REVIEW SUMMARY

CANCER

Tertiary lymphoid structures in cancer

Ton N. Schumacher* and Daniela S. Thommen*

BACKGROUND: Tertiary lymphoid structures (TLSs) are organized aggregates of immune cells that form postnatally in nonlymphoid tissues. TLSs are not found under physiological conditions but arise in the context of chronic inflammation, such as in autoimmune disease, chronic infection, and cancer. With few exceptions, the presence of TLSs in tumors correlates with better prognosis and clinical outcome upon immunotherapy, but, in spite of their presumed importance, the drivers of TLS formation in cancer and the contribution of these structures to intratumoral immune responses remain incompletely understood.

ADVANCES: TLSs resemble secondary lymphoid organs (SLOs) anatomically, and it was originally assumed that their formation would largely be induced by the same stimuli. However, the cell pools and signals that provide inductive stimuli for TLS formation are at least partially different. For instance, several observations suggest that tumor-specific T and B cell immunity may induce some of the molecular factors required for TLS formation and maintenance, and heterogeneity in these drivers may result in distinct TLS states.

It has been speculated that TLSs recapitulate SLO functions at the inflamed tissue site, and available evidence suggests that a contribution of TLSs to the strength of tumor-specific immune responses is plausible. However, whether such a contribution primarily involves the boosting of T cell responses generated in SLOs or the development of new T and B cell reactivities remains a key unanswered question. In addition, the presence of TLSs at the tumor site may offer the possibility for the generation of qualitatively distinct immune responses. Specifically, because TLSs are not encapsulated, exposure of TLS-resident immune cells to macromolecules from the inflamed microenvironment appears to be a realistic possibility, and this could potentially sculpt the nature of intratumoral immune responses. Finally, recent studies suggest a role for TLSs in the clinical response to immune checkpoint blockade, which may make these structures attractive therapeutic targets. However, the development of such strategies should take into account the possible consequences of ectopic formation of lymphoid tissue at other body sites.

OUTLOOK: The prognostic and predictive value of TLSs in cancer has strengthened the interest in these structures as potential mediators of antitumor immunity. Although TLSs have been identified in many cancer types, the markers used to define and characterize TLSs have often varied across studies, complicating efforts to compare predictive value and to assess TLS heterogeneity between cancer types. Thus, the development of standardized approaches to measure TLS number and composition is likely to further reveal their predictive and prognostic value in different disease settings. Related to this, a more comprehensive characterization of TLSs may potentially lead to the identification of a spectrum of TLS states, based on aspects such as cellular composition, location, maturation, and function. Similar to the definition of T cell states in cancer, which has substantially improved our understanding of the role of specific T cell populations in tumor-specific immunity, the molecular definition of TLS states may help to improve their value as prognostic and predictive markers. Finally, a better appreciation of TLS function and the potential contribution of TLSs to autoimmune toxicity will be important to maximize their value as therapeutic targets. ■

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Cite this article as T. N Schumacher, D. S. Thommen, *Science* **375**, eabf9419 (2022). DOI: 10.1126/science.abf9419

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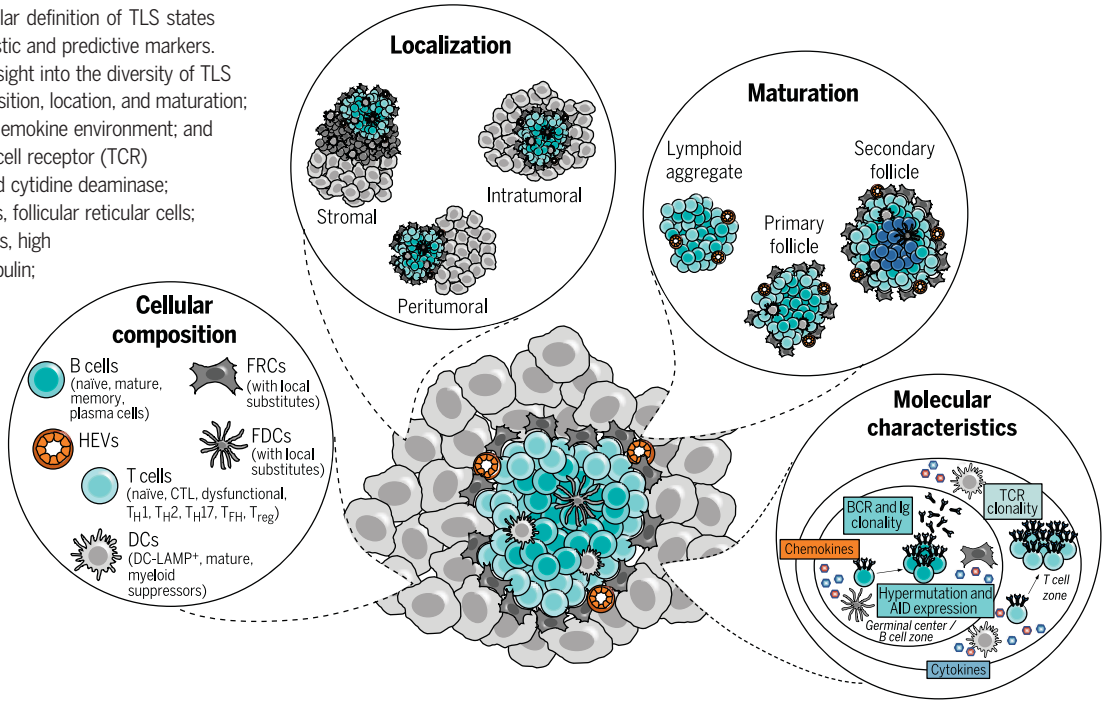
https://doi.org/10.1126/science.abf9419



Defining TLS states. The molecular definition of TLS states may advance their use as prognostic and predictive markers. Characteristics that will provide insight into the diversity of TLS states include their cellular composition, location, and maturation; properties of their cytokine and chemokine environment; and their B cell receptor (BCR) and T cell receptor (TCR) repertoires. AID, activation-induced cytidine deaminase; CTL, cytotoxic T lymphocyte; FRCs, follicular reticular cells; FDCs, follicular dendritic cells; HEVs, high endothelial venules; Ig, immunoglobulin; T_{FH}, T follicular helper cell; T_H, T helper cell; T_{reg}, regulatory T cell.

REVIEW

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Tertiary lymphoid structures in cancer

Ton N. Schumacher[1]* and Daniela S. Thommen[2]*

Ectopic lymphoid aggregates, termed tertiary lymphoid structures (TLSs), are formed in numerous cancer types, and, with few exceptions, their presence is associated with superior prognosis and response to immunotherapy. In spite of their presumed importance, the triggers that lead to TLS formation in cancer tissue and the contribution of these structures to intratumoral immune responses remain incompletely understood. Here, we discuss the present knowledge on TLSs in cancer, focusing on (i) the drivers of TLS formation, (ii) the function and contribution of TLSs to the antitumor immune response, and (iii) the potential of TLSs as therapeutic targets in human cancers.

Work over many years has documented that the presence of certain immune infiltrates in tumor lesions is associated with better prognosis in a number of cancer entities (*1*–*3*). In more recent years, efforts to increase tumor-specific T cell reactivity, either through infusion of ex vivo expanded intratumoral T lymphocytes (TILs) (*4*) or through blockade of immune checkpoint molecules on T cells (*5*–*7*), have provided causal evidence for a role of T cell immunity as a modifier of cancer growth. Furthermore, the observation that the presence of brisk immune infiltrates correlates with response to immune checkpoint blockade (ICB) (*8*–*11*) unites these two lines of research. Although the above data argue for the routine assessment of immune infiltrates in cancer lesions, there is increasing evidence that additional information may be gleaned from analysis of not just the presence but also the localization and interaction of immune cells at cancer sites.

A first, relatively straightforward refinement is the subdivision of T cells based on their location at the tumor border or in the tumor parenchyma (*9*). As may be expected, the presence of T cells in the tumor parenchyma is associated with improved clinical outcome, but whether this reflects increased attraction of T cells in those tumors that harbor an ongoing tumor-specific T cell response, or the active repulsion of T cells in other cancers, remains an important open question. Next to the location of intratumoral immune cells, the clustering of intratumoral immune infiltrates also appears of relevance. As a first example, an analysis of immune infiltrates in breast cancer has revealed that tumors with comparable immune infiltrates displayed distinct spatial distributions, referred to as mixed and compartmentalized organization (*12*). Importantly, compartmentalized organization, defined by the physical separation of clusters of immune cells and clusters of cancer cells, was associated with increased survival, independent from TIL density. Although in this study the prognostic potential was not formally coupled to the presence of tertiary lymphoid structures (TLSs), other recent studies have reported the association of TLSs in cancer lesions with improved prognosis (*13*, *14*), and with response to ICB (*15*–*17*), in a number of human malignancies. Collectively, these observations suggest that not only the presence of an immune infiltrate in a tumor but also the organization of tumor-infiltrating immune cells in TLSs may be crucial. Main questions that should be further addressed in the coming years concern the molecular processes that lead to TLS formation in cancer, the types of cancer-associated TLSs that exist, and the consequences of their presence for the generation or maintenance of tumor-specific immunity.

Composition and organization of immune infiltrates in cancer

TLSs, sometimes also referred to as tertiary lymphoid organs or ectopic lymphoid structures, are organized aggregates of immune cells that arise postnatally in nonlymphoid tissues. TLSs are not present under physiological conditions but form in chronically inflamed environments, for instance, in autoimmune diseases (*18*), allograft rejection (*19*), chronic inflammation (*20*), and cancer (*14*, *21*). TLSs have been reported in a number of cancer types such as non–small cell lung cancer (NSCLC), colorectal cancer (CRC), ovarian cancer, and melanoma (*22*–*26*). The occurrence of TLSs is likely to differ between cancer types, but with the presently available datasets, in which a number of different markers have been used to identify TLSs, a direct comparison has not been possible.

TLSs are characterized by an inner zone of $CD20^+$ B cells that is surrounded by $CD3^+$ T cells, similar to the lymph follicles in secondary lymphoid organs (SLOs) (*14*, *27*). Although the specific composition of TLSs may vary, within the T cell compartment, $CD4^+$ T follicular helper (T_{FH}) cells often represent the dominant subset (*28*), but $CD8^+$ cytotoxic T cells, $CD4^+$ T helper 1 (T_H1) cells, and regulatory T cells (T_{regs}) can also be present (*24*, *29*, *30*). Whereas B and T cell populations make up the bulk of TLS-associated immune cells, TLSs are also populated by distinct dendritic cell (DC) populations, for instance, $CD21^+$ follicular dendritic cells (FDCs), which are of mesenchymal origin and play a critical role in the selection of memory B cells during germinal center (GC) reactions in SLOs (*25*, *31*), or $CD83^+$ mature DCs [in some studies also described as dendritic cell–lysosomal associated membrane protein $(DC\text{-}LAMP)^+$ (*24*)], which predominantly localize in the T cell zone (*32*). The follicles can further contain scattered $CD68^+$ macrophages for clearance of apoptotic cells, similar to their role in SLOs (*33*). A dense stromal network, similar to the one formed by follicular reticular cells (FRCs) in SLOs, anchors the TLSs at the chronically inflamed tissue site (*34*). Finally, peripheral node addressin (PNAd)–positive high endothelial venules (HEVs) provide the specialized vasculature associated with TLSs that is thought to mediate lymphocyte recruitment (*31*).

Recently, an additional type of structured immune infiltrate in cancers has been described (*35*). Specifically, intratumoral immune or antigen-presenting cell (APC) niches in renal cell carcinoma have been defined as small, APC-dense regions with more than five MHC II^+ cells per 10,000 μm^2 that harbor tumor-reactive stem-like $CD8^+$ T cells, crucial mediators of durable immunotherapy responses in mouse models (*36*–*38*). Of note, the absence of APC niches was associated with tumor progression, consistent with the possibility that these structures may play a critical role in maintaining tumor control. Although APC niches are distinct from TLSs, with the latter consisting of larger organized aggregates densely packed with both B and T lymphocytes, it is presently unclear whether APC niches could reflect a very early stage of TLS formation.

The drivers of TLS formation

SLOs (including lymph nodes, spleen, tonsils, Peyer's patches, and mucosa-associated lymphoid tissue) are situated throughout the body to allow antigen sampling from different tissues and thereby promote the induction of adaptive immune responses. In settings of ongoing chronic inflammation, extranodal seeding of lymphoid tissue occurs, resulting in the formation of TLSs at organ sites. To understand the development of such TLSs, it may be useful

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to contrast it to the formation of SLOs during embryogenesis. The seeding and organization of SLOs, specifically of lymph nodes and Peyer's patches, results from a highly ordered series of events that involves an interplay between hematopoietic cells and nonlymphoid stromal cells, with critical roles for cytokines, chemokines, adhesion molecules, and survival factors as molecular components (*39*, *40*). SLO formation is initiated early during embryogenesis by the colonization of the lymph node anlagen by hematopoietic lymphoid tissue inducer (LTi) cells, $CD4^+$ $CD3^-$ $CD45^+$ innate lymphoid cells that differentiate from fetal liver precursors and are characterized by the expression of the RORγt and Id2 transcription factors (*41*). Clustering of LTi cells drives the initial steps of SLO formation in a tumor necrosis factor (TNF) family member–dependent fashion, with central roles for lymphotoxin α1β2 (LTα1β2) and, to some extent, TNF (*42*), and in the absence of either LTi cells or LTα1β2, formation of both lymph nodes and Peyer's patches is precluded (*41*). LTα1β2 and TNF bind to their respective receptors, LTβR and TNFR1, on mesenchymal lymphoid tissue organizer (LTo) cells, thereby promoting the expression of adhesion molecules such as vascular cell-adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1), mucosal addressin cell-adhesion molecule 1 (MAdCAM1), and PNAd, as well as the production of a set of chemokines known as lymphoid or homeostatic chemokines, including CC-chemokine ligand 19 (CCL19), CCL21, and CXC-chemokine ligand 13 (CXCL13) (*39*, *42*). Together, these molecules regulate the subsequent recruitment of immune cells to the lymphoid niche (*42*–*44*) and the vascularization by HEVs (*45*–*48*). Finally, compartmentalization of the nascent lymph follicle is achieved by the segregated expression of homeostatic chemokines with, for instance, $CCL19^+$ and/or $CCL21^+$ FRCs and $CXCL13^+$ FDCs guiding the distribution of lymphocytes that express the corresponding CCR7 and CXCR5 receptors, thereby allowing the formation of T cell and B cell zones (*42*, *49*–*51*). Of note, lymphoid chemokine secretion also induces a positive feedback loop that is crucial for the maintenance of the lymphoid niche, as signaling through CXCR5, which is expressed on B cells and on LTi cells, has been found to induce LTα1β2 expression (*45*).

TLSs display a pronounced anatomical resemblance to SLOs but, in most tissues, lack the surrounding capsule (*52*). This absence of encapsulation may permit direct access of their cellular components to the surrounding tissue but also creates the possibility of exposure of TLS-resident immune cells to macromolecules from the inflamed microenvironment. Although the formation of TLSs and SLOs was initially thought to be induced by the same molecular factors, with roles for LTα1β2-LTβR signaling and local expression of adhesion molecules and lymphoid chemokines, the cellular components involved are, at least partially, different, and the precipitating events that drive TLS generation are still only partly understood. In addition, a number of molecular inducers of TLSs that are independent of lymphotoxin signaling have been described [(*14*, *27*, *53*); see below]. Importantly, much of our understanding of the cellular and molecular processes that drive TLS formation has been obtained in models of autoimmune disease and chronic infection, and findings made in these disease models should thus only be seen as hypothesis-generating with regard to TLS formation in cancer tissue.

With respect to the upstream initiation of TLSs, it is, as of now, unclear whether bona fide LTi cells are required for the priming of the local mesenchyme or whether locally accumulated immune cells can substitute for LTi cells. In favor of the latter hypothesis, several immune cell populations—including T_H17 cells (*54*, *55*) and innate lymphoid cell-3 [ILC3 (*56*)], which both share the RORγt transcription factor with classical LTi cells, effector $CD8^+$ T cells and natural killer cells (*57*, *58*), B cells (*59*), and M1-polarized macrophages (*60*)—have all been reported to act as potential surrogate LTi cells in murine and human settings of either allograft rejection (*54*), autoimmunity (*55*), chronic inflammation (*59*, *60*), or cancer (*56*, *57*). Of note, unlike SLO formation, TLS induction may not always depend on lymphotoxin, as, for instance, interleukin-17 (IL-17) produced by T cells could induce CXCL13 and CCL19 expression in murine stromal cells in response to microbial stimulation, thereby promoting the formation of induced bronchus-associated lymphoid tissue (iBALT), a type of TLS that is formed in lung tissue (*61*). By the same token, lymphoid aggregates do develop in *LTα*−/− mice, although these structures do not show a segregation of T and B cell zones and lack HEVs (*62*), and thus may not be considered proper TLSs.

Similar to the presumed role of surrogate LTi cells in TLS generation, it is likely that certain local stromal and immune populations can act as surrogate LTo cells. Specifically, as has been shown for synovial fibroblasts from patients with rheumatoid arthritis, lymphotoxin and TNF signaling can induce tissue-resident fibroblasts to produce lymphoid chemokines such as CXCL13, CCL19, and CCL21, as well as survival factors including BAFF, IL-7, and April (*34*). In a B16-OVA melanoma model, a population of intratumoral cancer-associated fibroblasts could likewise act as LTo cells to induce TLS formation (*58*). Similarly, chemokine secretion by adipocytes and by vascular smooth muscle cells have been shown to explain TLS formation in the mesenteric adipose tissue of patients with Crohn's disease (*63*) and in atherothrombotic arteries (*64*), respectively. With respect to the role of different SLO-associated chemokines in TLS formation, local TLS formation in the pancreas could be induced by tissue-specific expression of chemokines such as CXCL13 (*51*), CCL21, CCL19, and CXCL12 in murine models (*50*), suggesting that the importance of these downstream chemokines is shared between SLOs and TLSs. Of note, although each chemokine was able to independently induce TLSs, their individual presence yielded structures with slightly distinct characteristics (see below). If cells located within inflamed cancer tissue can produce any of these chemokines in a lymphotoxin- and TNF-independent fashion, this may be predicted to allow TLS formation without a requirement for LTi cells. Of note, a number of intratumoral T cell subsets, including T_{FH} cells in breast cancer (*28*) and programmed cell death-1 (PD-1) bright $CD8^+$ T cells in NSCLC (*65*), as well as macrophages and B cells in ovarian cancer (*66*) and fibroblasts in triple-negative breast cancer (*67*) express CXCL13, suggesting that immune and stromal cells may be able to function as LTo cells and contribute to TLS formation and/or maintenance. Next to LTi and LTo cells, HEVs play a role in TLS formation because they can regulate lymphocyte entry and control the type of lymphocytes that are recruited to the lymphoid tissue through the expression of vascular addressins (*68*).

In support of a role for the local tissue context in determining TLS composition, transgenic expression of different cytokines and chemokines in murine models has been shown to induce TLSs with distinct characteristics. For instance, tissue-specific expression of CXCL13 induced B cell aggregates that lacked FDC networks (*51*), whereas TNF and CXCL12 expression induced small lymphocytic infiltrates consisting predominantly of B cells, few T cells, and, in the latter case, DCs (*50*, *69*). In addition, whereas the CCR7 ligands CCL19 and CCL21 were shown to induce similarly composed aggregates, the structures induced by CCL21 expression were both larger and more organized (*50*).

Differences in the detected TLS components have also been reported in distinct human cancer types (*21*, *27*, *70*), as, for instance, $DC\text{-}LAMP^+$ DCs have been described in TLSs in NSCLC but less in other cancer types (*22*, *24*). In addition, T_{FH} cells have mostly been documented in TLSs in breast cancer (*28*, *71*) and, more recently, in sarcoma (*17*). It is, however, important to note that much of the available data is derived from studies that used inconsistent markers to describe TLS components, and large-scale analyses using the same set of parameters, thereby allowing a rigourous assessment of TLS heterogeneity across cancers, are lacking as of now. A specific type of

heterogeneity for which a reasonable amount of evidence does exist relates to the extent of TLS maturation. Three maturation stages of lymphoid structures based on their structural similarity to SLOs have been defined in NSCLC, hepatocellular carcinoma (HCC), and CRC (*72*–*74*). The least-organized stage consists of dense lymphocytic aggregates without the presence of FDCs and with a lack of segregated T and B cell zones characteristic of bona fide TLSs. Primary follicle-like TLSs do contain FDCs but lack GC reactions. On the contrary, fully mature, secondary follicle–like TLSs also display active GCs, likely reflecting their full functional capacity.

Next to the evidence supporting the existence of different types of TLSs, arguably the most important difference between tumors is that whereas some are permissive for TLS formation, others are not, and it is important to understand under which conditions tumors do or do not support TLS formation. TLSs arise at sites of chronic inflammation, and several observations indicate that their formation is linked to antigen recognition by B and T cells at those sites. For instance, GC formation in SLOs, which is crucial for the generation of high-affinity, long-lived plasma cells and memory B cells (*75*), is regulated by the antigen-driven interaction between B cells and T_{FH} cells, and the fact that GC can form in TLSs suggests that a similar process of antigen recognition takes place in these structures. In human melanoma and ovarian cancer metastases, and in primary breast and gastric-esophageal cancers, clonal amplification of B cells and somatic hypermutation and isotype switching of immunoglobulins have been observed in microdissected TLSs, further reinforcing the concept of a local antigen-driven B cell response (*76*–*81*). Formation of TLSs has likewise been associated with the presence of antigen-specific T cell responses. Specifically, in NSCLC, the number of TLSs was shown to correlate with clonal dominance in both $CD8^+$ and $CD4^+$ T cells (*82*). In addition, tumor reactivity in human lung cancer was enriched in a subset of oligoclonal dysfunctional PD-1^{high} $CD8^+$ T cells and these PD-1^{high} T cells were predominantly observed in TLSs (*65*), consistent with the hypothesis that tumor-reactive T cell responses are present in TLSs. Of note, unlike other $CD8^+$ subsets, this PD-1^{high} $CD8^+$ subset has acquired the capacity to constitutively secrete CXCL13, one of the major chemoattractants involved in TLS formation (*65*). Furthermore, in patients with ovarian and uterine cancer, the presence of $CXCL13^+$ $CD103^+$ $CD8^+$ T cells correlated with TLS abundance and with predicted neoantigen burden (*83*). Collectively, these human data provide strong evidence for continued antigen recognition in TLSs and suggest that antigen-specific cells present in TLSs can produce the molecular factors required for TLS induction and maintenance. Definitive evidence for a role of antigen recognition in TLS formation has been obtained in murine tumor models. Specifically, work from Engelhard and colleagues has shown that the $CD8^+$ T cell pool is required for the efficient induction of lymph node–like vasculature, characterized by expression of PNAd and CCL21, in transplantable tumor models. In addition, PNAd expression on the intratumoral vasculature was higher in tumors that expressed a well-presented $CD8^+$ T cell antigen (*57*). Recent work by the same group furthermore demonstrated that intratumoral $CD8^+$ T cells and B cells jointly drive local fibroblast organization and TLS formation (*58*). Similarly, in a carcinogen-induced murine tumor model, HEV formation after T_{reg} depletion was dependent on $CD8^+$ T cells (*84*). Next to the role for antigen recognition in the formation of TLSs, such antigen recognition may also be be required for TLS maintenance, because TLS numbers rapidly go down after pathogen clearance in lungs of mice infected with influenza virus (*20*, *85*–*87*) or in patients after clearance of gastric *Helicobacter pylori* infection (*88*). As discussed further below, the link between antigen recognition and TLS formation complicates the interpretation of the association between TLS presence and clinical response to ICB.

The immune infiltrate in tumors in which TLSs are present is often skewed toward a T_H1 or cytotoxic effector state (*89*), with expression of genes relating to chemoattraction (*CXCL9*, *CXCL10*, *CXCL11*) and cytotoxicity (*GZMB*, *GZMH*, *GNLY*), and characterized by expression of a series of immune checkpoint molecules (*PDCD1*, *CTLA4*, *LAG3*, *HAVCR*, *TIGIT*) (*26*, *90*, *91*). The fact that expression of many of these molecules is induced by (chronic) T cell activation is consistent with the notion that antigen encounter forms a driver of TLS formation. However, it also remains possible that the presence of TLSs promotes such a T_H1 or cytotoxic effector cell–like response or that both processes are stimulated by a common upstream event. Of note, the presence of T_{regs} has been suggested to impede TLS formation by preventing HEV induction and immune infiltration in murine fibrosarcoma models (*84*, *92*). In addition to the emerging evidence suggesting that the nature of intratumoral T cell responses may influence TLS formation, it will be useful to determine how tumor cell–intrinsic characteristics can influence TLS formation. Work by Cabrita *et al.* did not observe an association between TLSs and either tumor mutational burden or specific driver mutations (*16*), but it will be important to further explore this relationship in additional cohorts, as well as the relationship between the presence of these tumor cell–intrinsic properties and the maturation state of TLSs. In breast cancer, evidence for FOXP1 expression by cancer cells as a determinant of their capacity to express lymphoid chemokines has been obtained (*93*). Although the above work identifies a number of factors that influence TLS formation in the tumor microenvironment, our understanding of the specific molecular determinants that create a local milieu that is or is not conducive to TLS formation is likely to be far from complete. As a framework to classify the different tumor microenvironments in which TLS formation does not occur, we propose to distinguish "restrictive tissue environments," in which TLS formation is actively suppressed, from "inadequate tissue environments," in which essential drivers, such as perhaps antigen, are lacking.

Role of TLSs in the regulation of tumor-specific immune responses

Because of their anatomical resemblance to SLOs, it has been suggested that TLSs recapitulate SLO functions at the inflamed tissue site. SLOs, specifically lymph nodes, foster the encounter of antigen-laden APCs from tissues and naïve lymphocytes from blood by providing a specialized niche that maximizes cell-cell contacts and thereby enables the generation of adaptive immune responses (*45*, *94*). Accumulating evidence suggests that adaptive immune responses can also be generated or boosted in TLSs. One of the main effector functions associated with B cells in TLSs is the production of disease-relevant antibodies that can mark antigen-expressing cells for opsonization, complement-mediated lysis, or antibody-dependent cellular cytotoxicity (*95*). GC formation in TLSs has been found to correlate with serum autoantibody concentrations, disease severity, and decreased organ function in several autoimmune diseases, including Sjögren's syndrome (*96*), myasthenia gravis (*97*), and Hashimoto's thyroiditis (*98*), suggesting a potential contribution of TLSs to disease progression. Furthermore, evidence that clonal proliferation, isotype switching, and B cell effector differentiation actively take place in TLSs is provided by the detection of activation-induced cytidine deaminase, the enzyme driving somatic hypermutation and class switching, and BCL6, the transcription factor contributing to GC entry and late-stage B cell maturation, in TLSs (*77*, *99*). Similarly, increased expression of activation markers has been observed on T cells within TLSs, as compared with other tumor-resident T cells in melanoma (*15*).

An important unresolved matter is whether TLSs mainly serve to reactivate or reeducate effector T cells, or whether they mostly support the priming of naïve T cells. Recruitment of effector T cells has been reported, particularly in the earliest phases of TLS formation (*34*), although this could also reflect the role of such effector T cells in TLS generation. Furthermore, TLS-associated T_H17 cells can acquire

phenotypic characteristics of T_{FH} cells in experimental autoimmune encephalomyelitis models (*55*), suggesting that a reeducation of effector populations may take place in TLSs. On the other hand, recruitment of naïve T cells to TLSs in pancreatic islets of nonobese diabetic mice has also been described. Of note, such naïve T cells do undergo proliferation in situ, consistent with local priming (*100*). Similarly, in the inflamed central nervous system, TLSs have been found to be involved in local priming of autoreactive T cell responses to endogenous myelin peptides (*101*). Additional evidence that TLSs can induce B and T cell responses in the absence of SLOs has been obtained using LTα-deficient mice that lack lymph nodes. Influenza A infection of such mice leads to the induction of iBALT at the time of viral clearance (*102*, *103*). Furthermore, T and B cell responses to viral antigens in these mice were qualitatively similar to responses initiated in lymph nodes but caused less immunopathology (*102*, *104*). In a modified vaccinia virus Ankara model, priming of antigen-specific T cell responses after blockade of lymphocyte egress from SLOs has been observed (*87*). Additionally, in a murine melanoma model, tumor-specific T cell responses driven by TLSs have been identified in the absence of SLOs and resulted in immune cell infiltration and tumor regression (*105*, *106*). Collectively, these data provide compelling evidence that TLSs can replicate SLO functions locally.

With canonical SLOs having evolved as sites to efficiently generate antigen-specific adaptive immune responses, one may wonder what the value is of replicating the process of lymphoid neogenesis at the inflamed tissue site. With detailed information on the function of TLSs presently lacking, a number of models may be proposed to explain why it may be advantageous to create a lymphoid niche at the site of infection or cancer during conditions of chronic inflammation (Fig. 1): (i) Speed: Local priming of T and B cell pools may lead to faster immune responses because it circumvents the trafficking of DCs and lymphocytes to and from SLOs. In line with this, entry of naïve T cells into tumors has been described and was dependent on the development of PNAd- and CCL21-expressing vasculature in mouse models (*57*). As a counterargument, considering that TLSs are particularly prominent in the context of a chronic inflammation, the argument of "speed" seems less than compelling. (ii) Efficiency: Generation of a local lymphoid niche may increase the likelihood of encounters between disease-associated antigens and rare matching lymphocytes, thereby, perhaps, enabling the induction of more vigorous or more broad immune responses. In this model, the functional and phenotypic properties of T and B cells induced in TLSs and SLOs may be identical, but the antigens that they target could partially differ. (iii) Control: Having a lymphoid niche that is in direct contact with the inflamed tissue site may provide an additional opportunity to steer the immune response. For instance, cytokines and/or metabolic factors produced in the surrounding tumor tissue may potentially percolate through the TLSs and thereby influence the nature of the immune response that is created. (iv) Survival: The presence of large numbers of dysfunctional PD-1^{high} $CD8^+$ T cells, a phenotype that has been linked to preferential tumor reactivity (*65*, *107*, *108*), in some TLSs is consistent with a model in which TLSs are important not only for the induction but also for the maintenance of immune responses. The secretion of survival factors by TLS-associated fibroblasts and other cell subsets supports lymphocyte homeostasis in TLSs (*34*, *109*) and, by analogy, may contribute to the long-term persistence of tumor-reactive T cells at tumor sites. To better understand the benefits, and possibly also detriments, of generating ectopic lymphoid niches, it will be important to determine whether immune responses induced in TLSs and SLOs are similar or distinct, with respect to either the antigens that they target or the properties of the antigen-specific lymphocyte pool that is created. Experimental approaches to induce the formation or disassembly of TLSs on command may be of value to dissect their effects on the tumor-resident immune cell pool.

Prognostic and predictive potential of TLSs

TLSs are associated with favorable prognosis in many cancer types (*27*), and the prognostic value of TLSs is often independent of TNM staging, as, for instance, documented in lung (*110*), colorectal (*74*), and pancreatic (*111*) cancer. TLS density as well as the presence of their components, such as T_{FH} cells, follicular B cells, DC-$LAMP^+$ mature DCs, and HEVs, have been shown to correlate with better survival in many different tumor types (*22*, *111*–*113*). In addition, multiple gene expression signatures associated with TLSs have shown positive prognostic value, including a plasma cell signature in ovarian cancer (*29*), a T_{FH} signature in head and neck squamous cell carcinoma (*114*), and various gene signatures associated with lymphoid chemokines (including *CCL5*, *CXCL9*, *CXCL10*, and *CXCL13*) in CRC (*26*), melanoma (*115*), and breast cancer (*28*, *116*). Furthermore, the presence of TLSs in tumors is frequently accompanied by a general increase in immune infiltration, as, for instance, shown in human NSCLC and in triple-negative breast cancer (*24*, *117*, *118*). Finally, the combination of TLSs and brisk intratumoral $CD8^+$ T cell infiltrates correlates with superior prognosis compared with $CD8^+$ T cell infiltration alone (*29*, *119*), an observation that has been used as an argument for a superior quality of the immune response generated in tumors that harbor TLSs.

Although it is tempting to interpret the observed correlations as evidence for a central role of TLSs in the induction or maintenance of tumor-specific immunity, it is important to realize that the formation of TLSs appears to depend on antigen recognition. As such, part of the observed prognostic value is likely to be explained by the fact that TLS formation indicates the presence of an ongoing immune response. To obtain further insight into this matter, it may be valuable to compare the cell states of tumor-reactive T cells that reside in lesions that harbor or lack TLSs. In addition, it may be of interest to identify (tumor) cell parameters that influence TLS formation in mouse models independent of antigen load, to subsequently test their effect on tumor control.

As discussed above, with the caveat that available studies in some cases vary with respect to the markers used for TLS identification, there are indications for TLS heterogeneity between cancer types as well as between patients (*21*, *27*), and work in chemokine-transgenic mouse models provides reasonable mechanistic support for this notion (*50*, *51*, *69*). It has been hypothesized that heterogeneity in TLS maturation state or location influences their prognostic value. In support of this, risk of recurrence was lower in patients with HCC or CRC harboring TLSs with primary or secondary follicle–like differentiation, compared with those with lymphoid aggregates (*73*, *74*). With regard to TLS location, most studies have reported the presence of peritumoral TLSs, but intratumoral TLSs have been described in HCC (*73*), germ cell tumors (*120*), and in lung metastases of renal cell carcinoma (*121*). In HCC, the presence of peritumoral TLSs was associated with a higher risk of cancer recurrence and unfavorable outcome as compared with intratumoral TLSs (*73*). However, in most cancers, no clear association between peri- or intratumoral location of TLSs and prognosis has been established. It should be noted though that the definition of peritumoral TLSs often does not differentiate between TLSs located in the stroma with clear separation from the tumor parenchyma and TLSs at the invasive margin (*122*), and it may be postulated that the association of these two subtypes of peritumoral TLSs with disease prognosis could differ.

Although TLSs are generally associated with good prognosis in most cancer types, their presence has been linked to tumor development or progression in some cancer types (*123*–*125*). A number of potential immunosuppressive mechanisms have been invoked to explain this observation. First, depletion of T_{regs}, which were predominantly present within TLSs, improved tumor control in a murine lung adenocarcinoma model, suggesting that TLS-associated T_{regs} may suppress endogenous antitumor T cell responses (*124*). Second, next

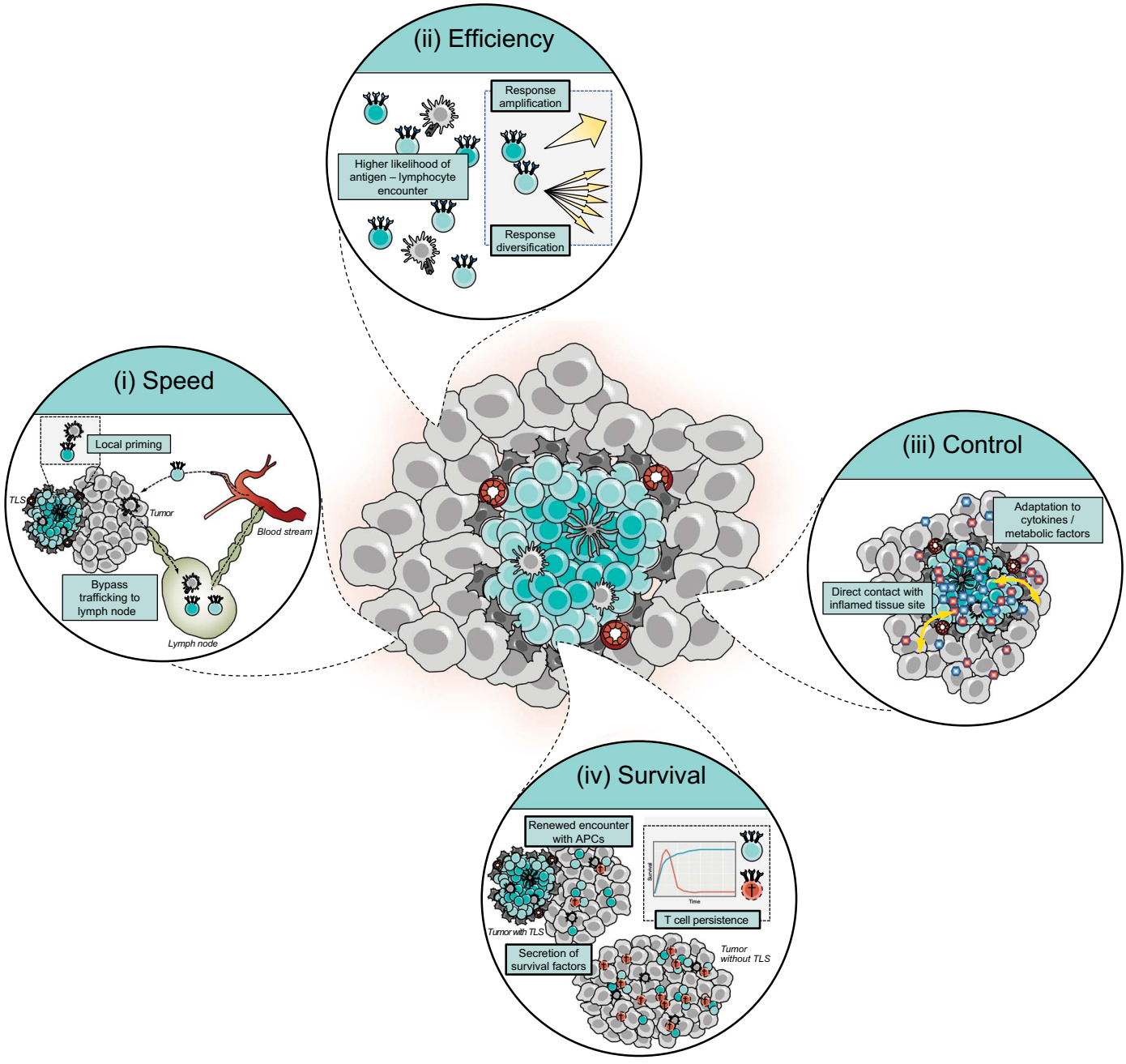

Fig. 1. Potential contributions of TLSs to antitumor immunity. The presence of TLSs in cancer tissue could support antitumor immune responses in different ways: (i) Speed: The priming of T and B cells at the tissue site may shorten the time to generate immune responses because it bypasses the trafficking of DCs and lymphocytes to and from SLOs. (ii) Efficiency: The formation of a local lymphoid niche may foster the induction of stronger or broader immune responses because lymphocytes may be more likely to encounter cognate antigen. (iii) Control: The direct exposure of TLS-associated immune cells to the inflamed tissue milieu may enable the fine-tuning of immune responses toward specific output signals. (iv) Survival: Lymphocyte homeostasis and survival could be promoted by survival factors that are secreted by TLS-associated cell populations or by repeated APC encounter by effector T cells.

to their possible role as producers of opsonizing tumor-specific antibodies, TLS-resident B cells may also suppress tumor-specific immunity, for instance, through IL-10 secretion (*126*, *127*). In addition, depending on the antibody isotypes produced and immune cell types present, tumor-specific antibodies may conceivably also dampen tumor-specific immune responses by signaling through inhibitory Fc receptors (*126*). Finally, TLSs have been proposed as microniches that may foster the transformation and outgrowth of malignant cells, based on the observation that HCC progenitor cells first appear in TLSs before egressing and forming liver tumors (*125*). Likewise, clusters of cancer cells have been detected within TLSs in human breast cancers, and their presence was associated with lymphatic invasion and higher nodal stage (*128*). Although the above observations provide evidence for heterogeneity in TLS composition that influences their prognostic value, direct evidence for the existence of "suppressive" TLSs that promote tumor progression is still limited. Methods to

specifically alter TLS properties in situ could offer a powerful approach to address such questions of causality.

A number of recent studies have also provided evidence for a predictive value of TLSs in response to ICB. Specifically, the presence of TLSs and brisk B cell infiltrates in pretreatment biopsies of melanoma, renal cell carcinoma, soft tissue sarcoma, and urothelial carcinoma has been shown to correlate with response to PD-1 or combined PD-1 plus cytotoxic T lymphocyte–associated protein 4 (CTLA-4) blockade (*15*–*17*, *129*). Similarly, a number of TLS components, including memory-like B cells and plasmablast-like cells, were enriched in pretreatment biopsies of ICB responders in melanoma (*130*). Furthermore, the presence of the PD-1^{high} dysfunctional CD8$^+$ T cells that predominantly localize within TLSs was shown to predict response to PD-1 blockade in late-stage NSCLC (*65*). Interestingly, TLS abundance correlated with programmed cell death ligand-1 (PD-L1) expression on immune cells (*131*, *132*) but not on tumor cells (*16*). In mice, combination therapy that led to the induction of TLSs also sensitized tumors to ICB in a checkpoint blockade–resistant tumor model and resulted in the generation of effector and memory T cells (*133*), suggesting that TLSs either directly contribute to the ICB response or report on a tumor microenvironment that is permissive to ICB.

Intriguingly, analysis of on-treatment tumor biopsies has shown that ICB treatment can also promote the formation of TLSs. After neoadjuvant ICB in high-risk melanoma and urothelial carcinoma, tumors of responding patients showed a higher number of TLS-associated B cells relative to matched pretherapy samples (*15*). In several studies exploring neoadjuvant PD-1 blockade in NSCLC and PD-1 plus CTLA-4 blockade in urothelial cancer, an increase in TLSs has been observed in regressing lesions (*134*, *135*). Similarly, ICB treatment increased the number and size of TLSs in a murine melanoma model, which correlated with superior tumor control (*58*). Considering the role of TLSs in promoting antigen-specific T and B cell responses, it may be speculated that ICB enhances not only TLS formation but also TLS functionality. Although a number of observations are consistent with a model in which ICB affects TLS functionality (*15*, *136*, *137*), the evidence is still circumstantial. Spatial analysis of tumors at very early time points (hours to days) after start of treatment, or analysis of ICB-treated ex vivo human tumor cultures (*138*), should be helpful to gain further insight into this matter.

Therapeutic induction of TLSs

In view of the reported association between TLSs and disease outcome in a number of settings, the induction of TLSs could form an attractive therapeutic strategy. The feasibility of local TLS induction by tissue-specific expression of TLS-associated cytokines and chemokines, including lymphotoxin (*139*), TNFα (*140*, *141*), LIGHT (*100*), CXCL13 (*51*), CCL21, CCL19, and CXCL12 (*50*), has been demonstrated in murine models. In addition, evidence that TLSs can be therapeutically induced and associate with tumor control was obtained in mouse models of breast and neuroendocrine pancreatic cancers, in which the combination of PD-L1 blockade with anti-angiogenic therapies resulted in the transformation of tumor blood vessels into HEVs followed by TLS formation, increased CD8$^+$ T cell stimulation, and tumor destruction (*133*, *142*). TLS induction independent of ICB has been observed in human cancers (Fig. 2), for instance, in patients with high-grade cervical intraepithelial neoplasia (CIN2/3), where TLS formation and clonal expansion of TLSs could be observed in regressing lesions after vaccination against the human papillomavirus oncoproteins (*143*). Similarly, therapeutic vaccination with an irradiated, allogeneic granulocyte-macrophage colony-stimulating factor–secreting pancreatic tumor vaccine (GVAX) in combination with cyclophosphamide led to TLS formation in pancreatic cancers in a large majority of patients (*144*). As a side note, the observed induction of TLSs upon these different types of vaccinations provides strong evidence that the strength of the antigen-specific immune responses forms a determinant of TLS formation in human disease. With respect to the effect of conventional therapies on TLS formation, induction of TLSs has been observed after neoadjuvant chemotherapy in NSCLC (*145*) and hepatoblastoma related to APC mutations (*146*). Notably, opposing findings were obtained in squamous cell lung cancer, in which neoadjuvant chemotherapy treatment resulted in impaired TLS maturation and loss of GCs (*72*). In addition, a similar observation has been made after steroid treatment, which is often coadministered with chemotherapy, in lung cancer (*72*) and urothelial cancer (*135*). At present, it is not entirely clear whether the negative effect on TLS organization depends

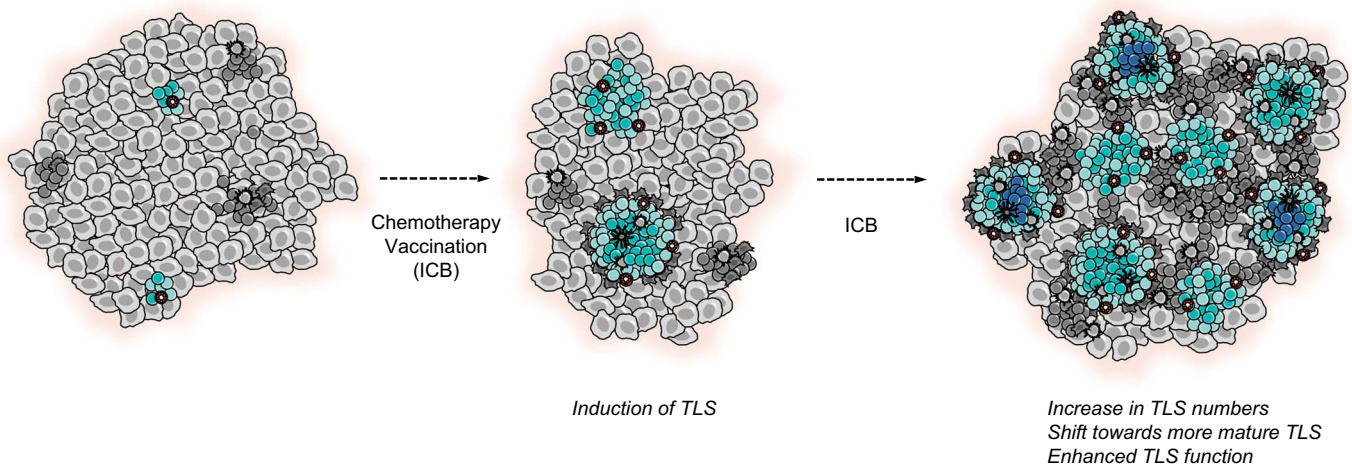

Fig. 2. Potential impact of cancer treatment on TLSs. Several therapeutic strategies have been found to induce or boost TLS formation in cancer. For instance, neoadjuvant chemotherapy was shown to promote de novo TLS development in NSCLC (*145*) and hepatoblastoma (*146*). Similarly, cancer vaccines were shown to promote TLS formation in pancreatic cancer (*144*) or CIN2/3 lesions (*143*). Although ICB has been shown to increase TLS numbers in several cancer types (*15*, *134*, *135*), it is unclear at present whether it can also induce de novo TLS formation. Additionally, ICB has been suggested to enhance TLS function in mice and humans by promoting the generation of effector and memory T cells (*133*), the activation of T_{FH} cells (*136*, *137*), and the induction of B cell class switching (*15*). Based on these observations, it is conceivable that ICB can also induce TLS maturation, although direct evidence is lacking as of now.

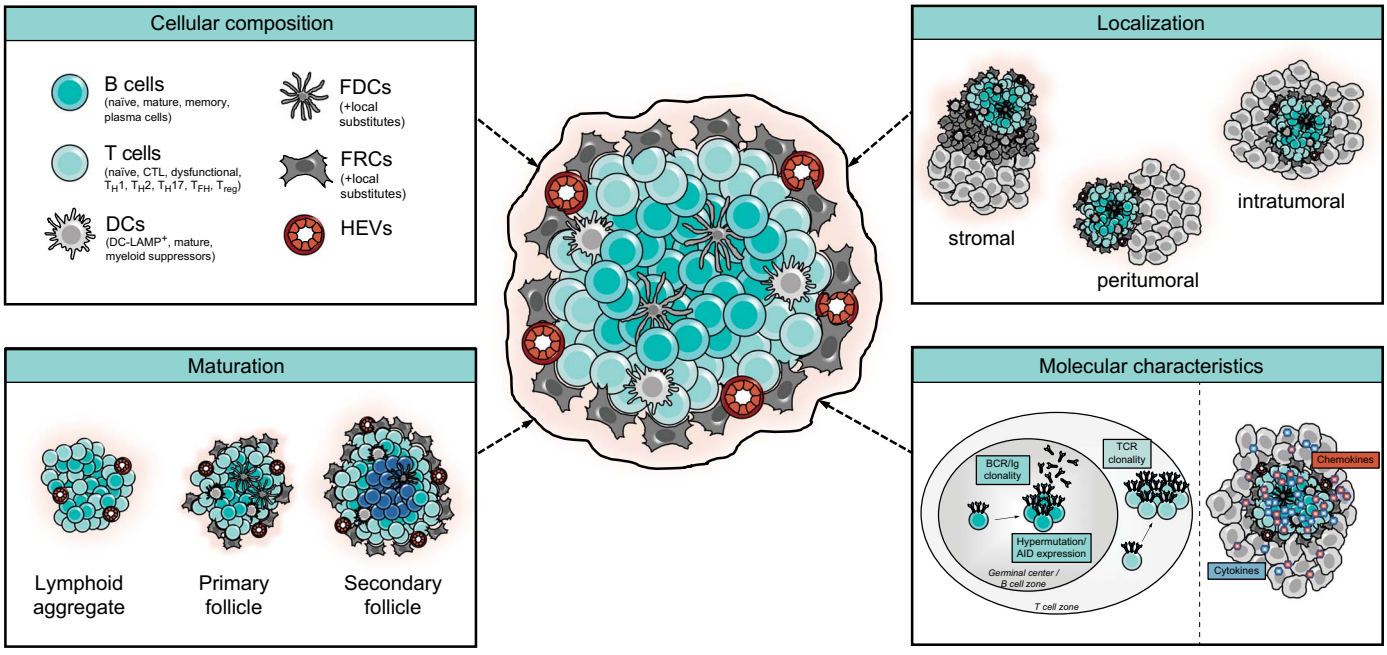

Fig. 3. Defining TLS states. A comprehensive molecular definition of TLS states using a consistent set of parameters should improve their value as prognostic and predictive biomarkers. Aspects that will provide more insight into the existence of TLS states include their cellular composition, location, and maturation; the molecular characteristics of their cytokine and chemokine milieu; and their B cell receptor (BCR) and T cell receptor (TCR) repertoires. AID, activation-induced cytidine deaminase; CTL, cytotoxic T lymphocyte; Ig, immunoglobulin.

on the type of chemotherapy used or on the concomitant treatment with steroids.

One important factor to consider is that although inducing or augmenting TLS function may improve tumor control, such interventions may at the same time boost autoreactive T and B cell responses at other tissue sites. Autoimmune reactions are observed as the main type of toxicity after ICB, and these so-called immune-related adverse events resemble the inflammatory processes that are often found in autoimmune diseases, including arthritis, myositis, thyroiditis, vasculitis, and colitis (*147*). Considering that TLSs have been found to support local inflammatory processes in many autoimmune diseases, it is conceivable that approaches that boost TLS numbers or TLS functionality could also increase ICB-induced autoimmune toxicity. Although, at present, few data exist on the role of TLSs in immune-related adverse events, an association between TLS formation and autoimmune myopathy upon PD-1 blockade has been reported. Specifically, biopsies from patients presenting with myalgia and muscle weakness after anti–PD-1 treatment revealed $CD8^+$ T cell–driven muscle tissue destruction that was associated with the formation of TLS-like structures expressing PNAd and CCL21 (*148*). Hence, induction or boosting of TLS function may promote not only antitumor responses but also the expansion of autoreactive T and B cells, and the risk-benefit ratio of such approaches therefore needs to be carefully evaluated.

Concluding remarks

Recent studies describing the prognostic and predictive value of TLSs in cancer have fueled interest in these structures as potential mediators of antitumor immunity. Based on available evidence, it is plausible that TLSs contribute to the strength of tumor-specific immune responses. However, whether this primarily involves the boosting of T cell responses generated in SLOs or the development of new T and B cell reactivities remains a key unanswered question. Similarly, the presence of TLSs at the tumor site offers a clear possibility for the generation of qualitatively distinct immune responses through the effects of local tissue factors. However, direct evidence for a distinct nature of immune responses that are formed or boosted in TLSs is presently lacking. Although TLSs have been described in numerous cancer types and their prognostic value is beyond doubt, the usage of consistent markers to define and characterize TLSs should form an area of future attention to maximize the value of these structures as potential biomarkers. Related to this, a more comprehensive characterization of TLSs would likely help provide a definition of a spectrum of "TLS states," based on aspects such as cellular composition, location, maturation, and function (Fig. 3). Much like the definition of T cell states has helped the field to better understand their role in cancer control, the molecular definition of TLS states could improve their value as prognostic and predictive markers. Finally, a more detailed understanding of TLS function and their potential role in autoimmune toxicity will be helpful to appreciate their value as therapeutic targets.

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ACKNOWLEDGMENTS

Funding: This work was supported by European Research Council (ERC) Advanced Grant SENSIT (grant agreement no. 742259), the Stevin Award, and the Jeantet-Collen Prize for Translational Medicine (to T.N.S.) and by the Dutch Cancer Society (KWF Young Investigator Grant 12046) (to D.S.T.). **Competing interests:** The authors declare that they have no competing interests.

10.1126/science.abf9419

Tertiary lymphoid structures in cancer

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Science **375** (6576), eabf9419. DOI: 10.1126/science.abf9419

Tertiary lymphoid structures in cancer
Tertiary lymphoid structures (TLSs) are lymphoid formations that are found in nonlymphoid tissues. TLS can develop in inflamed tissues and are associated with chronic inflammatory disorders, autoimmunity, and cancer. In the setting of tumors, TLSs facilitate the influx of immune cells into the tumor site and have therefore attracted interest as a means of improving anticancer immunity and favorable treatment response in patients. Schumacher and Thommen review the biology of TLSs and outline recent advances in TLS research. They discuss how TLSs are detected and defined, the mechanism(s) of formation in cancer, and the potential of targeting TLSs for therapeutic benefit. —PNK

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