil-to-lymphocyte ratio, *EBVnGC* EBV-negative gastric carcinoma, *ICI* Immune checkpoint inhibitors

prognostic outcomes. Of note, in gastric cancer patients, a reported synergy between NLRs and TLSs has demonstrated improved prognostic stratification [91]. Furthermore, in terms of predictive value, TLSs have the potential to inform clinical decision-making and guide treatment strategies by assessing the overall survival risk in patients with untreated tumors. This is exemplified in the study of advanced soft tissue sarcoma (STS) patients, where TLSs served as predictive biomarkers for evaluating the efficacy of Pembrolizumab. The findings indicated that the subset of patients with abundant TLSs exhibited significantly higher response rates and progression-free survival (PFS) compared to the overall patient cohort in the prior PEMBROSARC study. This underscores the predictive value of TLSs in tailoring immunotherapy approaches for advanced STS [92]. Moreover, ongoing clinical trials evaluating the efficacy of checkpoint blockade (ICB), such as trials NCT04705818 (Study Registration Dates: 20210111, the Clinical Trial Registry: Institut: Bergonié) and NCT03475953 (Study Registration Dates: 20180220, the Clinical Trial Registry: Institut: Bergonié), along with those exploring drug combination therapy NCT04874311 (Study Registration Dates: 20210504, the Clinical Trial Registry: Institut: Bergonié), have integrated the presence of TLSs in tumor samples as inclusion criteria for participants. This underscores the increasing recognition of TLSs as a relevant factor in shaping treatment strategies in clinical trials.

7 TLSs and cancer therapy

The current consensus acknowledges the efficacy of tumor therapies that activate anti-tumor immunity over the long term. In the context of tumor treatment, the impact of TILs and TLSs on patient outcomes is a subject of interest. Notably, in patients with human lung squamous cell carcinoma, the density of TLSs emerged as a robust independent prognostic marker in untreated individuals. However, in those undergoing neoadjuvant chemotherapy, despite similar TLSs density, the impaired formation of GCs rendered TLSs density non-prognostic. Further investigations revealed that corticosteroid therapy during chemotherapy adversely influenced TLSs development, eliminating their prognostic significance [31]. Contrastingly, in $HER2^+$ breast cancer patients treated with adjuvant chemotherapy and trastuzumab, TLSs were associated with a favorable prognosis [82]. Mouse experimental models exploring TLSs function also provided insights into treatment modalities. For instance, treatment with the interferon gene (STING) agonist was found to promote TLSs formation in the pro-inflammatory TME in a mouse model of melanoma [39].

Tumor therapies that stimulate the development of TLSs play a pivotal role in enhancing anti-tumor immune responses, leading to improved survival rates for patients. In hepatoblastoma associated with adenomatous polyposis coli (APC-HB), a study revealed the generation of TLSs in 11 patients who underwent cisplatin-based neoadjuvant chemotherapy, contrasting with 5 patients who did not receive this treatment. The presence of TLSs in the former group was linked to a favorable prognosis [93]. Similarly, studies on cancer vaccines yielded promising results. Therapeutic vaccines targeting the E6 and E7 proteins of HPV16 and HPV18 induced the formation of mature TLSs in the stroma and localized expansion of clonal T cells within tumor lesions in patients with high-grade cervical intraepithelial neoplasia (CIN2/3). In contrast, TLSs were rarely observed in unvaccinated patients, highlighting the potential of cancer vaccines to stimulate TLSs development [94].

The success of ICB in melanoma treatment [95] and chimeric antigen receptor T-cell therapy (CAR-T) for hematologic tumors [96], among other breakthroughs, have attracted great interest in tumor immunotherapy. Notably, patients responsive to immunotherapy often exhibit a higher abundance of TLSs compared to non-responders [97]. Furthermore, the cellular composition of TLSs may vary in response to treatment efficacy. In the context of PD-1/CTLA-4 immunotherapy for uroepithelial carcinoma, non-responders displayed an enrichment of $FOXP3^+$ T-cell-low TLSS clusters, while treated patients exhibited an increased frequency of macrophage-low-containing TLSs compared to those without immunotherapy [98]. Specific cell populations within TLSs, such as $CD20^+CD22^+ADAM28^+$ B cells, have been identified as contributors to a positive response to immunosuppressive therapy [99]. The maturation of TLSs also emerges as a potential predictor of tumor immunotherapy efficacy. A comprehensive retrospective analysis of anti-PD1/PD-L1 antibody-treated tumor patients revealed that the presence of mature TLSs correlated with improved objective remission rates (ORR), disease-free survival (DFS), and overall survival (OS) [100]. TLSs and their cellular composition have been closely linked to the ability of tumor patients to respond to PD-1, influencing the reactivation of intratumoral immune cells [101]. Despite its remarkable efficacy, tumor immunotherapy can elicit a range of adverse effects in patients. TLSs, while promoting antitumor immune responses, may also play a role in the development of these adverse effects. For instance, a study on three patients with inflammatory myopathies following PD-1 inhibitor therapy found TLSs formation in the lesions, suggesting their involvement in lymphocyte infiltration and a potential role in adverse responses [102]. In conclusion, the presence, cell composition and maturation of TLSs may,

in certain cases, serve as indicators of the efficacy of tumor therapy for patients.

TLSs constitute vital components in anti-tumor immunity, and their induction has emerged as a promising focus for tumor immunotherapy. In addition to the previously discussed approaches for triggering TLSs production in tumor immunotherapy, alternative methods for prompting TLSs neogenesis have been documented. Breakthroughs in experimental TLSs induction within animal models offer a valuable opportunity to delve into the intricate interactions among immune cells during antitumor immune responses. For example, introducing Helicobacter hepaticus (*Hhep*), an intestinal microbe previously absent, into colorectal cancer (CRC) mice induced $CD4^+$ T follicular helper (Tfh) cells, fostered B cell-dominated anti-tumor immunity, and facilitated the formation of mature TLSs [103]. Additionally, a recent report highlighted a nano-vaccine's capacity to enhance anti-tumor responses by promoting TLSs formation in a mouse model mimicking nasopharyngeal carcinoma [104].

Given the pivotal role of stromal cells in lymphoid neogenesis, extensive exploration has taken place to understand their capacity as inducers of tertiary lymphoid structures (TLSs). For instance, the subcutaneous injection of lymph node stroma (sLN) in mice has been demonstrated to provoke TLSs production. The presence of induced TLSs has proven to enhance anti-tumor immunity, particularly against MC38 colorectal cancer xenografts in mice [105]. Acknowledging the clinical limitations associated with stromal cell-induced lymphoid neogenesis, alternative "cell-free" induction methods have been explored. In a noteworthy study, a slow-release gel containing LT-α1β2, CCL19, CCL21, CXCL12, CXCL13, and soluble RANK ligand was embedded in a collagen sponge scaffold. This concoction was transplanted into the kidney capsule of mice and subsequently removed after three weeks. The result was the formation of artificial TLSs (artTLSs), comprising B-cell areas, T-cell areas, FDC and FRC networks, and HEV, within the cytokine-containing scaffold. Transplantation of these artTLSs into immunodeficient mice stimulated robust immune responses upon antigenic stimulation [106]. Similarly, 3D printed porous scaffolds loaded with immunomodulators have demonstrated the ability to recruit more immune cells than traditional hydrogels, facilitating the formation of artTLSs and effectively exerting anti-tumor effects [107]. The development of individualized artTLSs-scaffolded tumor vaccines holds promise as a potential strategy for future cancer vaccine therapy.

8 Conclusions

Currently, the specific mechanisms governing the formation of tumor-associated TLSs remain incompletely elucidated. Standardized and normalized methods for characterizing and quantifying TLSs are yet to be established. The precise mechanism underlying lymphoid neogenesis of TLSs in tumors is not well understood, and the factors contributing to the heterogeneity of TLSs in tumors remain unknown. The ongoing efforts to apply TLSs in tumor immunotherapy are still in the early stages. Despite these challenges, the widely reported anti-tumor effects of TLSs in solid tumors, and their influence on the cells in the TME, suggest a broad potential for application in anti-tumor immunotherapy. TLSs exhibit associations with patient prognosis across various tumor types and can, to a certain extent, reflect the efficacy of cancer treatment. They possess the potential to serve as valuable biomarkers, offering significant prospects for guiding clinical decision-making and treatment selection. Beyond their prognostic value, TLSs act as a potent source of anti-tumor immunity, making their induction a promising target for tumor immunotherapy. Therefore, gaining a more comprehensive understanding of TLSs' functions and their potential role in tumor immunotherapy holds great promise for advancing precision immunotherapy and offering renewed hope for incurable cancer patients.

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