Occurrence of Tertiary Lymphoid Tissue Is Associated with T-Cell Infiltration and Predicts Better Prognosis in Early-Stage Colorectal Cancers

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Abstract

Purpose: Tumor-infiltrating T lymphocytes (TIL) play a key role in the clinical outcome of human colorectal cancer; however, the dynamics of their recruitment along colorectal cancer clinical progression have not been fully elucidated. Tertiary lymphoid tissue (TLT) is an ectopic organized lymph node–like structure that typically forms at sites of chronic inflammation and is involved in adaptive immune responses. Its occurrence in cancer is sporadically documented and its role and clinical relevance is largely unknown.

Experimental Design: The occurrence of TLT, the correlation with TILs, and the clinical relevance were evaluated retrospectively, in a cohort study involving a consecutive series of 351 patients with stage II and III colorectal cancer. The role of TLT in lymphocyte recruitment was assessed in a preclinical model of colorectal cancer.

Results: In both human colorectal cancer and in a murine model of colorectal cancer, we identified organized TLT, highly vascularized (including high endothelial venules), and correlated with the density of CD3⁺ TILs. Intravenous injection in mice of GFP splenocytes resulted in homing of lymphocytes to TLT, suggesting an active role of TLT in the recruitment of lymphocytes to tumor areas. Accordingly, TLT density and TIL infiltration correlated and were coordinated in predicting better patient’s outcome among patients with stage II colorectal cancer.

Conclusions: We provide evidence that TLT is associated with lymphocyte infiltration in colorectal cancer, providing a pathway of recruitment for TILs. TLT cooperates with TILs in a coordinated antitumor immune response, when identifying patients with low-risk early-stage colorectal cancer, thus, representing a novel prognostic biomarker for colorectal cancer.

Introduction

Immune infiltration is a fundamental component of solid tumors (1), its involvement in cancer progression being documented by preclinical and clinical studies. The adaptive immune response plays an active role in controlling cancer growth and dissemination. In fact, T-cell infiltration correlates with favorable prognosis in various common neoplastic diseases, including colorectal cancer (2–5), emerging as a potential immune biomarker of outcome and promising therapeutic target. In colorectal cancer, tumor-infiltrating lymphocytes (TILs) are associated with favorable prognosis (2–4, 6–8), independently by tumor-node-metastasis (TNM) stage (3, 6, 8), or only in stage II colorectal cancer (4). Surprisingly, despite the documented association between TILs and the clinical outcome, the dynamics of T-cell recruitment and activation along colorectal cancer progression have not been fully elucidated.

Tertiary (or ectopic) lymphoid tissue (TLT) is a vascularized immune compartment that forms at sites of exacerbated inflammatory reactions and is potentially involved in the promotion of adaptive immune responses (9, 10). Similarly to secondary lymphoid organs, the function of TLT is dependent on its structural organization, with
Translational Relevance

Our findings reveal that tertiary lymphoid tissue (TLT) is associated with lymphocyte infiltration in colorectal cancer, contributing to tumor-infiltrating lymphocyte (TIL) recruitment; TLT cooperates with TILs in a coordinated antitumor immune response and in predicting better outcome in patient, thus, representing a novel prognostic biomarker for human colorectal cancer. Improved understanding of the complexity of immune infiltration along the progression of colorectal cancer and the identification of immune biomarkers might help in the design of tailored immune-based therapeutic approaches, targeting the immune system according to the stage of disease. The organized accumulation of lymphocytes into ectopic lymphoid tissue in colorectal cancer is a positive prognostic factor and may then represent a target for therapeutic intervention in stage II colorectal cancer, with the aim to stimulate the immune system against tumor progression.

B-cell follicles, T-cell areas, and specialized hematic and lymphatic vessels, suggesting the possibility that it behaves as a functional immune site (11, 12). TLT is found in the inflamed area of several autoimmune diseases (13), chronic inflammatory conditions (14–16), and some tumor types (17–21). In these clinical settings, TLT improves the adaptive immune response to persisting antigens expressed in the target organ (9, 10). In solid tumors, a role for TLT in the organization of the local immune response and in lymphocyte recruitment has been suggested (22), but solid evidence has not been provided yet (23, 24). In human colorectal cancer, discrete aggregates of lymphocytes at the tumor invasive margins have been described and referred to as Crohn’s-like reaction (25–27); however, its functional role and the connection with the dispersed lymphocytic infiltrate with prognostic value, also in regard to the type of genetic instability, is surprisingly unexplored. Being a paradigm for the complex relationship between chronic inflammation, T-cell–mediated immunity, and cancer progression (28, 29), colorectal cancer, represents an ideal clinical setting to define the association between ectopic lymphoid tissue, a frequent manifestation of chronic inflammatory conditions, and infiltration of T cells with antitumor properties.

As in secondary lymphoid organs, the formation of TLT relies on few molecular mediators released by resident stromal cells, including members of the lymphotxin family (30) and lymphorganogenic chemokines (CXCL13 and CCL21), responsible for lymphoid cell recruitment (30–33). During the process of adult lymphoid neogenesis, similar molecular mediators and cellular interactions induce formation of specialized vessels, including high endothelial venules (HEV) and lymphatics, which support traffic of lymphocytes from the blood to lymphoid tissues and lymph nodes (34). The presence of HEV in ectopic tissues indicates the possibility for naïve and central memory T lymphocytes to be recruited, thanks to their expression of L-selectin (CD62L) and its specific binding to peripheral node addressin (PNAd), selectively expressed on HEV (35). In fact, the formation of HEV has been suggested to mediate rapid recruitment of lymphocytes into chronically inflamed tissues, including some tumor tissues (36).

In the present study, we investigated the contribution of TLT to CD3+ T-cell infiltration in colorectal cancer. In a retrospective cohort study, we identified highly organized lymphocyte aggregates representing bona fide TLT and we defined its prognostic relevance with respect to CD3+ T-cell infiltration and demographics, clinical, and histopathologic variables and their interactions. In a preclinical model of colitis-associated colorectal cancer, we functionally addressed the question whether TLT is involved in the recruitment of T lymphocytes into colon cancer tissue. A quantitative analysis, not subjected to estimation of pathologist has not been performed to date for lymphoid tissue in colorectal cancer. Thus, a clinically relevant definition of TLT in human colorectal cancer is expected to have important implications in the standardized assessment of lymphocyte infiltration in human colorectal cancer and in the design of clinical trials aimed to test novel immunotherapeutic approaches.

Patients and Methods

Patients

Tissue specimens from 351 stage II and III patients without any sign of metastatic disease at diagnosis who consecutively underwent radical surgical resection for pT3 or pT4 colorectal cancer, were retrieved from the previous series (4). Patients’ demographics, clinical, and histopathologic data were available and obtained from the Institutional Intranet; please refer to Supplementary Table S1 for the list of the variables assessed. The absence of metastasis at diagnosis was assessed in all patients by combining histopathologic findings, surgical records, and perioperative imaging. To study the prediction of disease recurrences according to the state of immune infiltration, patients with pT1 or pT2 colorectal cancer, who have a very low risk of progression at diagnosis, and patients with perioperatively detected metastases were excluded. To exclude potential confounders in the study design, stage I patients were not enrolled in the cohort studied for their unlikely occurrence of disease recurrence, whereas patients with stage IV colorectal cancer were not included because they are characterized by the presence of distant metastasis, which is an outcome event of our analysis. Patients who underwent neoadjuvant radiotherapy for rectal cancer were excluded from the study, because of the possibility of interference with the assessment of the local immune response. Chemotherapy treatment was administered and allocated by a nonrandom assignment according to adjuvant protocols in use at the time of surgery.
Study design
Tissue specimens of patients with colorectal cancer who consecutively underwent radical surgical resection for pT3 or pT4 colorectal cancer at the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) from January 1997 to November 2005 were retrospectively studied. Investigators who were blinded to the results of the morphologic analysis assembled a clinical retrospective database by collecting demographics, clinical, and histopathologic data from the institutional intranet (Supplementary Table S1). These variables, together with the median values of TLT and TIL IRA (immunoreactive area), were used as predictors of the outcome of patient. The outcome of patients who undergo radical resection of colorectal cancer is a variable affected by an event defined as any local tumor recurrences or any metachronous distant organ metastases and named disease-free survival (DFS). To detect or exclude any postsurgical tumor recurrences, patients underwent thoracoabdominal computed tomography, abdominal ultrasonography, and chest radiography, which were done according to common protocols for surveillance. The observation period started immediately after the surgical procedure. The mean follow-up period of the cohort studied was 4.71 years (SD = 2.63 years) for DFS. The detection of tumor recurrence or death was computed from diagnosis until data were censored on May 30, 2010. To further assess any possible biases, interaction analyses in predicting the prognosis of patient were performed for all the variables assessed to detect any effect modifier (P < 0.10).

Immunohistochemistry and microsatellite status
From each patient enrolled in the study, 2-μm thick tissue slidest from formalin-processed and paraffin-embedded tumor sections were processed for immunohistochemistry. After deparaffinization and rehydration, sections were immersed in an antigen retrieval bath, incubated with 3% H2O2 for 15 minutes. Slides were autostained (IntelliPATH FLX; Biocare Medical) with primary antibodies raised against CD3 (clone F7.2.38; Dako), CD20 (clone L26; Dako), PNAd (MECA-79; BD Pharmingen), Lyve-1 (ab14917; Abcam), CD21 (clone EP3093; Abcam), α-smooth muscle actin (α-SMA) (clone 1A4; R&D Systems), and CXCL13 and CCL21 (AF801 and AF457; R&D Systems). A 30-minute incubation with the DAKO Envision system (Dako) or the Anti-Goat Polymer Kit (BioCare) followed. Diaminobenzidine tetrahydrochloride (Dako) was used as chromogen. Nuclei were lightly counterstained with a freshly made hematoxylin solution (Medite). Presence of fibrosis was assessed on 2-μm thick sections stained for 20 minutes with 0.1% Sirius red in saturated picric acid (Sigma-Aldrich). The sections were further washed in water, mounted, and analyzed under an optical microscopy. Microsatellite status was assessed preliminarily for all cancers included in the study by testing instability at mononucleotide repeats, as previously described (37, 38). The Ethics Committee of the Humanitas Clinical and Research Center approved the study, and written informed consent was obtained by the referring physician, at the time of surgery by each patient. Slides were digitized using a computer-aided image analysis system (Olympus dotSlide).

TLT exhibited a distinct structural organization in colorectal cancer, outlined by an area composed of CD3+ cells and a compartment of CD3-negative lymphoid cells (B cells). To quantify TLT, an expert pathologist, who was blinded to any patient clinical data, randomly selected three noncontiguous microscopic areas located at the tumor invasive front occupied by TLT. Computer-assisted measurement of the selected areas was obtained as the percentage ratio between TLT area and the total digitized tissue surface. For each histologic section, the mean values obtained in three different regions were calculated and used for the subsequent statistical analysis. CD3+ TIL IRA was quantified as previously described (4). Median values of the overall distribution of TLT and TIL IRA (2.68% and 2.06%, respectively) were chosen as representative cutoff to perform statistical analyses.

Statistical analysis
The association between the extent of TLT density and CD3+ TILs, baseline characteristics and tumor features of patient was estimated by Pearson simple linear regression analysis. A Cox proportional hazards model was developed to assess the role of TLT density and other demographic, clinical, and histopathologic features, in predicting the occurrence of disease-free–specific survival. Time to follow-up was stopped at the time of the death of patient for any case unrelated to colorectal cancer disease, and this case was not considered an event of outcome. To assess for confounders, COX multivariate analysis was performed by entering only variables and their significant interactions with a P value less than 0.20 at univariate analysis. Interactions between variables were calculated by analyzing their multiplicative term in the Cox model. By a backward stepwise elimination approach, nonsignificant variables, and their nonsignificant interactions, were removed from the model. Interacting variables at multivariate analysis (P < 0.10) were then tested for subgroup analysis accordingly. Differences in median values of TLT density between subsets of colorectal cancer and DFS were tested by the Mann–Whitney U test and by the Cuzick trend test. Kaplan–Meier curves of DFS were plotted, whereas the log-rank test was used to compare the curves of each subgroup of patients with colorectal cancer. For each test, only two-sided P values lower than 0.05 were considered statistically significant. All the analyses were done using Epi Info (Version 3.4.3), StatsDirect Statistical software (Version 2.5), and GraphPad Prism software (Version 4.1).

Quantification of HEV in human colorectal cancer
Consecutive tumor slides from 20 patients with colorectal cancer were stained with antibodies raised against PNAd. For each tumor slide, the absolute numbers of PNAd-positive vessels within each intratumoral follicle...
and follicles associated with the normal mucosa were quantified.

**Mice and murine models of colorectal cancer**

Eight-week-old C57BL/6j and mice were purchased from Charles River Laboratories; eGFP/C57BL/6 mice from The Jackson Laboratory. Procedures involving animals and their care were conformed to institutional guidelines in compliance with national and international laws and policies. Mice were housed in a specific pathogen-free animal facility of the IRCCS Humanitas Clinical and Research Center in individually ventilated cages. In the AOM/DSS (azoxymethane/dextran sodium sulphate) model, mice developed adenomas as a result of the combined treatment with the carcinogen AOM (10 mg/kg; Sigma-Aldrich) and sequential administration of the mucosal irritant DSS [3 rounds of 2% DSS (MW = 36,000–50,000; MP Biomedicals)] in the drinking water. Adenomas develop in the distal colon overtime during the different treatments and are evaluated after 10 weeks.

Additional details are included in the Supplementary Methods.

**Results**

**Human colorectal cancer contains aggregates of T cells with features of TLT**

The lymphocytic reaction in colorectal cancer tissues includes dispersed TILs and discrete lymphoid aggregates, the latter referred to as Crohn-like reaction (25–27), which has not been characterized yet. By staining colon cancer specimens with an anti-CD3 antibody, we identified aggregates of lymphocytes displaying a distinct structural organization, compared with generic clusters of TILs (Fig. 1A). These organized structures had features of TLT, with compartmentalized T (Fig. 1B, left) and B (Fig. 1B, middle) areas, sometimes with germinal centers, and a network of CD21⁺ follicular dendritic cells (FDC; Fig. 1B, right). Notably, TLT was often localized at the invasive front of the tumor, in stromal regions containing a considerable amount of cells expressing α-SMA (Fig. 1C, left), a marker of activated fibroblast cells usually associated with local inflammatory/fibrotic response. The lymphomagenic chemokines CCL21 (Fig. 1C, middle) and CXCL13 (Fig. 1C, right) were also found inside lymphoid tissue, suggesting an active recruitment of T and B cells and plasticity of these structures. Staining of collagen fibers confirmed the fibrosis surrounding TLT (Fig. 1D, left). TLT contained PNAd⁺ HEVs (Fig. 1D, middle) and Lyve-1-positive lymphatic vessels (Fig. 1D, right), thus, confirming that lymphoid aggregates in human colorectal cancer have features of TLT.

**Density of TLT in human colorectal cancer correlates with increased density of TILs and associates with increased number of HEVs**

To analyze the relationship between TLT and TILs in human colorectal cancer, we systematically evaluated the density of TLT in 351 tissue specimens from deeply invading (pT3/pT4) patients with colorectal cancer without evidence of distant organ metastasis at diagnosis, by staining with an anti-CD3-specific antibody and quantifying the area of TLT by computer-assisted image analysis. We quantified TLT at the invasive front of the tumor, which represents the tumor–host interface (Fig. 1A). Notably, in the cohort of 351 patients with colorectal cancer, whole-tissue visualization of CD3 infiltration showed a higher density of TLT in tumors containing high density of CD3⁺ TILs (Fig. 2A, left), compared with tumors containing low density of CD3⁺ TILs (Fig. 2A, right). In fact, the density of TLT linearly correlated with the density of dispersed tumor-infiltrating CD3⁺ T cells (R = 0.32; P < 0.001; Supplementary Table S2; Fig. 2B).

Because lymphoid tissue contained PNAd⁺ HEVs, specialized vessels with a key role in the traffic of T cells (Fig. 1D), we aimed to explain the correlation of TLT and TILs in human colorectal cancer, by quantifying the distribution of HEV in colorectal cancer specimens. HEVs were present mostly in the context of TLT and very rarely found in the surrounding tumor tissue. Comparison between lymphoid tissue associated with the normal mucosa (Fig. 2C, left) and TLT at the invasive front of the tumor (Fig. 2C, right) showed an increased number of HEV associated with lymphoid follicles in the tumor compartment (P < 0.05; Fig. 2D), indicating that the process of lymphoid neogenesis in human colorectal cancer includes formation of HEV, which might allow recruitment of T cells.

**Density of TLT associates with a better prognosis in patients with colorectal cancer**

We then investigated the clinical significance of TLT in relationship with TILs in a retrospective cohort study. Considering the overall cohort, TLT IRA% at the invasive tumor front ranged from 0% to 23.98%, with a median value of 2.68% (second-third quartiles, 0.75%–5.99%). TLT was present in 276 (78.6%) of 351 tumors and the distribution was skewed toward low values. Distributions of TLT according to the patient histopathologic characteristics are described in Supplementary Table S2. We recorded 84 events of colorectal cancer disease relapse (DFS) in 351 patients with stage II and III colorectal cancer.

Unadjusted univariate analysis showed that high TLT density (≥median) significantly correlated with better outcome [HR, 0.62; 95% confidence interval (CI), 0.40–0.97; Supplementary Table S3]. Importantly, to control for confounders, we performed multivariate Cox analysis that revealed an interaction between higher densities (≥median) of both TLT and TILs with nodal status in predicting patients relapse (P = 0.07 and P = 0.03, respectively), which suggests that the ability of TLT and TILs to predict patients relapse may change according to nodal status (Supplementary Table S3). Therefore, we performed a subgroup analysis revealing that in patients with node-negative colorectal cancer (n = 185), a high density of TLT (≥median, 2.68%) and of TILs (≥median, 2.06%) was associated with better prognosis compared with patients with a low density of TLT and TILs (log-rank test; P = 0.02 and P = 0.02, respectively; Fig. 3A and B, left); conversely,
TLT and TIL densities were irrelevant to predict the prognosis of patients with node-positive colorectal cancer ($n = 166$; log-rank test; $P = 0.46$ and $P = 0.64$, respectively; Fig. 3A and B, right). Moreover, we further confirmed that the prognostic behavior of TLT and TILs varies with disease extent in a different analysis, performed with continuous values. Similarly to TILs (Fig. 3C, right), among patients with node-negative colorectal cancer, TLT density was significantly lower in patients who relapsed ($n = 26$) than in patients with no evidence of disease recurrence [$n = 159$; N0 (no relapse) vs. N1 (relapse; $P = 0.43$); N2 (no relapse) vs. N2 (relapse; $P = 0.15$)], thus, behaving as a prognostic biomarker only in early-stage patients (Fig. 3C, left).

To address the question whether the immune response comprising of TLT and TILs has no impact at all in stage III colorectal cancer, we performed a stage-by-stage analysis, showing the ability of nodal status to identify relapses of patient with colorectal cancer among subgroups of TLT density (Table 1). Similarly to TILs, nodal involvement was associated with worst outcome in subgroups of patients with colorectal cancer with intermediate TLT density (0.1%–4.2%, $P = 0.001$; 4.2%–8.4%, $P = 0.05$) but not in patients with colorectal cancer with very low or very high...
immune values (0%, \(P = 0.16; \geq 8.32%, \ P = 0.33\); Table 1). Consequently, only very low and very high TLT densities (0%; \(\geq 8.32\%\)) were associated with better outcome in both stage II (\(P = 0.04\)) or stage III (\(P = 0.02\)) colorectal cancer and might, therefore, operate as stage-independent predictors of survival (Table 1).

These results suggest that the prognostic behavior of TLT is similar to that of TILs and prompted us to test which one was the best prognostic marker. However, because these biomarkers identified overlapping populations, which were mutually dependent in predicting prognosis (data not shown), we developed two stepwise, backward Cox multivariate analysis models (model A and model B), to test the independency of their prognostic performance, with respect to other demographics, clinical, histopathologic features, and microsatellite status in stage II colorectal cancer (Table 2). Model A and model B revealed that higher densities of TLT and TILs are both independent prognostic markers of better prognosis in stage II colorectal cancer, compared with other tumor features (Table 2). Results obtained so far prompted us to hypothesize that TLT and TILs have a comparable prognostic impact. To better understand their prognostic function, we analyzed the correlation of TLT and TILs distribution according to disease progression. Figure 3D showed that TLT correlates with TILs density (\(\geq \text{median}\)) only in patients who did not experience relapse (\(P = 0.001\)), but not in those who relapsed (\(P = 0.28\); Fig. 3D), thus, suggesting that the two biomarkers are coordinated in mediating the antitumor response only among patients with a good prognosis.

Our data have shown that microsatellite instable (MSI) patients with colorectal cancer had a tendency to better prognosis compared with those with microsatellite stable (MSS) phenotype, although not statistically significant (HR, 0.56; 95% CI, 0.27–1.16; \(P = 0.12\); Supplementary Table S3). To further address whether the prognostic value of MSI might differ according to the density of TLT, we performed Kaplan–Meier curves with subgroups analyses (Supplementary Fig. S1). The analysis showed that MSI colorectal cancer was not significantly associated with prognosis in both patients with TLT density low (<median) or high (\(\geq \text{median}\); \(P = 0.27; P = 0.36\), respectively), thus, being independent variables in predicting prognosis (Supplementary Fig. S1).

**TLT is involved in lymphocyte infiltration in a preclinical model of colorectal cancer**

Despite CD3\(^{+}\) T-cell density is recognized as a prognostic marker for patients with colorectal cancer (3, 4),...
the dynamics of CD3⁺ T-cell recruitment and activation at the tumor site have not been clarified. The coordination between TLT and TILs in predicting prognosis in human colorectal cancer prompted us to better clarify the association of TLT and T-cell infiltration. We took advantage of a preclinical model of inflammation-driven carcinogenesis (AOM/DSS; ref. 39), which would recapitulate the formation of TLT associated with chronic inflammatory conditions (23). Organized accumulations of lymphoid cells are present in murine colon mucosa of AOM/DSS mice, both adjacent to the normal crypts (Supplementary Fig. S2A, left) and in the tumor region (Supplementary Fig. S2A, right). The aggregates are comprised of mostly B lymphocytes and include an area of T cells (Supplementary Fig. S2B) and a network of FDCs (Supplementary Fig. S2C, left). The lymphoid chemokine CXCL13 is expressed at high levels in the aggregates (Supplementary Fig. S2C, right), consistent with the predominant presence of B cells. During inflammation-driven colon carcinogenesis, lymphoid tissue significantly increased compared with control mice (Supplementary Fig. S2D), consistently with the local induction of TLT in chronically inflamed tissues. To define the association of TLT with T lymphocytes in colorectal cancer, we compared CD3⁺ T cells within TLT of control and AOM/DSS mice (Fig. 4A, left and middle). Quantification of the T-cell infiltrate indicated that the number of CD3⁺ T cells in lymphoid tissue significantly increased in AOM/DSS mice (Fig. 4A, right). Thus, as evidenced by our previous clinical analysis, this result further confirmed in a preclinical model that TLT associates with increased T-cell infiltration in colorectal cancer.

To test the hypothesis that TLT is actively involved in the recruitment of lymphocytes, we intravenously injected GFP⁺ splenocytes into control mice and mice subjected to the AOM/DSS protocol. After 24 hours, GFP⁺ cells localized in TLT of AOM/DSS mice,
very few or none were observed in lymphoid tissue of control mice (Fig. 4B, left and middle). Whole-tissue analysis evidenced a significant increase in the density of GFP<sup>+</sup> cells in colorectal cancer–associated lymphoid tissue (Fig. 4B, right), thus, confirming that TLT in the tumor mediates recruitment of lymphocytes.

### Table 1. Prediction of risk for disease relapse by nodal status in 351 pT3/pT4 colorectal cancer

<table>
<thead>
<tr>
<th>Relapse</th>
<th>No (n = 267)</th>
<th>Yes (n = 84)</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 TILs density</td>
<td>&lt;1% N0</td>
<td>32</td>
<td>11</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>33</td>
<td>17</td>
<td>1.39 (0.65–2.97)</td>
</tr>
<tr>
<td></td>
<td>1%–5% N0</td>
<td>77</td>
<td>11</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>52</td>
<td>25</td>
<td>2.91 (1.43–5.91)</td>
</tr>
<tr>
<td></td>
<td>5%–10% N0</td>
<td>32</td>
<td>2</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>15</td>
<td>15</td>
<td>12.12 (2.75–53.38)</td>
</tr>
<tr>
<td></td>
<td>≥10% N0</td>
<td>18</td>
<td>2</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>8</td>
<td>1</td>
<td>1.12 (0.10–12.36)</td>
</tr>
<tr>
<td>TLT density</td>
<td>No TLT N0</td>
<td>25</td>
<td>8</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>25</td>
<td>17</td>
<td>1.81 (0.78–4.20)</td>
</tr>
<tr>
<td></td>
<td>0%–4.16% N0</td>
<td>62</td>
<td>12</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>45</td>
<td>31</td>
<td>2.90 (1.48–5.65)</td>
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<tr>
<td></td>
<td>4.16%–8.32% N0</td>
<td>42</td>
<td>4</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>N1–N2</td>
<td>20</td>
<td>7</td>
<td>3.27 (0.95–11.17)</td>
</tr>
<tr>
<td></td>
<td>≥8.32% N0</td>
<td>30</td>
<td>2</td>
<td>1.00 ref.</td>
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<tr>
<td></td>
<td>N1–N2</td>
<td>18</td>
<td>3</td>
<td>2.41 (0.40–14.45)</td>
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</tbody>
</table>

**NOTE:** Colorectal cancer were subgrouped according to the density of CD3<sup>+</sup> TILs and TLT at the tumor invasive front. 
Abbreviations: N0, no lymph node involvement; N1, one to three nodes involved; N2, more than or equal to four nodes involved.

### Table 2. The multivariate Cox hazard model for predictive factors of disease relapse in 185 stage II pT3/pT4 colorectal cancer

<table>
<thead>
<tr>
<th>Relapse</th>
<th>Model A</th>
<th>Model B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (n = 159)</td>
<td>Yes (n = 26)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>TLT density</td>
<td>&lt;Median</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>≥Median</td>
<td>88</td>
</tr>
<tr>
<td>TIL density</td>
<td>&lt;Median</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>≥Median</td>
<td>83</td>
</tr>
<tr>
<td>Local invasion&lt;sup&gt;a&lt;/sup&gt;</td>
<td>pT3</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>pT4</td>
<td>10</td>
</tr>
<tr>
<td>Tumor cell type&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Adenocarcinoma</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>Variants</td>
<td>6</td>
</tr>
</tbody>
</table>

**NOTE:** Multivariate analysis was performed by introducing TLT density (≥median) in model A and TIL density (≥median) in model B and by entering all other variables with a P value less than 0.20 at univariate analysis. By a backward stepwise elimination approach, nonsignificant variables, and their nonsignificant interactions, were removed from the model.
<sup>a</sup>pT3, invading through the muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissues. pT4, directly invading adjacent organs or perforating visceral peritoneum.
<sup>b</sup>Variants mucinous or medullary.
Whole-mount tissue analysis of TLT in murine colons allowed to visualize a dense network of vessels surrounding TLT, which included CD31+ blood vessels and Lyve-1+ lymphatic vessels (Fig. 4C, left and middle). Quantification of the volume of vessels draining TLT evidenced a significant increase in AOM/DSS mice compared with control mice (right).
control mice (Fig. 4C, right). Among CD31+ vessels, lymphoid tissue contained PNAd+ HEVs (Fig. 4D, left); CD3+ T cells were localized inside HEV and in close proximity to the vessel wall (Fig. 4D, middle). The number of PNAd+ HEV within lymphoid tissue of AOM/DSS mice was higher compared with control mice (Fig. 4D, right), thus, confirming that TLT formation in colon cancer associates with expansion of a vascular network and can sustain lymphocyte recruitment.

Discussion

The recognition of the key role of tumor-infiltrating leukocytes in cancer has triggered the efforts toward a better definition of the complexity of the immune response in tumors, with the relevant clinical perspective to identify novel prognostic biomarkers, which might help in the design of immune-based therapeutic approaches. In this scenario, CD3+ lymphocytes have recently emerged as a robust immune biomarker in several solid tumors, including colorectal cancer. In our study, we show that CD3+ T-infiltrating lymphocytes at the tumor invasive front of colorectal cancer can localize in organized aggregates, comprised of T- and B-cell areas and a network of FDCs, thus with features of TLT, in which clinical relevance in relationship to TILs has been so far unexplored.

Intratumor TLT was associated with an increased density of TILs and to a dense vascular network, including HEV and lymphatic vessels, required to ensure proper traffic of lymphocytes within lymphoid organs, and thus, suggesting that TLT has the capability to sustain traffic of T cells. The presence of the chemokines CCL19 and CXCL13 within TLT strongly supports the hypothesis that lymphoid tissue is relevant to mediate active recruitment of lymphocytes into the tumor, which was then confirmed by intravenous injection of GFP splenocytes in mice.

The clinical relevance of tumor-infiltrating T cells in human colorectal cancer has been extensively documented (2–6, 8, 26, 27). Previous analyses of T-cell infiltration in patients with colorectal cancer claimed CD3+ TILs as better indicator of prognosis than TNM tumor staging (3). However, on accurate analysis, CD3+ TILs retained prognostic significance only in patients with nodal-negative colorectal cancer (4), thus, identifying CD3+ TILs as a prognostic biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer and suggesting the possibility to implement the TNM-based system with one biomarker for stage II colorectal cancer. In our study, we show that CD3+ TILs as better TLT strongly supports the hypothesis that lymphoid tissue is relevant to mediate active recruitment of lymphocytes into the tumor, which was then confirmed by intravenous injection of GFP splenocytes in mice.

Our stage-by-stage analysis showed that only very high or very low TLT densities have prognostic impact in stage III and behave as independent predictors of survival. Essentially, although the antitumor impact of immune infiltration is more relevant in stage II colorectal cancer, only the strongest immune responses have an impact on the prognosis in stage III. However, the number of patients with colorectal cancer identified by very high cutoff values is proportionally scarce and thus, limits its clinical impact. The generation of threshold values and combined immune values is a critical issue in the assessment of the prognostic abilities of immune cells and should be carefully managed. In previous studies, the use of these cutoffs together with combined values obtained from markers identifying overlapping immune cell populations has led to claim the futility of pathologic staging (3, 8). This strategy fostered statistical analysis but identified a very small benchmarking population of patients with colorectal cancer devoid of TILs and with a dismal prognosis (3, 8), with limited clinical prognostic relevance when addressing surveillance strategies in the overall population of colorectal cancer.

The type of genomic instability has been proposed as an important variable to be included in the design of studies on immune cells and prognosis (40). MSI patients with colorectal cancer have a better prognosis over MSS (41, 42) together with a higher lymphocytic reaction at the tumor site (4, 6, 26, 37). Our data further addressed this issue by showing that the prognostic value of TLT density is independent by MSI, thus, not being influenced by their lower metastatic potential.

Protocol variability for quantification of immune cells, together with inconsistent statistical design is a critical factor contributing to discrepancy of results among studies. Importantly, although the assessment of TILs, irregularly and heterogeneously dispersed within the tissue is challenging, TLT are easy to detect under an optical microscope and by image analysis, being organized as cellular aggregates. A worldwide concerted action (Immunoscore) is ongoing, aimed at assessing the actual clinical usefulness of a standardized methodologic assessment of T-cell infiltration (43, 44). The results presented here further suggest that the quantification of organized TLT is a feasible and easy approach to this issue in the context of TNM staging and that TLT and TILs are coordinated in their clinical relevance in early-stage human colorectal cancer. Thus, TLT assessment should also be considered when the prognostic value of TILs is investigated.

We found that TLT and TILs populations are highly overlapping in their extent and prognostic abilities because these biomarkers were mutually dependent in predicting the prognosis of patient. However, we provided phenomenologic evidence that the antitumor algorithm represented by TLT and TIL densities is coordinated only when identifying colorectal cancer patients who were not relapsing. Thus, it is conceivable to hypothesize that T-cell recruitment and the mounting of an efficient T-cell antitumor immune reaction is favored by the
presence of a local immune environment like TLT at the tumor site, whereas the pathways of activation of these two immune players seem to differ among colorectal cancers with an aggressive behavior. According to their cellular composition, rich in T and B cells, their structural organization and their intratumor localization, it is reasonable to hypothesize that TLT at the invasive front in human colorectal cancer might collect T cells in close proximity to cancer cells, potentially improving the efficiency of the antitumor response.

B cells are a relevant component of TLT. In human cancer, B cells are known to promote tumor immunity by several mechanisms, including production of antibodies directed to tumor-specific antigens, antigen presentation, and enhancement of T-cell antitumor activity (45, 46) all functions being highly favored by the presence of an immune site. In this regard, our data suggest that also a humoral immune response organized at the tumor site, within TLT, might be a player in the generation of an antitumor immune response with prognostic relevance.

Overall, the occurrence and modulation of TLT may be particularly significant in the development and the responsiveness of novel immunotherapeutic approaches. In this scenario, we also provided further phenomenologic evidence to the idea that nodal invasion is crucial in determining the efficiency and the coordination of adaptive antitumor responses (47). Nevertheless, the lack of murine models properly reproducing the progression of colorectal cancer across its stages of disease might explain at least in part recent failures in translating immunotherapeutic approaches to clinical practice. Therefore, we suggest that TNM stage of disease should be a critical variable in the design and the assessment of clinical trials aimed to test the responsiveness of novel immunotherapeutic strategies in colorectal cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the article.

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