Occurrence of tertiary lymphoid tissue is associated to T cell infiltration and predicts better prognosis in early stage colorectal cancers

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STATEMENT OF TRANSLATIONAL RELEVANCE

Our findings reveal that tertiary lymphoid tissue is associated to lymphocyte infiltration in CRC, contributing to TIL recruitment; TLT cooperates with TILs in a coordinated anti tumor immune response and in predicting better patient’s outcome, thus representing a novel prognostic biomarker for human CRC. Improved understanding of the complexity of immune infiltration along the progression of CRC 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 CRC is a positive prognostic factor and may then represent a target for therapeutic intervention in stage II CRC, with the aim to stimulate the immune system against tumor progression.
ABSTRACT

**Purpose.** Tumor infiltrating T lymphocytes (TILs) play a key role in the clinical outcome of human colo-rectal cancer (CRC); however, the dynamics of their recruitment along CRC 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 stage II and III CRC patients. The role of TLT in lymphocyte recruitment was assessed in a preclinical model of CRC.

**Results.** In both human colo-rectal cancer and in a murine model of CRC, 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 stage II CRC patients.

**Conclusions.** We provide evidence that tertiary lymphoid tissue is associated to lymphocyte infiltration in CRC, providing a pathway of recruitment for TILs. TLT cooperates with TILs in a coordinated anti-tumor immune response, when identifying low risk early-stage CRC patients, thus representing a novel prognostic biomarker for CRC.
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 to favorable prognosis in various common neoplastic diseases, including colo-rectal cancer (CRC) (2-5), thus emerging as a potential immune biomarker of outcome and promising therapeutic target. In CRC, tumor infiltrating lymphocytes (TILs) are associated to favorable prognosis (2-4, 6-8), independently by TNM stage (3, 6, 8), or only in stage II CRC (4). Surprisingly, despite the documented association between TILs and the clinical outcome, the dynamics of T cell recruitment and activation along CRC 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 B-cell follicles, T-cell areas and specialized haematic 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 CRC, 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 lymphotoxin 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 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 colo-rectal cancer. In a retrospective cohort study, we identified highly organized lymphocyte aggregates representing *bona fide* tertiary lymphoid tissue and we defined its prognostic relevance with respect to CD3+ T cell infiltration and demographics, clinical and histopathological variables and their interactions. In a preclinical model of colitis-associated CRC, 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 pathologist’s estimation has not been performed to date for lymphoid tissue in CRC. Thus, a clinically relevant definition of TLT in human CRC is expected to have important implications in the standardized assessment of lymphocyte infiltration in human CRC and in the design of clinical trials aimed to test novel immunotherapeutic approaches.
MATERIALS 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 (CRC), were expanded from the previous series (4). Patients’ demographics, clinical and histopathological 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 histopathological 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 CRC, 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, while stage IV CRC patients 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 CRC patients who consecutively underwent radical surgical resection for pT3 or pT4 colorectal cancer (CRC) 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 morphological analysis assembled a clinical retrospective database by collecting demographics, clinical and histopathological data from the institutional intranet (Supplementary Table S1). These variables, together with the median values of TLT and TIL IRA, were tested as predictors of patient’s outcome. 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 thoraco-abdominal computed-tomography (CT) abdominal ultrasonography, and chest radiography, that 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 tumour 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 patient’s prognosis were performed for all the variables assessed in order to detect any effect modifier (P<0.10).

Immunohistochemistry and microsatellite status. From each patient enrolled in the study, 2 µm thick tissue slides from formalin processed and paraffin embedded tumour 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-SMA (clone 1A4, R&D) CXCL13 and CCL21 (AF801 and AF457, R&D). A 30 minutes incubation with the DAKO Envision system (Dako) or Anti-Goat Polymer kit (Biocare) followed. Diaminobenzidine tetrahydrochloride (DAB) (Dako) was used as chromogen. Nuclei were lightly counterstained with a freshly made haematoxylin solution (Meditex). Presence of fibrosis was assessed on 2 µm-thick sections stained for 20 min 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 screened preliminarily for all cancers included in the study by testing instability at mononucleotide repeats, as previously described (37, 38). 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, Olympus, Italy).
TLT exhibited a distinct structural organization in CRC, 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 non-contiguous microscopic areas located at the tumour 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 histological section, the mean values obtained in three different regions were calculated and used for the subsequent statistical analysis. CD3+ TIL IRA (immunoreactive area) 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 cut-off to perform statistical analyses.

**Statistical analysis.** The association between the extent of TLT density and CD3+ TILs, patient’s baseline characteristics and tumour features was estimated by Pearsons’ simple linear regression analysis. A Cox proportional hazards model was developed to assess the role of TLT density and other demographic, clinical and histopathological features, in predicting the occurrence of disease specific survival (DFS). Time to follow-up was stopped at the time of patient’s death for any case unrelated to CRC 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, non-significant variables, and their non-significant interactions, were removed from the model. Interacting variables at multivariate analysis (P<0.10) were then tested for subgroups analysis accordingly. Differences in median values of TLT density between subsets of CRC and DFS were tested by the Mann–Whitney U test and by Cuzick's trend test. Kaplan-Meier curves of DFS were plotted, while log-rank test was used to compare the curves of each subgroup of CRC patients. 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 CRC. Consecutive tumor slides from 20 CRC patients 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 to the normal mucosa were quantified.

Mice and murine models of CRC

8-week-old C57BL/6J and mice were purchased from Charles River (Calco, Italy); eGFP/C57Bl/6 mice from Jackson Lab. 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 model, mice developed adenomas as a result of the combined treatment with the carcinogen azoxymethane (AOM, 10 mg/kg; Sigma-Aldrich) and sequential administration of the mucosal irritant dextran sulphate (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 Methods are included in the Supplementary Method File.
RESULTS

Human CRC contains aggregates of T cells with features of tertiary lymphoid tissue. The lymphocytic reaction in CRC tissues includes dispersed tumor infiltrating lymphocytes (TILs) and discrete lymphoid aggregates, the latter referred to as Crohn’s 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 to generic clusters of TILs (Figure 1A). These organized structures had features of TLT, with compartmentalized T (Figure 1B, left) and B (Figure 1B, middle) areas, sometimes with germinal centers, and a network of CD21+ follicular dendritic cells (FDC) (Figure 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 (Figure 1C, left), a marker of activated fibroblast cells usually associated to local inflammatory/fibrotic response. The lymphorganogenic chemokines CCL21 (Figure 1C, middle) and CXCL13 (Figure 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 (Figure 1D, left). TLT contained PNAd+ HEV (Figure 1D, middle) and Lyve-1 positive lymphatic vessels (Figure 1D, right), thus confirming that lymphoid aggregates in human CRC have features of tertiary lymphoid tissue.

Density of TLT in human colo-rectal cancer correlates with increased density of tumor infiltrating lymphocytes and associates to increased number of high endothelial venules. To analyze the relationship between TLT and TILs in human CRC, we systematically evaluated the density of TLT in 351 tissue specimens from deeply invading (pT3/pT4) CRC patients 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 (Figure 1A). Notably, in the cohort of 351 CRC patients, whole-tissue visualization of CD3 infiltration showed a higher density of TLT in tumors containing high density of CD3+ TILs (Figure 2A, left), compared to tumors
containing low density of CD3+ TILs (Figure 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 and Figure 2B).

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

**Density of TLT associates with a better prognosis in CRC patients.** 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 tumour 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 towards low values. Distributions of TLT according to the patient histopathological characteristics are described in Supplementary Table S2. We recorded 84 events of CRC disease relapse (DFS) in 351 stage II and III CRC patients.

Unadjusted univariate analysis showed that high TLT density (≥ median) significantly correlated with better outcome (HR 0.62, 95% CI 0.40-0.97; Supplementary Table S3). Importantly, to control for confounders, we performed multivariate Cox analysis which 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). We therefore performed a subgroup analysis revealing that in patients with node-negative CRC (n=185), a high density of TLT (≥ median, 2.68%) and of TILs (≥ median, 2.06%) was associated
with better prognosis compared to patients with a low density of TLT and TILs (log-rank test, \( P=0.02 \) and \( P=0.02 \), respectively) (**Figure 3A-B, left**); conversely, TLT and TIL densities were irrelevant to predict the prognosis of patients with node-positive CRC (\( n=166 \)) (log-rank test, \( P=0.46 \) and \( P=0.64 \), respectively) (**Figure 3A-B, right**). Moreover, we further confirmed that the prognostic behaviour of TLT and TILs varies with disease extent in a different analysis, performed with continuous values. Similarly to TILs (**Figure 3C, right**), among node-negative CRC patients, 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 N0 (Relapse) (\( P=0.03 \))] (**Figure 3C, left**). Conversely, TLT density did not differ in nodal positive patients (N1 and N2) with or without tumour recurrence [N1 (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 (**Figure 3C, left**).

To address the question whether the immune response comprising of TLT and TILs has no impact at all in Stage III CRC, we performed a stage-by-stage analysis, showing the ability of nodal status to identify CRC patient’s relapses among subgroups of TLT density (**Table 1**). Similarly to TILs, nodal involvement was associated with worst outcome in subgroups of CRC patients with intermediate TLT density (0.1-4.2%, \( P=0.001 \); 4.2-8.4%, \( P=0.05 \)) but not in CRC patients 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 (\( P=0.02 \)) CRC and might therefore operate as stage-independent predictors of survival (**Table 1**).

These results suggest that the prognostic behaviour of TLT is similar to that of TILs and prompted us to test which one was the best prognostic marker. However, since these biomarkers identified overlapping populations, which were mutually dependent in predicting prognosis (data not shown), we developed two step-wise, backward Cox multivariate analysis models (model A and model B), to test the independency of their prognostic performance, with respect to other demographics, clinical, histopathological features and MS-Status in stage II CRC (**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 CRC, compared to other tumour 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 analysed the correlation of TLT and TILs distribution according to disease progression. Figure 3D showed that TLT correlates with TILs density (≥median) only in patients who did not experience relapse (P= 0.001), but not in those who relapsed (P=0.28) (Figure 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) CRC patients had a tendency to better prognosis compared to 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 Meyer curves with subgroups analyses (Supplementary figure S1). The analysis showed that MSI CRC was not significantly associated with prognosis in both patients with TLT density low (<median) or high (≥median), (P=0.27, P=0.36, respectively), thus being independent variables in predicting prognosis (Supplementary figure S1).

**Tertiary lymphoid tissue is involved in lymphocyte infiltration in a preclinical model of CRC.** Despite CD3+ T cell density is recognized as a prognostic marker for CRC patients (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 CRC 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) (39), which would recapitulate the formation of tertiary lymphoid tissue associated to 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 Figure S2A, left) and in the tumor region (Supplementary Figure S2A, right). The aggregates are comprised of mostly B lymphocytes and include an area of T cells (Supplementary Figure S2B) and a network of follicular dendritic cells (Supplementary Figure S2C, left). The lymphoid chemokine CXCL13 is expressed at high levels in the aggregates (Supplementary Figure S2C, right), consistent with the predominant presence...
of B cells. During inflammation-driven colon carcinogenesis, lymphoid tissue significantly increased compared to control mice (Supplementary Figure S2D), consistently with the local induction of TLT in chronically inflamed tissues. To define the association of TLT with T lymphocytes in CRC, we compared CD3+ T cells within TLT of control and AOM/DSS mice (Figure 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 (Figure 4A, right). Thus, as evidenced by our previous clinical analysis, this result further confirmed in a preclinical model that TLT associates to increased T cell infiltration in CRC.

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, while very few or none were observed in lymphoid tissue of control mice (Figure 4B, left and middle). Whole tissue analysis evidenced a significant increase in the density of GFP+ cells in CRC-associated lymphoid tissue (Figure 4B, right), thus confirming that TLT in the tumour mediates recruitment of lymphocytes.

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 (Figure 4C, left and middle). Quantification of the volume of vessels draining TLT evidenced a significant increase in AOM/DSS mice compared to control mice (Figure 4C, right). Among CD31+ vessels, lymphoid tissue contained PNAd+ high endothelial venules (Figure 4D, left); CD3+ T cells were localized inside HEV and in close proximity to the vessel wall (Figure 4D, middle). The number of PNAd+ HEV within lymphoid tissue of AOM/DSS mice was higher of, compared to control mice (Figure 4D, right), thus confirming that TLT formation in colon cancer associates to 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 towards 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 colo-rectal cancer. In our study, we show that CD3+ T infiltrating lymphocytes at the tumor invasive front of CRC can localize in organized aggregates, comprised of T and B cell areas and a network of follicular dendritic cells, thus with features of tertiary lymphoid tissue, whose clinical relevance in relationship to TILs has been so far unexplored.

Intratumor TLT was associated to 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 CRC has been extensively documented (2-6, 8, 26, 27). Previous analyses of T cell infiltration in CRC patients claimed CD3+ TILs as better indicator of prognosis than TNM tumor staging (3). However, on accurate analysis, CD3+ TILs retained prognostic significance only in nodal-negative CRC patients (4), thus identifying CD3+ TILs as a prognostic biomarker for Stage II CRC and suggesting the possibility to implement the TNM-based system with one developed upon the densities of CD3+ TILs. As to TLT, here we took advantage of a computer assisted image analysis, which is objective and statistically more relevant, providing continuous distributions of immune cell densities. By these means, we showed for the first time that, in accordance with TILs, TLT are not predominant over tumor staging in predicting patient outcome and their prognostic relevance varies with the state of disease progression at diagnosis. Thus, they may contribute to tumor control at the early stages of the
disease, while they progressively lose this capability along with tumor progression and occurrence of lymph node metastasis.

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, while the antitumor impact of immune infiltration is more relevant in Stage II CRC, only the strongest immune responses have an impact on the prognosis in Stage III. However, the number of CRC patients identified by very high cut-off 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 employment of these cut-offs together with combined values obtained from markers identifying overlapping immune cell populations has led to claim the futility of pathological staging (3, 8). This strategy fostered statistical analysis but identified a very small benchmarking population of CRC patients devoid of TILs and with a dismal prognosis (3, 8), which does not provide clinical prognostic relevance when addressing surveillance strategies in the overall population of CRC.

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 CRC patients have a better prognosis over MSS (41, 42) together with a higher lymphocytic reaction at the tumour 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, while 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 methodological 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 CRC. 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, since these biomarkers were mutually dependent in predicting patient's prognosis. However, we provided phenomenological evidence that the antitumour algorithm represented by TLT and TIL densities is coordinated only when identifying CRC 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 favoured by the presence of a local immune environment like TLT at the tumour site, while the pathways of activation of these two immune players seem to differ among CRCs with an aggressive behaviour. According to their cellular composition, rich in T and B cells, their structural organization and their intra tumor localization, it is reasonable to hypothesize that TLT at the invasive front in human CRC might collect T cells in close proximity to cancer cells, potentially improving the efficiency of the anti-tumor 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 antitumour 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 phenomenological evidence to the idea that nodal invasion is crucial in determining the efficiency and the coordination of adaptive anti-tumor responses (47). Nevertheless, the lack of murine models properly reproducing the progression of CRC 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 CRC.
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REFERENCES


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<table>
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<th>TLT density</th>
<th>Relapse</th>
<th>Cox Hazards Model</th>
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<tr>
<td></td>
<td>No TLT (n=257)</td>
<td>Yes (n=84)</td>
</tr>
<tr>
<td>0 - 4.16 %</td>
<td>N0 25</td>
<td>8</td>
</tr>
<tr>
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<td>N1-N2 25</td>
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<tr>
<td>4.16 - 8.32 %</td>
<td>N0 42</td>
<td>4</td>
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<td>N1-N2 20</td>
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<td>≥ 8.32 %</td>
<td>N0 30</td>
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<td>N1-N2 18</td>
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Table 1. Prediction of risk for disease relapse by nodal status in 351 pT3/pT4 CRC.

CRC were subgrouped according to the density of CD3+ TILs and TLT at the tumor invasive front.

N0: no lymph node involvement. N1: 1-3 nodes involved. N2: more than or equal to 4 nodes involved.
### Table 2. Multivariate Cox hazard model for predictive factors of disease relapse in 185 Stage II pT3/pT4 CRC.

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, non-significant variables, and their non-significant interactions, were removed from the model.

**a:** pT3: invading through the muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues. pT4: directly invading adjacent organs or perforating visceral peritoneum.

**b:** Variants mucinous or medullary.

<table>
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<tr>
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<th>Relapse</th>
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<th>Model B</th>
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<tr>
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<td>No</td>
<td>Yes</td>
<td>HR (95% CI)</td>
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<td></td>
<td>(n=159)</td>
<td>(n=26)</td>
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FIGURE LEGENDS

Figure 1. Characterization of aggregates of T cells with features of tertiary lymphoid tissue in human CRC. (A) A representative image obtained from a virtual digital slide of a colo-rectal tumor specimen. Staining with anti-CD3 antibody allows identification of the T lymphocytic reaction, including the presence of lymphoid aggregates at the invasive front (asterisks). Panels on the right are magnifications of the lymphoid aggregates. (B) Lymph-node like follicles (dotted lines) in human CRC specimens are composed of CD3+ T cells (left), CD20+ B cells (middle) and networks of CD21+ follicular dendritic cells (right). Sections in left and middle panels are consecutive sections and show the compartmentalization between B and T cells in the follicle. (C) Intratumor lymphoid tissue is prevalently associated to tumor stroma, containing -SMA positive cells (left). The lymphoid chemokines CCL21 (middle) and CXCL13 (right) are present inside lymphoid follicles. (D) Staining with Sirius Red evidenced the presence of collagen fibers closed to vascular structures (asterisks) (left). Vessels within lymphoid tissue include PNAd+ HEV (middle) as well as Lyve-1+ lymphatic vessels (right), suggesting traffic of naïve and memory lymphocytes. Sections in I and J are consecutive sections, showing HEV and lymphatic vessels in the same follicle. Dot lines indicate follicle contour. Scale bars 1 mm (A); 200 μm (B, C left; D, left and right); 100 μm (C, middle and right; D, middle).

Figure 2. Correlation of tumor-associated TLT with increased density of T cells and formation of high endothelial venules (HEV) in human CRC. (A) Representative images obtained from virtual digital slides of two colo-rectal tumor specimens with a low (left) and high (right) density of infiltrating T cells and follicles. Staining with anti-CD3 antibody allows identification of lymphoid tissue and infiltrating lymphocytes. Asterisks indicate lymphoid tissue. (B) Correlation between CD3+ TILs (% IRA: immunoreactive area) and TLT density (% IRA) in 351 colo-rectal cancer patients. P value by Pearson’s simple linear regression analysis. (C) Increased number of HEV associated to TLT in the tumor compartment. Comparison between lymphoid tissue associated to the normal mucosa (left) and TLT at the invasive front of the tumor (right).
Quantification of HEV in tumor lymphoid tissue compared to lymphoid follicles associated to normal mucosa. HEV were quantified by computer assisted image quantitative analysis on slides from 20 paraffin embedded cancer tissue specimens stained with anti PNAd antibody. Bars represent the number of HEV in each follicle (D). Two-tailed p values by t test. * P< 0.05. Scale bars 1 m (A); 200 μm (C).

**Figure 3. Clinical relevance of TLT in 351 CRC patients.** (A-B) Kaplan-Meier curves showing disease-free survival (DFS), according to TLT and TIL density. A high density of TLT (≥median, 2.68%) and of TILs (≥ median, 2.06%) is associated with better outcome in patients with node-negative CRC (P=0.02 and P=0.02 respectively, n=185) (A-B, left), but not in those with node-positive CRC (P=0.46 and P=0.64 respectively, n=166) (A-B, right). (C-D) Coordination of TLT and TIL immune infiltration. The prognostic behaviour of TLT (C, left) and TILs (C, right) varies with disease extent. Among node-negative CRC patients, TLT density is significantly lower in patients who relapsed (n=26) than in patients with no evidence of disease recurrence (n=159) (P=0.03), while does not differ in nodal positive patients with or without tumour recurrence. Distribution of TLT IRA% according to the CD3+ density (≥median) in relapsing and not relapsing stage II CRC patients. TLT and TILs correlate only among patients that do not relapse (D). P (C, left): N0(noR)vsN0(R) P=0.03; N1(noR)vsN1(R) P=0.43; N2(noR)vsN2(R) P=0.15. P (C, right): N0(noR)vsN0(R) P=0.01; N1(noR)vsN1(R) P=0.74; N2(noR)vsN2(R) P=0.51.

**Figure 4. Tertiary lymphoid tissue mediates increased lymphocyte infiltration in a preclinical model of CRC.** (A) Expansion of TLT associates to increased number of CD3+ T cells. Staining with anti-CD3 antibody in lymphoid tissue (dotted circle) of control mice (left) and AOMDSS mice (middle). Quantification of CD3+ cells in colon tissues of mice was performed by computer-assisted image analysis and indicates increased number of CD3+ T cells in the TLT of AOMDSS mice, compared to control mice (right). (B) Tertiary lymphoid tissue is actively involved in the recruitment of lymphocytes. GFP+ splenocytes were intravenously injected in control and AOM/DSS mice and whole colons analysed by immunofluorescence after 24 hours.
Representative images of GFP+ cells in a lymphoid follicle (dotted circle) of control (left) and AOM/DSS (middle) mice. Quantification of GFP+ cells was performed on 20 μm thick sections from the whole colon; each dot represents the density of GFP+ cells in the TLT analysed (right). One representative of 2 experiments performed (n = 3 mice, Ctrl; n = 5 mice AOM/DSS; bars represent SEM). (C) The vessel network draining lymphoid tissue (B220+ B cells) in the colon mucosa of control (left) and tumor-bearing mice (middle) includes CD31+ blood vessels and Lyve1+ lymphatic vessels. Morphometric analysis on colon whole mounts indicates vessel expansion around and inside lymphoid tissue of AOMDSS mice (right) (n = 6 mice, Ctrl; n = 6 mice AOM/DSS; bars represent SEM).  (D) TLT contains functional high endothelial venules (HEV). Staining with and anti-PNAd antibody indicates presence of HEV in lymphoid tissue (left). CD3+ T lymphocytes circulate into PNAd+ HEV, and localize in closed proximity to the vessel wall (asterisks) (middle). The number of HEV increases in lymphoid tissue of tumor bearing mice (right). Scale bar 200 μm (A; B; C); 100 μm (D, left); 50 μm (D, middle). Two-tailed P values by t test. ** P< 0.005; *** P< 0.001.
Figure 1
Figure 2
Figure 3
Figure 4
Occurrence of tertiary lymphoid tissue is associated to T cell infiltration and predicts better prognosis in early stage colorectal cancers

Giuseppe Di Caro, Francesca Bergomas, Fabio Grizzi, et al.

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