

Characteristics of tertiary lymphoid structures in primary cancers

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Abbreviations: ADC, adenocarcinoma; APC, antigen-presenting cell; DC, dendritic cell; HEV, high endothelial venule; NSCLC, non-small-cell lung cancer; LT, lymphotoxin; LT_i, lymphoid tissue inducer; OS, overall survival; SCC, squamous cell carcinoma; SLO, secondary lymphoid organ; TIL, tumor-infiltrating lymphocyte; TLS, tertiary lymphoid structure; Treg, regulatory T cell

Tumors are sustained by complex networks of interactions between malignant cells, stromal cells and tumor-infiltrating immune cells. These networks differ from patient to patient in terms of nature, composition and organization as well as with regard to the precise localization of tumor-infiltrating cells. Of note, the heterogeneity of the immunological component of the tumor microenvironment, as opposed to its mere abundance, has been shown to influence disease outcome. However, a key question remains: where does the activation of tumor-specific T cells take place? The recently described, tumor-associated lymph node-like entities termed “tertiary lymphoid structures” exhibit a structural organization that is reminiscent of secondary lymphoid organs, and thus may imprint the local immune contexture. Here, we discuss how cancer-associated tertiary lymphoid structures impact on the tumor micro-architecture, immune microenvironment, and ultimately, patient survival.

Introduction

A tumor is a complex network of interacting cellular compartments, comprising malignant cells as well as endothelial, stromal, and immune cells. This so-called “tumor interactome,” which differs from patient to patient, is intimately associated with tumor progression and disease outcome.¹

Tumors have been shown to establish an immunosuppressive microenvironment that robustly inhibits, if not completely neutralizes, immune effector cells. This is instrumental for malignant cells to replicate uncontrolled and spread throughout the entire body. Several lines of evidence from mouse and human studies have reported that the immune system can control oncogenesis and tumor progression in spite of such an immunosuppressive pressure, at least under selected circumstances.^{2,3} More

particularly, an association between an abundant infiltration of neoplastic lesions by T cells with improved clinical outcome has been reported in patients affected by multiple types of solid neoplasms.² However, the mechanisms that govern the shaping of an efficient immune contexture within the tumor remain poorly understood. Moreover, the site(s) at which the activation of tumor-specific effector cells takes place remain(s) unknown. The dogma states that professional antigen-presenting cells (APCs) sample and process antigens within the tumor, then migrate through lymphatic and/or blood vessels to tumor-draining lymph nodes, where they present processed peptides to antigen-specific T and B cells.⁴ However, an increasing body of evidence has demonstrated that adaptive immune responses that efficiently protect the host against infectious agents can be initiated independently of secondary lymphoid organs (SLOs).^{5,6} In this setting, antigen presentation appears to take place in ectopic lymph node-like structures that have been named tertiary lymphoid structures (TLSs). TLSs share many structural characteristics with SLOs. In particular, T and B lymphocytes are segregated into 2 distinct and adjacent regions surrounded by specialized blood vessels called high endothelial venules (HEVs), which allows the extravasation of lymphocytes from the blood to the tissue. The T-cell rich area comprises clusters of T cells and mature dendritic cells (DCs). A B-cell follicle is also present, encompassing a ring of naïve B cells around a germinal center that mainly contains B cells, but also T cells, follicular DCs, and macrophages.

The immunological functions of TLSs in cancer have been poorly investigated. Here, we will review novel insights on the impact of cancer-associated TLSs on the micro-architecture of the tumor microenvironment, i.e., its vasculature and global immune contexture, and how the presence of TLSs ultimately affects patient survival.

The Tumor Microenvironment: A Permissive Milieu for Lymphoid Neogenesis?

TLSs have first been described in humans in many non-neoplastic inflammatory contexts, such as autoimmune diseases, viral and bacterial infections, graft rejection, and several idiopathic diseases.⁵⁻⁷ The inducible nature of TLSs was first highlighted by Kratz and colleagues in a transgenic mouse model expressing

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Table 1. Studies reporting the presence of tertiary lymphoid structures in human neoplasms

Cancers	Cellular composition of lymphoid aggregates/TLS	Studied cases	Stage of the disease	References
Breast carcinoma	T cells (including CD4 ⁺ T cells), mature DCs	32 patients	carcinoma in situ to grade III	Bell et al., 1999
	T cells, B cells (GC B cells and naive B cells), FDCs	3 patients	grade II to III	Coronella et al., 2002
	T cells, B cells, PCs, FDCs	4 patients	ND	Nzula et al., 2003
	lymphocytes (hematoxylin counterstaining)	191 patients	grade II to III	Gobert et al., 2009
	T cells, B cells, HEVs	146 patients	grade I to III	Martinet et al., 2011
	T cells (Tfh, CD4 ⁺ T cells and few CD8 ⁺ T cells), B cells (GC B cells), FDCs	70 patients	grade I to III	Gu-Trantien et al., 2013
	T cells, B cells, mature DCs, HEVs	146 patients	grade I to III	Martinet et al., 2013
Colorectal carcinoma	T cells (including CD4 ⁺ T cells, memory T cells, few CD8 ⁺ T cells), B cells, mature DCs	17 patients	ND	Suzuki et al., 2002
	T cells, mature DCs	40 patients	grade I to IV	McMullen et al., 2010
	T cells, B cells, FDCs	ND	ND	Bergomas et al., 2011
	T cells, B cells (including B cell precursors), FDCs	21 patients	grade 0 to IVA	Coppola et al., 2011
	T cells, B cells, HEVs	5 patients	ND	Martinet et al., 2011
	T cells, B cells, mature DCs	25 patients	ND	Remark et al., 2013
Colorectal carcinoma liver metastasis	mature DCs	70 patients	ND	Miyagawa et al., 2004
Colorectal carcinoma lung metastasis	T cells, B cells, mature DCs	140 patients	stage IV	Remark et al., 2013
Lung carcinoma	T cells (including CD4 ⁺ T cells and few CD8 ⁺ T cells), B cells (including GC B cells), mature DCs, FDCs	74 patients	stage I to II	Dieu-Nosjean et al., 2008
	no NK cells	86 patients	stage I to III	Platonova et al., 2011
	T cells (including memory T cells and few naïve T cells), mature DCs, HEVs	15 patients	stage I to III	de Chaisemartin et al., 2011
	T cells, B cells, HEVs	5 patients	ND	Martinet et al., 2011
Melanoma	memory T cells, mature DCs	82 patients	stage IA to IIIA	Ladányi et al., 2007
	T cells (including CD4 ⁺ and CD8 ⁺ T cells, rare FoxP3 ⁺ cells), B cells, mature DCs	21 patients	stage IV	Messina et al., 2012
	T cells, B cells, HEVs	18 patients	ND	Martinet et al., 2012
	T cells (including CD8 ⁺ T cells), B cells (including AID ⁺ GC B cells), mature DCs, FDCs, HEVs	29 patients	stage IIIB to IV	Cipponi et al., 2012
Mucosal-Associated Lymphoid Tissue lymphoma	T cells, B cells (including naïve B cells, AID ⁺ GC B cells, marginal zone B cells, malignant B cells), FDCs	18 patients	low grade	Bombardieri et al., 2007
	T cells, B cells (including naïve B cells, AID ⁺ GC B cells, marginal zone B cells, malignant B cells), FDCs	20 patients	ND	Barone et al., 2008
Ovary carcinoma	T cells, B cells, HEVs	18 patients	ND	Martinet et al., 2011
Renal cell carcinoma	T cells, B cells, mature DCs	24 patients	ND	Remark et al., 2013
Renal cell carcinoma lung metastasis	T cells, B cells, mature DCs	52 patients	stage IV	Remark et al., 2013

Abbreviations: DC, dendritic cell; FDC, follicular DC; GC, germinal center; HEV, high endothelial venule; ND, not determined; TLS, tertiary lymphoid structure.

lymphotoxin (LT) α in pancreatic islets (RIP-LT α model). These authors proposed the term “lymphoid neogenesis” for the process governing the development of TLSs.⁸ In contrast to Kratz and colleagues, Moyron-Quiroz et al. observed the genesis of TLSs in

a LT α -deficient mice,^{9,10} although these structures appeared less organized than in wild-type animals, primarily consisting of B cells but containing few T cells, no follicular DCs, and no HEVs. These data suggest that LT α participates in the organization of

TLs but it is not an absolute requirement for lymphoid neogenesis. In the RIP-LT α model, LT α induced the expression of multiple lymphoid chemokines, including chemokine (C-C motif) ligand 19 (CCL19), CCL21, and chemokine (C-X-C motif) ligand 13 (CXCL13).¹¹ In addition, the ectopic expression of CCL19, CCL21, and CXCL13 promoted the local recruitment of T and B cells into TLS,^{7,12} and the absence of one of these chemokines was sufficient to abrogate the development of TLSs in the lungs of mice infected by the influenza virus.¹³ Thus, even if each chemokine can act as a TLS inducer, their concomitant expression seems mandatory for a complete program of lymphoid neogenesis. Subsequently, lymphoid chemokines were shown to sustain lymphoid neogenesis by participating in a self-amplificatory loop involving their own expression and that of LT α , as clearly demonstrated in SLOs.

It is well known that lymphoid tissue inducer (LTi) cells play a critical role in the initial phases of lymphoid organogenesis. Thus, many studies have investigated the role of LTi cells in TLS neogenesis. It seems that TLS induction can be initiated independently from LTi cells, as TLSs can emerge in their absence.^{7,14} Thus, lymphoid neogenesis is likely to involve additional players that can substitute for LTi cells. Some immune cells have indeed the capacity to activate local resident mesenchymal stromal cells through LT α and tumor necrosis factor α (TNF α) receptors. Moreover, the expression of LT α β ₂ by T cells can be induced by CCL21 and CCL19, whereas B cells can be stimulated to secrete LT α β ₂ by CXCL13.¹⁵⁻¹⁷ Nevertheless, the precise stimulus initiating the development of TLSs, in particular cancer-associated TLSs, has not been clearly identified to date.

Several studies showed that TLSs are induced in a chronic inflammatory environment and their presence is associated with the exacerbation of local immune responses. Although the tumor microenvironment presents many similarities with such a setting (including the chronicity of the inflammation and the presence of functional APCs), it differs by at least one major aspect, its immunosuppressive nature.^{18,19} One could suspect that this immunosuppressive microenvironment would represent a major obstacle for the emergence of TLSs and/or for their functionality.

The presence of intratumoral lymphoid aggregates with varying levels of organization (i.e., comprising or not a B-cell follicle and/or HEVs) has been described in patients affected by primary colorectal cancer,²⁰⁻²⁴ breast carcinoma,²⁴⁻³⁰ melanoma,³¹⁻³⁴ mucosal-associated lymphoid tissue (MALT) lymphoma,^{35,36} ovarian carcinoma,²⁴ and lung cancer^{24,37-39} (Table 1). TLSs have been observed even in the context of metastatic disease, especially in colorectal carcinoma,⁴⁰ renal cell carcinoma,⁴⁰ and melanoma patients,^{31,34} demonstrating that lymphoid neogenesis can occur and/or be maintained at all disease stages. However, distinct levels of TLS organization have been observed in metastatic melanoma³¹ and primary lung cancer (unpublished data from our laboratory). Such differences may reflect different stages in the program of lymphoid neogenesis, as previously suggested in non-transformed inflammatory settings.^{10,41} Finally, the density of TLSs is highly heterogeneous even among tumors of a given type. This heterogeneity might also reflect the diversity of individuals tumor

microenvironments, being more or less permissive to lymphoid neogenesis.

TLs and HEVs Crosstalk in a Finely Regulated Manner Within the Tumor Microenvironment

In many tumors, one of the major hurdles against the efficacy of chemo- and immunotherapy (including anticancer vaccines) is the limited accessibility of immune cells, notably T cells, to the neoplastic lesion. Several mechanisms have been proposed to underlie this phenomenon including the disruption of the architecture and structure of intratumoral blood vessels. The presence of an abnormal vasculature, associated with reduced vessel density, has been reported in multiple cancers, suggesting some sort of disturbance in the neoangiogenic program. Two main types of blood vessels co-exist in tumors, CD34⁺ (or CD31⁺) blood vessels and peripheral node addressin (PNAd)⁺ HEVs, each of which can differentiate into the other under physiological and pathophysiological conditions. One study suggested that no correlation exists between the densities of blood vessels and that of HEVs in breast carcinoma, suggesting that their developmental programs can proceed independently from each other.²⁴ Strikingly, the intratumoral density of these 2 different types of vessels has an opposite prognostic value in cancer patients. Indeed, high densities of CD34⁺ (or CD31⁺) blood vessels are associated with tumor growth and poor clinical outcome in breast,⁴² colorectal,⁴³ and lung⁴⁴⁻⁴⁶ cancer patients. These data support a model according to which neoangiogenesis is stimulated by the tumor microenvironment, promoting the formation of vascular emboli and support the metastatic spread of neoplastic cells to distant sites. In contrast, the presence of HEVs, which have been observed in melanoma^{31,33} and breast cancer^{24,30} lesions, correlated with long-term patient survival.^{24,33,47} Moreover, the density of HEVs closely correlated with that of tumor-infiltrating T and B cells,^{24,33,47} and HEVs were shown to co-localize with cancer-associated TLSs.^{24,30,31,33,38} In lung cancer, the PNAd ligand CD62L, is expressed by most lymphocytes found within TLSs (with the exception of germinal center B cells), but not by lymphocytes present in the other areas of the neoplastic lesion.³⁸ HEVs are vessels specialized in the extravasation of lymphocytes from the bloodstream to SLOs. Given the strong analogies between SLOs and TLSs, we suggest that HEVs may actively participate in the recruitment of circulating lymphocytes to intratumoral TLSs. Recently, Girard and colleagues demonstrated that DCs play a key role in the regulation of the entry of lymphocyte into SLOs as they maintain a fully mature HEV endothelium in a LT β receptor-dependent manner.^{48,49} Mature DCs are exclusively detected in the T cell-rich areas of TLSs, adjacent to HEVs.^{30,31,33,38} Thus, it is tempting to speculate, but remains to be formally demonstrated, that DCs may regulate lymphocyte trafficking not only in SLOs but also in cancer-associated TLSs.

The mechanisms governing the extravasation of lymphocytes into SLOs are well characterized, and involve 3 main families of molecules: adhesion molecules, chemokines, and integrins. In lung cancer patients, a specific set of adhesion molecules expressed by HEVs have been selectively associated with the presence of TLS: intercellular adhesion molecule (ICAM)2, ICAM3,

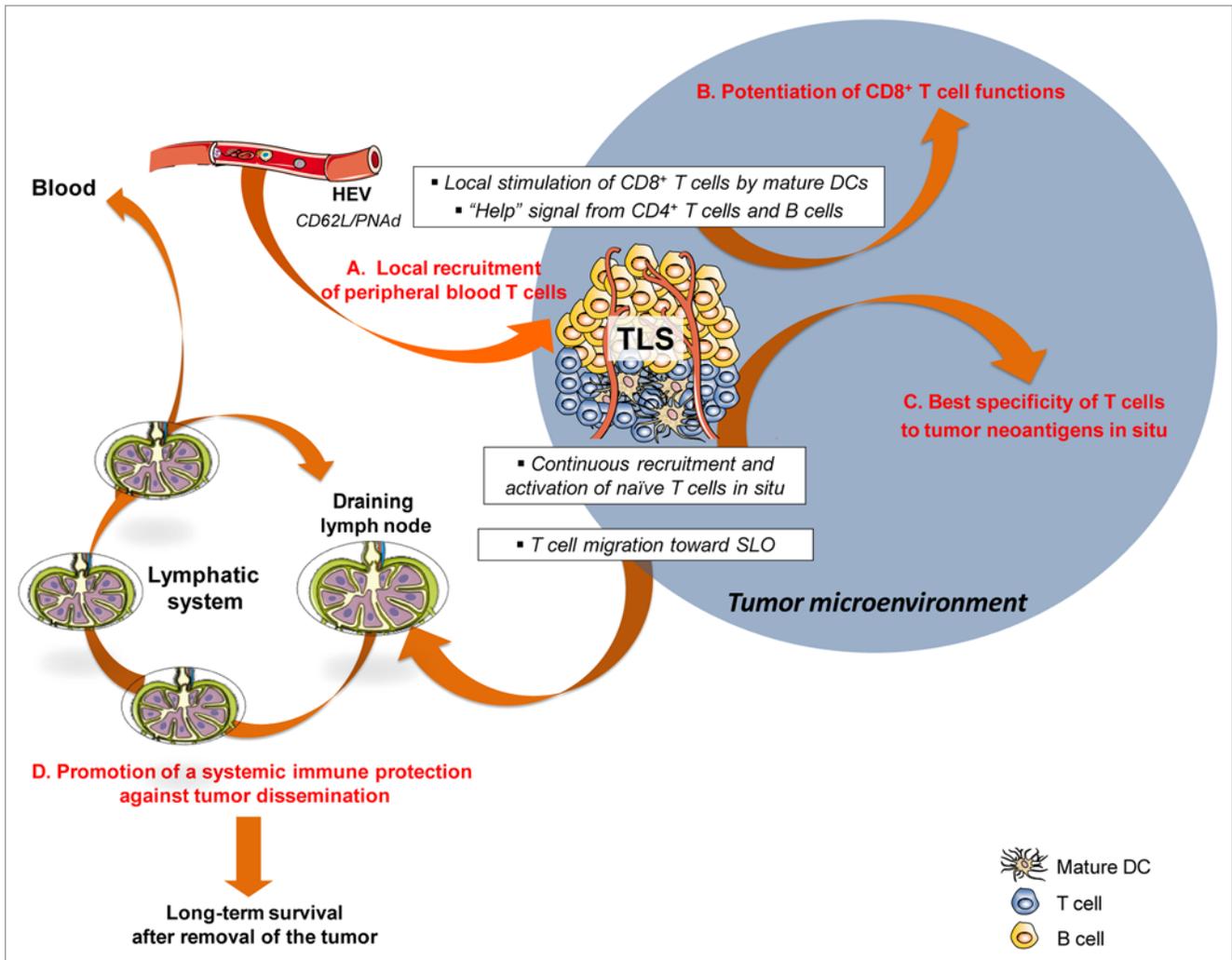


Figure 1. Role of tertiary lymphoid structures in the initiation of local and systemic protective immune responses against primary neoplastic lesions and metastases. DC, dendritic cell; HEV, high endothelial venule; SLO, secondary lymphoid organ; TLS, tertiary lymphoid structure.

vascular cell adhesion molecule 1 (VCAM1), mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) and PNAd.³⁸ In mouse and rat models, intratumoral endothelial cells were shown not to express ICAM1 and VCAM1, as opposed to endothelial cells in distant tissues,^{50,51} in accordance with the downregulation of VCAM1 on the surface of endothelial cells cultured in presence of neoplastic cells.⁵² HEVs expressing ICAM1, VCAM1, MAdCAM1, and PNAd have been also observed in the close proximity of lymphoid aggregates in LT-induced chronic inflammatory lesions.⁸ Moreover, the expression of ICAM2, VCAM1, and PNAd has also been observed in lymphoid aggregates found in non-inflamed human lungs,^{53,54} suggesting that TLSs could represent a favorable microenvironment for the differentiation and maintenance of blood vessels into functional HEVs. ICAM2 is involved in the arrest of circulating T cells along the HEV endothelium of SLOs,⁵⁵ and may have the same function within TLSs. VCAM1 is induced upon inflammation, and contributes to the migration of circulating memory T cells to tissues.⁵⁶ Thus, VCAM1 may represent a key molecule for the recruitment of

memory T cells, which are the main T-cell components of lung cancer-associated TLSs.³⁸ In contrast, MAdCAM1 is a gut-homing molecule that regulates T-cell migration,⁵⁷ and has not previously shown to contribute to TLS. This suggests that HEVs from lung cancer-associated TLSs exhibit an adhesion molecule profile that differ from that of TLSs surging in non-neoplastic settings.

Altogether, these observations corroborate the hypothesis that TLSs may represent a privileged site for the maturation and maintenance of functional HEVs, unlike the other areas of the tumor microenvironment. TLS-serving HEVs may provide a gateway for the recruitment of circulating T cells into the tumor, and thus represent an interesting target for anticancer immunotherapy (Fig. 1A).

Chemokines, an Orchestra Conductor of TLS Organization

The migration of lymphocytes into SLOs is dependent on the expression of various lymphoid chemokines, including CCL19,

CCL21, and CXCL13.^{12,14,58} The overexpression of these chemokines has been documented in the TLSs of lung and breast carcinoma patients.^{27,38} Moreover, in several murine models, the injection of cancer cells engineered to express either CCL19 and/or CCL21 has been shown to induce a robust infiltration of developing neoplastic lesions by T cells and DCs.⁵⁹⁻⁶³ This has been associated with a reduction in tumor growth and, at least in some cases, with the induction of a systemic antitumor immune response linked to the control of metastatic dissemination.^{59,64} In human lung cancers, CCL19⁺ cells are predominantly mature DCs, which are selectively present in TLSs.³⁸ In the lung of mice infected by the influenza virus, the depletion of DCs correlated with a dramatic decrease in TLS density, and the few remaining TLSs were significantly disorganized.^{65,66} The maintenance of TLSs may be partly mediated by DCs via the recruitment of CCR7⁺ cells, including newly activated DCs and naïve and central-memory T cells, as we observed in lung cancer-associated TLSs.³⁸ Recently, DCs have also been shown to play a critical role in the induction of lymphangiogenesis within TLSs in a LT- and CCL21-dependent manner.⁶⁷ As observed in various inflammatory diseases, lung cancer-associated lymphatic vessels express CCL21. Thus, it is tempting to speculate that CCL21⁺ lymphatic vessels in the proximity of TLS may represent a major path for immune cells (e.g., activated DCs and central-memory T cells) to reach tumor-draining lymph nodes and establish a systemic protection against metastatic dissemination.

In human lung cancers, CXCL13 was detected on follicular DCs that homed specifically to the germinal centers of B-cell areas within TLSs.³⁸ Therein, follicular DCs co-localized with a subset of TLS T cells expressing CD4 and chemokine (C-X-C motif) receptor 5 (CXCR5), hence displaying a phenotype of follicular helper T cells.³⁸ Interestingly, CXCL13⁺ follicular helper T cells have recently been detected within breast carcinoma-associated TLSs, and their density was associated with long-term patient survival.²⁷ The density of CXCR5⁺ follicular B cells also appears to be correlated with a favorable clinical outcome among lung cancer patients (Dieu-Nosjean et al., unpublished data) suggesting that the expression of CXCL13 in cancer-associated TLSs may promote the local initiation of a protective humoral immune response through the recruitment of CXCR5⁺ cells into the germinal center.

CCL17 and CCL22 are also overexpressed in lung cancer-associated TLSs, in agreement with the higher proportion of CCR4⁺ T cells among TLS-associated T cells as compared with T cells found in other areas of the neoplastic lesion.³⁸ CCL17 and CCL22 operate by a shared receptor (CCR4), which is expressed by many T-cell subpopulations including T_H2, T_H17, T_H22 T cells, regulatory T cells (Tregs), and effector T cells under inflammatory conditions.⁶⁸ Nonetheless, the role of the CCL17-CCL22/CCR4 in cancer remains controversial. In some murine models, the intratumoral injection of CCR4 ligands induces tumor regression with a concomitant recruitment of effector T cells,⁶⁹⁻⁷² whereas in others studies a deleterious effect associated with a strong influx of Tregs has been reported.^{71,73,74} However, it should be noted that the prognostic value of tumor-infiltrating Tregs in cancer patients is also a matter of debate.² This is likely

due to the limited specificity of biomarkers for human Tregs, such as the transcription factor forkhead box P3 (FOXP3), which is also an indicator of the T-cell activation status. Gobert et al. have shown that Tregs are present in the stroma of breast tumors as well as within intratumoral lymphoid aggregates.²⁹ The migration of Tregs to lymphoid structures is mediated by the CCL22/CCR4 axis. Tregs can be activated by mature DCs, and their high density in lymphoid aggregates is correlated with short-term survival of breast cancer patients. Murine models have illustrated that a high density of intratumoral Tregs correlates with low densities of HEVs, suggesting that HEV neogenesis is inhibited by Tregs.^{75,76} However, in melanoma patients, no correlation was observed between the density of HEVs and that of Tregs.³³ This apparent discrepancy suggests that further investigations are required in order to better define the role of TLS-associated Tregs in oncogenesis and tumor progression.

Interleukin (IL)-16 is also expressed by T and B cells infiltrating lung cancer-associated TLSs.³⁸ In an asthma model, IL-16 has been shown to operate as a chemoattractant for various CD4⁺ cells, including CD4⁺ T cells, DCs and myeloid cells.^{77,78} IL-16 can also prime CD4⁺ T cells to respond to IL-2 by proliferating, becoming activated and/or differentiating into memory cells.⁷⁹ Thus, the role of IL-16 within TLSs may be of particular importance for the functionality of tumor-infiltrating T lymphocytes.

Thus, the expression of a defined set of chemokines by cancer-associated TLSs strongly suggests that some immune cell populations could be selectively recruited into TLSs with a putative key impact on the cell-to-cell cross-talk and global intratumoral immune contexture.

The Presence of TLS Impacts on the Local Immune Microenvironment

The selective recruitment of immune cells, especially naïve and central-memory CD62L⁺ T cells, to TLSs led us to hypothesize that TLSs may represent a privileged site for the differentiation and activation of tumor-infiltrating lymphocytes. To obtain further insights into these issues, we have compared the characteristics of the local immune microenvironment with the density of TLSs in patients with lung cancer (Goc et al., manuscript submitted). Patients were stratified into two groups according to the presence of a high or a low density of mature DCs, a cell population that is selectively detected within TLSs and hence is used as a specific TLS marker. Tumor bearing high densities of mature DCs (DC^{hi} tumors) contained increased amounts of CD4⁺ and CD8⁺ T cells (encompassing naïve, central-memory and effector-memory cells) as well as of activated T cells, as compared with tumors scarcely infiltrated by mature DCs (DC^{lo} tumors). Elevated TLS densities also correlated with the overexpression as well as the coordination of genes linked to T_H1 polarization, cytotoxicity, activation, and immune effector functions. In contrast, genes involved in T_H2 polarization, inflammation, immunosuppression, and angiogenesis were not differentially expressed by DC^{hi} and DC^{lo} tumors. These data indicate that high TLS densities are associated with a specific intratumoral contexture that is characterized by the coordination of adaptive

cellular immune responses (Fig. 1B). This suggests that TLS might directly impact on the intratumoral immune contexture in lung cancer patients. Similar findings have been reported in lung metastases of colorectal carcinoma but not renal cell carcinoma, in agreement with the differential density of TLSs found in these cancer types.⁴⁰ Of note, the protective effects of a T_H1 and cytotoxic immune signature have been widely demonstrated in mice,⁸⁰ and correlate with a favorable disease outcome in patients affected by most solid cancers (exception made for renal cell carcinoma and ocular melanoma²). However, the possibility that such immune signature may be associated with the density of TLSs has never been reported.

One of the remaining key questions is whether TLSs are directly involved in the induction of antitumor immune responses, or, vice versa, whether they relay locally an intense immune reaction initiated in SLOs. Several lines of evidence support the former possibility. First, it has been demonstrated that antitumor immune responses can develop within neoplastic lesions in mice lacking SLOs. This demonstrates that effective T-cell priming can be achieved within intratumoral TLSs independently of SLOs.^{81,82} Second, the presence of TLSs has been correlated with the generation of tumor-specific T cells, suggesting that TLSs could act as a functional site for the induction of protective antitumor immunity.^{82,83} Similar findings have also been obtained in models of infectious diseases,^{9,66} indicating that TLSs may represent an active site for the induction of adaptive immune responses in multiple settings.

In summary, it seems that the presence of a favorable (or at least a permissive) tumor microenvironment allows for the creation of TLSs, which is critical for the local coordination and polarization of protective antitumor immune responses.

TLS as a Novel Biomarker in Cancer

The majority of prognostic studies has highlighted a positive impact of intratumoral T cells on the survival of patients affected by solid tumors.^{2,84} The high reproducibility of these data strongly support the idea that one should take into account immune criteria for the prognostic staging of neoplastic lesions.⁸⁵ Several studies have indeed reported a favorable clinical outcome for lung cancers patients whose neoplastic lesions contain a high density of CD8⁺ T cells (Refs. 86,87 and Goc et al., submitted manuscript). Moreover, a high density of mature DCs (a specific marker of TLS) has been correlated with the long-term survival of patients affected by early-stage, advanced and metastatic lung cancer (Refs. 37,40 and Goc et al., submitted manuscript).

Thus the survival of lung cancer patients appears to be positively influenced by the abundance of tumor-infiltrating mature DCs and CD8⁺ T cells. This raises the question of evaluating the clinical impact of these tumor-infiltrating cells in combination. The stratification of lung cancer patients (at all disease stages) according to low or high densities of intratumoral mature DCs and CD8⁺ T cells ($DC^{lo}/CD8^{lo}$, $DC^{lo}/CD8^{hi}$, $DC^{hi}/CD8^{lo}$, and $DC^{hi}/CD8^{hi}$ patients) provided several interesting hints (Goc et al., submitted manuscript). First, patients with a high density of mature DCs, regardless of the density of CD8⁺ T cells ($DC^{hi}/$

$CD8^{lo}$ and $DC^{hi}/CD8^{hi}$ groups), were at lower risk of death than their DC^{lo} counterparts, indicating that elevated amounts of intratumoral mature DCs are sufficient to favorably impact on clinical outcome. This said, it should be noted that most of DC^{hi} tumors are also highly infiltrated by CD8⁺ T cells. In this context, the scarcity of $DC^{hi}/CD8^{lo}$ tumors suggests a causal link between the presence of TLSs and that of tumor-infiltrating CD8⁺ T cells. This observation further supports the hypothesis that intratumoral TLSs represent an active site for the recruitment, activation, proliferation, and priming of tumor-infiltrating T cells. Second, patients with low intratumoral amounts of both mature DCs and CD8⁺ T cells ($DC^{lo}/CD8^{lo}$ group) turned out to have a poor clinical outcome as compared with the three other groups of patients ($DC^{lo}/CD8^{hi}$, $DC^{hi}/CD8^{lo}$, and $DC^{hi}/CD8^{hi}$ patients). This demonstrates that the DC/CD8 score allows for the identification of a subgroup of patients with a very high risk of death. Finally, patients with a high density of tumor-infiltrating CD8⁺ T cells but a low density of mature DCs ($DC^{lo}/CD8^{hi}$ individuals) exhibited an intermediate risk of death. These data suggest that a high density of tumor-infiltrating CD8⁺ T cells in the absence of a robust tumor infiltration by mature DCs is not sufficient to predict favorable disease outcome among lung cancer patients. The presence of mature DCs appears therefore to be required to license the positive prognostic value of tumor-infiltrating CD8⁺ T cells. In absence of TLSs, high density of intratumoral CD8⁺ T cells may be less efficient in maintaining long-term protective antitumor immunity. Similar results have been obtained with regard to tumor-infiltrating B cells and CD8⁺ T cells in ovarian cancer patients.^{88,89}

The presence of TLSs may imprint the behavior of tumor-infiltrating CD8⁺ T cells. This assumption is in agreement with the involvement of TLSs in the local activation of CD8⁺ T cells, which has been demonstrated in different murine models.^{66,83,90} Several mechanisms might explain the survival benefit related to elevated densities of cancer-associated TLSs. Mature DCs, which may have engulfed a large spectrum of tumor-associated antigens (TAAs), are very efficient at presenting processed peptides to antigen-specific T cells (Fig. 1B). In addition, the recruitment of naïve T cells into TLSs is highly favorable for the continuous activation of new T-cell clones specific for newly arising TAAs (Fig. 1C). Moreover, the local stimulation of intratumoral T cells by mature DCs might potentiate their effector functions and limit their sensitivity to anergy and/or exhaustion.^{91,92} Finally, CD4⁺ T cells, which represent the major T-cell subset of TLSs, could deliver important help signals to CD8⁺ T cells. In a murine model of pancreatic cancer, it has been illustrated that the “help” coming from CD4⁺ T cells can also limit the induction of tolerance and the depletion of CD8⁺ T cells, promote the survival of effector-memory T cells and optimize their effector activity.^{89,93}

It should be noted that the improved prognosis associated with a high density of tumor-infiltrating mature DCs was observed among lung cancer patients who underwent surgical tumor resection, and hence who were deprived of all tumor-infiltrating immune cells. This suggests that the prognostic value of intratumoral mature DCs is most likely related to the development of systemic antitumor immunity, in turn

originating from local adaptive immune responses orchestrated within TLSs. As such, the presence of lymphatic vessels close to TLSs may participate in the emigration of some immune cell populations, including central-memory T cells, to tumor-draining lymph nodes, a key pathway for the establishment of robust systemic immunity against micro-metastasis (Fig. 1D).

Conclusions

The presence of TLSs has been documented in many solid tumors, in both mice and humans. Interestingly, TLSs manifest a huge variability in terms of density and cellular organization, indicating that the nature of tumor microenvironment is critical for lymphoid neogenesis. In this context, one of the challenges for the future is to precisely determine the conditions that

provide an optimal environment for the induction and maintenance of cancer-associated TLSs.

In addition, the presence of particular vessels, namely, HEVs, around TLSs strongly suggests that this microenvironment represents an ideal gateway for the entry of circulating lymphocytes into TLSs, a step that is critical for the initiation of adaptive immune responses, as demonstrated in SLOs. Moreover, TLSs can imprint the local immune contexture, hence influencing disease outcome in cancer patients. Thus, the density of CD8⁺ T cells predicts long-term survival among lung cancer patients only in presence of TLS-associated DCs. These data need to be confirmed in patients affected by other solid tumors. Nonetheless, the presence of TLSs provides per se a survival benefit to cancer patients. We speculate that lymphoid neogenesis might represent

an interesting target for eliciting and/or boosting antitumor immunity in cancer patients. Moreover, TLSs may be used in the future as a novel biomarker for the identification of cancer patients with a high risk of relapse. A deeper comprehension of the cellular and molecular mechanisms whereby TLSs confer long-term protection to cancer patients is essential to develop efficient strategies for their therapeutic manipulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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