Combined low densities of FoxP3$^+$ and CD3$^+$ tumor-infiltrating lymphocytes identify stage II colorectal cancer at high risk of progression

AUTHORS.
Tommaso Cavalleri$^1$, Paolo Bianchi$^1$, Gianluca Basso$^1$, Giuseppe Celesti$^1$, Fabio Grizzi$^2$, Paola Bossi$^3$, Luana Greco$^1$, Calogero Pitrone$^1$, Emanuele Valtorta$^4$, Gianluca Mauri$^{4,10}$, Mauro Truini$^4$, Filippo Gustavo Dall’Olio$^5$, Giovanni Brandi$^6$, Andrea Sartore-Bianchi$^{4,10}$, Luigi Ricciardiello$^7$, Valter Torri$^8$, Lorenza Rimassa$^9$, Salvatore Siena$^{4,10}$, Alberto Mantovani$^{2,11,12}$, Alberto Malesci$^{13,14}$, Luigi Laghi$^{1,14,15,16}$

Author information:
1 Laboratory of Molecular Gastroenterology, Department of Gastroenterology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy
2 Department of Immunology and Inflammation, Humanitas Clinical and Research Center, Rozzano, Milan, Italy
3 Department of Pathology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy
4 Niguarda Cancer Center, Grande Ospedale Metropolitano Niguarda, Milan, Italy
5 Division of Oncology, Sant’Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy
6 Department of Experimental, Diagnostic and Specialty Medicine, Sant’Orsola-Malpighi Hospital, Bologna, Italy
7 Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy
8 Laboratory of Methodology for Biomedical Research, Department of Oncology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy
9 Medical Oncology and Hematology Unit, Humanitas Cancer Center, Humanitas Clinical and Research Center, Rozzano, Milan, Italy
10 Università degli Studi di Milano, Dipartimento di Oncologia ed Emato-Oncologia, Milano, Italy
11 Department of Biotechnologies and Translational Medicine, Humanitas University, Rozzano, Milan, Italy
12 The William Harvey Research Institute, Queen Mary University of London, London, United Kingdom
13 Department of Internal Medicine, Humanitas University, Rozzano, Milan, Italy
14 Department of Gastroenterology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy
15 Hereditary Cancer Genetics Clinic, Department of Gastroenterology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy
16 On behalf of Alleanza contro il Cancro (ACC) Colorectal cancer Working Group

*These authors equally contributed to this work
Keywords: colorectal cancer outcome, tumor infiltrating lymphocytes, FoxP3, CD3, microsatellite instability

Running Title: FoxP3+ and CD3+ TILs and Stage II CRC.

Corresponding Authors:

Dr. Luigi Laghi  
Dept. of Gastroenterology  
IRCCS Humanitas Clinical and Research Center  
Via Manzoni, 56  
20089 Rozzano – Milan  
ITALY  
e-mail: luigi.laghi@humanitas.it  
phone: +39. 02. 8224. 4572  
fax: +39. 02. 8224. 4590

Prof. Alberto Malesci  
Dept. of Gastroenterology  
IRCCS Humanitas Clinical and Research Center  
Via Manzoni, 56  
20089 Rozzano - Milan  
ITALY  
e-mail: alberto.malesci@humanitas.it  
phone: +39. 02. 8224. 4542  
fax: +39. 02. 8224. 4590

Words count: 3074

Total number of figures and tables: 5

Conflicts of interest: All the authors declare no conflicts of interest.
ABSTRACT

The densities of CD3$^+$ and CD8$^+$ tumor-infiltrating lymphocytes (TILs), combined with TNM (tumor-node-metastasis) staging, have prognostic value for nonmetastatic colorectal cancer (CRC) patients. We compared the prognostic value of CD3$^+$ and FoxP3$^+$ TILs at the invasive front, TNM classifiers, and microsatellite (MS) status in a trial set of patients with stage II-III CRC ($n = 413$), by recursive partitioning with a classification and regression tree (CART). Significant prognostic factors and interactions were re-assessed by logistic regression and Cox proportional-hazards modeling in the trial and a validation set ($n = 215$) of patients with stage II CRC. In the trial set, CART indicated that TIL numbers were of value only in predicting recurrence risk for stage II cancers, where low densities of FoxP3$^+$ TILs ranked first and low densities of CD3$^+$ TILs further stratifying risk. Multivariate analysis showed that TILs interacted with tumor stage (FoxP3$^+$, $P = 0.06$; CD3$^+$, $P = 0.02$) and MS instability (FoxP3$^+$; $P = 0.02$). In stage II MS-stable cancers, concomitant low densities of both FoxP3$^+$ and CD3$^+$ TILs identified patients with the highest progression risk in the trial (HR 7.24; 95%CI, 3.41-15.4; $P < 0.001$) and the validation (HR 15.16; 95% IC, 3.43-66.9; $P < 0.001$) sets. FoxP3$^+$ and CD3$^+$ TIL load in CRC was more informative than other prognostic factors before the cancer progressed to lymph nodes. This prognostic information about TILs, including FoxP3$^+$ cells, suggests that randomized controlled trials might be refined to include interactions between TNM status, molecular classifiers, and post-surgical treatments.
INTRODUCTION

Translational studies have clarified the role of the local immune response in controlling the progression of colorectal cancer (CRC). High intra- and peri-tumoral densities of CD3+ T-cells, CD8+ cytotoxic lymphocytes and memory CD45RO+ cells predict a better postsurgical outcome [1, 2]. Densities of CD3+ and CD8+ cells at these locations defined an immunoscore assessing the extent of the anti-tumor response in early CRC that was more informative than the analysis of a single T-cell population at a given location [3]. With a retrospective analysis of CRC cohorts [4], an international consortium presented Immunoscore as a reliable predictor of cancer recurrence in patients with nonmetastatic CRC and an improvement upon the prognostic power of conventional TNM (T, size and spread of primary cancer; N, number of metastatic lympho nodes; M, metastases spread to distant organs) staging [5].

Although not included in Immunoscore, high densities of FoxP3+ tumor infiltrating lymphocytes (TILs) predict a favorable CRC outcome [6-13]. The positive prognostic value of FoxP3+ TILs is counter-intuitive, given that the expression of the Foxp3+ transcription factor is typical of T-regulatory cells (T-regs), an immunosuppressive population associated with poor prognosis in other cancers [14]. Before FoxP3+ TILs can function as an immune biomarker predictive of CRC outcome, their prognostic value needed to be analyzed. First, densities of FoxP3+ TILs might be influenced by the tumor microsatellite (MS) status [6, 8, 15]. Indeed, FoxP3+ TILs had no prognostic value for CRC with MS-instability (MSI) in a study stratified by tumor mismatch-repair status [7]. Second, the prognostic value of FoxP3+ TILs is unclear in the context of nodal involvement, which may reflect tumor immune evasion [16]. In patients with stage II/III CRC, FoxP3+ TILs presented as a stage-independent prognostic factor, although stages II and III were not separately analyzed [6]. Finally, the interaction of FoxP3+ with other T cells is unclear. Densities of FoxP3+ TILs predicted survival of patients with early CRC independently of, and more accurately than, densities of CD8+ and CD45RO+ TILs in one study [6], but were less informative than densities of CD45RO+ TILs in...
another study [8]. FoxP3+ TILs were also reported to significantly interact with the prognostic value of CD8+ or CD3+ TILs [9, 11, 12]. The aim of this study was to see whether densities of FoxP3+ cells at the invasive tumor front can add to the prognostic significance of CD3+ TILs in a patient series of pT3/pT4 CRC stratified by nodal involvement and MS status.

PATIENTS AND METHODS

Study population. The trial set included formalin-fixed, paraffin-embedded (FFPE) tumor specimens from 413 consecutive patients with stage II-III pT3-T4 CRC who had received radical surgery at the Humanitas Clinical and Research Center, from 1997 to 2006. The external validation set included tissues of stage II CRC from 215 consecutive patients who had undergone surgery from 2010 to 2015 at the St. Orsola-Malpighi Hospital in Bologna (n=74), or from 2008 to 2014 at the Grande Ospedale Metropolitano Niguarda in Milan (n=141). The absence of metastasis at diagnosis was assessed definitively in all patients by combining histopathological findings, surgical records, and perioperative imaging (abdominal CT and chest radiography in all patients). The observation period started immediately after surgery. To monitor postsurgical tumor recurrences, thoraco-abdominal CT, abdominal ultrasonography, and chest radiography were done according to common protocols for surveillance. Patients with rectal cancer treated with neo-adjuvant radiotherapy were not included in the study. Five-fluorouracil chemotherapy was administered on clinical grounds and not in the context of prospective trials. MS-status was systematically assessed by analysis of mononucleotide repeats. MSI assignment was based on the analysis of repeats in mononucleotides BAT26 and BAT25. DNA was purified from paraffin sections of formalin-fixed tissue with a neoplastic cell content above 50%. After DNA extraction by proteinase-K digestion, BAT26 and BAT25 loci were amplified by fluoresceinated primers, and PCR products analyzed by capillary electrophoresis (ABI PRISM 310 DNA Sequence; PE Applied Biosystems). Finally, the MSI phenotype tumours were investigated for MMR protein defects by immunohistochemistry [17, 18].
Ethics approval and consent to participate. The study was conducted in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects. Samples from both the internal and external set were obtained complying with protocols approved by the local Ethical Committee and Institutional Review Board at Humanitas Clinical and Research Center (approval no. 1052 and further acknowledgment of 14 May 2013). All patients provided their informed consent to the referring physician or to other clinicians involved in the study at each participating center.

Immunohistochemistry. Formalin-fixed, paraffin-embedded, and 2-μm thin sections of tumour were deparaffinized and exposed to an antigen-retrieval system (1 mmol/L ethylenediamine tetraacetic acid, pH 8, for 30 min at 98°C). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature; non specific staining was reduced with Background Sniper (Biocare Medical, The Netherlands) for 15 min at room temperature. Then, slides were incubated for 60 min at room temperature in moist chamber with the specific antibodies for CD3 (1:50, clone F7238; Dako, Italy) and FoxP3 (dilution 1:100, clone 236/E7, Abcam, UK), or mouse IgG (Dako, Italy) for negative controls. To reveal bound antibodies the slides were exposed to MACH 4 Universal HRP Polymer system (Biocare Medical, the Netherlands) following manufacturer’s instructions, followed by incubation with DABchromogen X50 (Dako, Italy) as chromogen. Finally, nuclei were lightly counterstained with a freshly made hematoxylin solution (Harris Hematoxylin, DiaPath, Italy).

Image analysis. From whole tissue slides, we obtained digital images of three randomly chosen, non-adjacent, non-overlapping areas at the tumor invasive front. The selected areas had to include 50% of tumor gland and 50% of bordering stroma. The observer who selected the areas of interest was blinded to tumor microsatellite status and to any patient clinical data. The Image-Pro Premier 9.2 (Media Cybernetics, Rockville, MD, USA) analysis software calculates on the total digital captured area the percentage of TIL immune-reactive area (IRA%) on the basis of red, green, and
blue color segmentation. For each specimen, the average IRA% from the three different areas was calculated and used for subsequent statistical analysis.

**Statistical analysis.** We weighed the relationship between IRA% of CD3\(^+\) and FoxP3\(^+\) TILs by linear regression, and assessed their association with patient demographics, clinical-pathological features at diagnosis by Wilcoxon/Mann-Whitney test.

For evaluating CD3\(^+\) and FoxP3\(^+\) TIL densities as prognostic factors, we first used recursive partitioning analysis to identify foretelling variables. These variables included patient demographics and clinical-pathological tumor features, assessed by the CART (Salford Systems, San Diego, CA, USA) software, which also identifies cutoff values for continuous variables. CART, commonly used in data mining, was used to build a model predicting the likelihood of recurrence (dependent variable) testing several independent variables (i.e., demographics, clinical, pathological, molecular features, and TIL densities) [19, 20]. The algorithm weights each independent variable in predicting the dependent one, moving along a tree-like decision structure. In the decision tree, each node hierarchically identifies the independent variable with the best predictive value. From each node, branches emerge, as long as predictive variables can be identified. Afterward, the prognostic values of CD3\(^+\) and FoxP3\(^+\) TILs densities were re-tested by logistic regression, and the variables significantly associated with disease relapse at univariate analysis were entered in a multivariate model including possible interactions. Independent predictors of disease-free survival (DFS) were tested in a Cox proportional-hazards [21] model to evaluate progression. DFS was calculated from diagnosis until March 1\(^{st}\), 2013, which was the date of data censoring for the trial set, and December 31\(^{st}\) 2017 for the validation set. To analyze the survival of CRC patients grouped according to TIL density, we plotted Kaplan-Meier curves and obtained the relative Log-rank tests. All statistical analyses except for recursive partitioning were managed by STATA (version 13.1). Two-sided \(P\) values of \(< 0.05\) were considered statistically significant.

**RESULTS**
**Densities of FoxP3⁺ and CD3⁺ TILs by patient demographics and tumor features.**

Variable densities of CD3⁺ TILs were detectable at the invasive front of 97.3% of tissues from the trial set (402 of 413), and FoxP3⁺ TILs were detected in 82.3% (340 out of 413) of tissues. Linear regression analysis showed no significant correlation (p=0.26) between the density of FoxP3⁺ TILs (median, 0.37%; 2nd-3rd quartile, 0.13-0.68) and the density of CD3⁺ TILs (median, 2.42%; 2nd-3rd quartile, 0.92-5.78).

Densities of FoxP3⁺ and CD3⁺ TILs by patient demographics and tumor molecular/pathological features are detailed in Supplementary Table S1. MSI correlated with a higher density of CD3⁺ TILs (P < 0.001) and with lower densities of FoxP3⁺ cells (P < 0.001). No FoxP3⁺ immuno-reactivity was detectable in 21 of 66 (31.8%) of these tumors.

**Data mining for the prognostic impact of TILs in stage II/III CRC.**

CART analysis (Fig. 1) identified TNM staging as the highest hierarchical node (odds ratio (OR), 2.97; 95%CI, 1.85-4.77; p<0.001) in the prognostic tree, stage III accounting for 70 of 102 (68.6%) postoperative recurrences. Densities of FoxP3⁺ TILs or CD3⁺ TILs had no predictive value in stage III CRC, in which only nodal status had further predictive value (N2 vs. N1; OR, 3.56; 95%CI, 1.93-6.58; P < 0.001). In contrast, both FoxP3⁺ and CD3⁺ TILs had a place in the prognostic tree of stage II CRC. Low densities of FoxP3⁺ cells ranked first in this decisional branching (OR 5.20; 95%CI, 2.26-11.9; P < 0.001), identifying 23 of 32 (71.9%) recurrences. Secondary branching of low-FoxP3⁺ tumors by CD3⁺ TILs further improved the identification of recurrences (OR, 4.46; 95%CI, 1.58-12.6; P < 0.001). The combination of low densities for both Foxp3⁺ and CD3⁺ TILs predicted 16 of 32 (50.0%) recurrences with 20 of 179 (11.2%) false positives, and thus 83% accuracy.

**Analytical assessment of prognostic variables and of their interactions.**

At logistic regression analysis (Supplementary Table S2), postoperative recurrences were significantly associated with lower (below median) densities of FoxP3⁺ (OR, 2.24; 95%CI, 1.41-3.56; P < 0.001) and CD3⁺ TILs (OR, 1.59; 95%CI, 1.01-2.50; P = 0.04). Other variables...
significantly associated with recurrences included stage III \((P < 0.001)\), pT4 local invasion \((0.03)\), and angio-invasion \((p=0.002)\). A protective effect was observed for MSI \((P = 0.05)\). At multivariate analysis, densities of both FoxP3+ and CD3+ TILs interacted with tumor stage \((P = 0.06\) and \(P = 0.02\), respectively), densities of FoxP3+ cells significantly interacting also with the MS-status of stage II CRC \((0.03)\). A stratified analysis showed that below-median densities of FoxP3+ and CD3+ TILs were associated with disease progression in patients with stage II MS-stable (MSS) CRC, but not in patients with MSI or stage III cancer (Table 1).

ROC curve analysis confirmed that densities of TILs can predict postsurgical progression in patients with stage II MSS CRC (Fig. 2), the area under the curve (AUC) being 0.77 for FoxP3+ cells and 0.71 for CD3+ cells. The estimation of the cutoffs returned values of IRA% matching those adopted by the CART. ROC analysis also confirmed the absence of any predictive value of TILs in stage II MSI cancers (AUC: 0.45 for FoxP3+; 0.53 for CD3+ TILs), and in stage III tumors (AUC: 0.55 and 0.53, respectively).

At Cox proportional-hazards model [21] (Table 2), low densities of FoxP3+ and CD3+ TILs, as defined by ROC cutoffs, were both independent predictors of poor disease-free survival (DFS) in stage II MSS CRC (FoxP3+: hazard ratio (HR), 5.61; 95%CI, 2.38-13.2; \(P < 0.001\) - CD3+: HR, 5.76; 95%CI, 2.16-15.35; \(P < 0.001\)). Deep local invasion (pT4) was the only additional predictor of recurrence (HR, 3.88; 95%CI, 1.29-11.7; \(P = 0.02\)). Tumors with high densities of both FoxP3+ and CD3+ TILs had no recurrence. The outcome of cancers with a discordant pattern of TILs (high/low or low/high densities) was better than that of tumors with low densities for both FoxP3+ and CD3+ TILs. As a result, coexisting low densities of both TIL markers provided the strongest predictor of poor outcome (HR, 7.24; 95%CI, 3.41-15.4; \(P < 0.001\), vs. all other combinations). The recurrence rate of cancers with low densities for both FoxP3+ and CD3+ TILs (50%) exceeded the sum of the recurrence rate of tumors with discordant densities (15%), as observed in additive models of biological interaction.
Kaplan-Meier curves (Fig. 3) recapitulated statistical analysis by showing that stage II and MSS CRC (Fig. 3A) harboring both low-density FoxP3+ and low-density CD3+ TILs, had a 5-year DFS lower than 60%, which was worse than that of tumors with high densities for both cell types (100% 5-year DFS; $P < 0.001$) or discordant densities (85% 5-year DFS, $P < 0.001$). Conversely, TILs densities did not predict DFS of patients with stage III MSS CRC (Fig. 3B), nor that of subjects with MSI cancer (Fig. 3C).

**Data validation in the external set of stage II MSS CRC.**

In the external validation set of stage II MSS CRC, demographics and tumor pathological features showed no significant association with densities of FoxP3+ and CD3+ cells (Supplementary Table S3). ROC curve analysis showed that AUC of FoxP3+ and CD3+ TILs (0.78 and 0.71, respectively) were superimposable to those computed from corresponding tumors of the trial set (Supplementary Fig. S1). Cox multivariate analysis employing trial set cutoffs confirmed that both FoxP3+ (HR 5.15; 95%CI, 1.94-13.7; $P = 0.001$) and CD3+ (HR 2.78; 95%CI, 1.23-6.28; $P = 0.001$) TILs are independent predictors of recurrence (Supplementary Table S4). Again, the combination of low-density FoxP3+ and low-density CD3+ TILs predicted the worst outcome (HR 5.31; 95%CI, 2.45-11.5; $P < 0.001$), as confirmed by Kaplan-Meier curves (Fig. 3D).

The prognostic value of TILs was independent of adjuvant therapy. Kaplan-Meier curves of 327 patients with stage II MSS CRC (a cohort arrived at by merging the trial and validation sets) showed that combined information about density of FoxP3+ and CD3+ TILs predicted DFS in treated ($n = 119$, 36.4%) and untreated patients (Supplementary Fig. S2).

**DISCUSSION**

Studies have shown that the extent of the local immune response to CRC is a determinant of the patient outcome [1-3, 5, 16, 22]. The present study demonstrates that postoperative recurrences are better predicted by densities of FoxP3+ TILs at the invasive front of stage II MSS CRC than by CD3+ TILs. By introducing the concept of a synergistic interaction of FoxP3+ and CD3+ TILs in
determining the protective effect of the local immune response, this work supports the inclusion of the density of FoxP3+ cells as a prognostic variable.

Following a meta-analysis supporting the prognostic value of FoxP3+ TILs [23], our study demonstrates the prognostic value of FoxP3+ TILs at the tumor front of stage II CRC through the analysis of whole-tissue sections and by statistical analysis. The statistical analysis included recursive partitioning, which weighs the impact of a candidate marker by allowing for interactions with other prognostic variables [24]. The results were confirmed by conventional multivariate and interaction models. The lack of correlation between the densities of FoxP3+ and CD3+ TILs along with their different association with MSI do not suggest that infiltration by FoxP3+ cells is a simple homeostatic response to an effective T-cell recognition. Rather, our data suggest individual recruitment of FoxP3+ and CD3+ TILs, which then synergistically interact in protecting against cancer progression [25].

Even though high density of FoxP3+ TILs correlates with improved outcomes in patients with stage II CRC, FoxP3+ TILs are also associated with immunosuppressive functions. Indeed, FoxP3 is expressed by activated effector T cells [26] and by T cells that, once sorted from CRC, inhibit IFNγ production and T-cell proliferation [27]. The latter are bona fide immunosuppressive Tregs. Colonic microflora can also divert T-cell killing activity away from cancer cells [28]. Therefore, it has been proposed that FoxP3+ Tregs trimmed by the colonic milieu may attenuate the Th17-mediated pro-inflammatory and tumor-enhancing response induced by bacterial exposure [10, 15], thus freeing other TILs to target antigens expressed by cancer cells. Consistent with this view, T-regs induced tumor regression in a mouse model of intestinal polyposis [29]. In addition to their regulatory functions modulating the immune response to the colonic commensal microflora, FoxP3+ cells could activate effector and memory TIL abilities rather than suppressor functions [30]. Effector and memory TILs are otherwise associated with Treg cells shaped by microenvironmental stimuli, and require a complex mix of phenotypic features for their proper identification [31].
Various factors slow the introduction of immune-based prognostic markers into the clinical routine. Consensus on which T-cell subsets and locations best serve for prognosis in CRC is lacking. Indeed, a meta-analysis confirmed the prognostic value of the immune infiltrate but did not validate the individual impact of T-cell subtypes and sites [32].

Densities of CD3+ and CD8+ cells within the tumor and at the invasive margin were proposed as informative for CRC prognostication [3] and adopted by the Immunoscore consortium [4, 5]. Our results endorse the inclusion of FoxP3+ cell into the panel of markers aimed at predicting recurrences of stage II CRC.

The most controversial issue is the prognostic value of TILs densities across TNM stages. In the present study, TILs stratified patient survival across stage II and III, but neither CD3+ nor FoxP3+ TILs had prognostic value in stage III CRC. This stands in conflict with studies reporting that CD3+ and FoxP3+ TILs have prognostic value independent of CRC stage [2, 7, 8, 33] and to some extent in conflict with the Immunoscore results [5], in which stage III CRCs were under-represented, and increasing nodal involvement modified patient outcome independently of the Immunoscore with multivariate analysis. In a scenario in which the interaction between nodal invasion and prognostic value of TILs is overlooked, it would be assumed that the magnitude of the local immune reaction controls tumor progression across stages with unmodified efficiency. Our results contradict this notion and support the integration of immunometric data with the TNM system. Furthermore, other immune cells, such as tumor-associated macrophages, tertiary lymphoid tissue [34], and neutrophils, modify cancer cell behavior [35] and may predict the outcome for patients with stage III CRC [36].

At present, neither discordant biological theories nor the lack of clarity on the hierarchical positioning of the immune markers vs. the TNM system should delay the implementation of studies needed to complete the validation of the best immunoscore for stage II CRC. Neither FoxP3+ nor CD3+ cells have a prognostic impact in MSI CRC, so that the exclusion of these cancers strengthens the predictive power of TILs. TIL analysis in stage II CRC would be prognostically efficient even
in the absence of MSI screening, due to the low recurrence rate of mismatch-repair deficient cancers, explained by persistent renewal of neo-antigens to improve immune surveillance [37]. At any event, universal screening for MSI is currently an advocated standard [38, 39], so that TNM classification would be best empowered by the parallel inclusion of TILs and MS-status assessment. Features that pose the greatest risk of excluding patients with stage II CRC from such assessment include inadequate sampling of lymph nodes, poorly differentiated histology, pT4 invasion, and perforation [40]. None of these confers a risk of recurrence superior to that reported for immune markers by large reference studies. Accordingly, we found that the prognostic impact of low-FoxP3⁺-CD3⁺ TILs in stage II MSS CRC exceeded the weight of pT4. This reinforces the concept that only the introduction of a prognostic immunoscore can optimize the benefits of adjuvant therapy in stage II CRC. Although the limited number of patients with stage II cancers treated with adjuvant therapy does not allow for conclusive answers, our results suggest that the immune response is more relevant than chemotherapy in determining the outcome of stage II CRC. Thus therapeutic efforts and innovative approaches should focus on patients with low TIL loads. Such approaches might include the use of interleukins (IFN-γ) and/or antibodies (anti-CTL4 or anti-PD-1) to boost the immune response of patients with MSS CRC. Patients with MSI CRC might benefit not only from anti-PD1 therapies but also from vaccination strategies based on frameshifted peptides. Our results from implementation of Immunoscore and including information about FoxP3⁺ TILs suggest the value of further testing within randomized controlled trials, such as those of the TOSCA trial [41] and of the IDEA collaboration [42].

ACKNOWLEDGMENTS

LL would like to acknowledge and thank Alleanza Contro il Cancro (ACC), on behalf of the Colorectal cancer working group.
FUNDING

Authors at IRCCS Humanitas Clinical and Research Center (TC, PBi, LG, GB, LL) are supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), grant “Macrophages and colorectal cancer response to 5-fluoruracil: chemo-macrophage interaction mediating tumor cell killing” AIRC Investigator Grant IG 16092 (2015-2018). Luigi Laghi also acknowledges Alleanza Contro il Cancro (ACC) for supporting the fellowship of dr. Calogero Pitrone. Alberto Mantovani is supported by AIRC Investigator Grant IG 19014 and AIRC 5x1000 21147.

Authors at Niguarda Cancer Center (GM, MT, ASB, SS) are supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), grants Targeting Resistances to Molecular Therapies in Metastatic Colorectal Carcinomas – Special Program Molecular Clinical Oncology 5 x 1000 (2015-2017) and AIRC Investigator Grant IG 20685 (2017-2023); CORDIS Community Research and Development Information Service, Horizon 2020 Project ID 635342, grant Molecularly Guided Trials with Specific Treatment Strategies in Patients with Advanced Newly Molecular Defined Subtypes of Colorectal Cancer (MoTriColor); Ministero della Salute, Codice Progetto NET 02352137, grant Genomic-Based Triage for Target Therapy in Colorectal Cancer; and Fondazione Oncologia Niguarda Onlus, grant Terapia Molecolare dei Tumori.

The funding sources did not have access to the raw data and had no role in study design; data collection, analysis, or interpretation; or writing of the report. The corresponding author had full access to all the data and final responsibility for the decision to submit the Article for publication.
References

Table 1. Tumor densities of FoxP3\(^+\) and CD3\(^+\) TILs as predictors of CRC post-surgical recurrence, stratified by tumor stage and MS-status

<table>
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<th></th>
<th>Stage II</th>
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<th>Stage III (n=202)</th>
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<td>MSI (n=41)</td>
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<tr>
<td></td>
<td>Rate of recurrence (%)</td>
<td>OR (95%CI)</td>
<td>p</td>
<td>Rate of recurrence (%)</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td><strong>FoxP3(^+) TILs</strong></td>
<td>above median</td>
<td>4/86 (4.7)</td>
<td>1.00 ref.</td>
<td>2/21 (9.5)</td>
<td>1.00 ref.</td>
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<td></td>
<td>below median</td>
<td>24/84 (28.6)</td>
<td>8.75 (2.74-28.0)</td>
<td>&lt;.001</td>
<td>2/20 (10.0)</td>
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<tr>
<td><strong>CD3(^+) TILs</strong></td>
<td>above median</td>
<td>5/85 (5.9)</td>
<td>1.00 ref.</td>
<td>2/21 (9.5)</td>
<td>1.00 ref.</td>
</tr>
<tr>
<td></td>
<td>below median</td>
<td>23/85 (27.1)</td>
<td>6.78 (2.26-20.4)</td>
<td>.001</td>
<td>2/20 (10.0)</td>
</tr>
<tr>
<td><strong>Local Invasion</strong></td>
<td>pT3</td>
<td>24/160 (15.0)</td>
<td>1.00 ref.</td>
<td>2/33 (6.1)</td>
<td>1.00 ref.</td>
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<tr>
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<td>pT4</td>
<td>4/10 (40.0)</td>
<td>4.89 (0.94-24.4)</td>
<td>.05</td>
<td>2/8 (25.0)</td>
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<td><strong>Nodal status</strong></td>
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<tr>
<td></td>
<td>N2</td>
<td>32/131 (24.4)</td>
<td>1.00 ref</td>
<td>38/71 (53.5)</td>
<td>3.48 (1.87-6.46)</td>
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</tbody>
</table>

\(^a\)logistic regression, multivariate analysis
NA, not applicable
NS, variable not qualified (p>.10) for being entered into multivariate analysis of stage III CRC
Table 2. Low densities\(^a\) of FoxP3\(^+\) and CD3\(^+\) TILs, and their combination, to predict disease-free survival (DFS) in stage II MSS CRC.

<table>
<thead>
<tr>
<th></th>
<th>Rate of recurrence (%)</th>
<th>HR (95%CI)(^b)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FoxP3(^+) TILs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high</td>
<td>7/109 (6.4)</td>
<td>1.00, ref.</td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>21/61 (34.4)</td>
<td>5.61 (2.38-13.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td><strong>CD3(^+) TILs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high</td>
<td>5/87 (5.8)</td>
<td>1.00, ref.</td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>23/83 (27.7)</td>
<td>5.76 (2.16-15.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td><strong>FoxP3(^+)/CD3(^+) TILs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high/high</td>
<td>0/58 (0.0)</td>
<td>1.00, ref.</td>
<td></td>
</tr>
<tr>
<td>high/low</td>
<td>7/51 (13.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>low/high</td>
<td>5/29 (17.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>low/low</td>
<td>16/32 (50.0)</td>
<td>7.24 (3.41-15.4)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

\(^a\) defined by optimal cutoffs at ROC curves (Fig. 2): 0.23% IRA for Fox P3\(^+\) cells, 1.86% IRA for CD3\(^+\) cells

\(^b\) multivariate Cox regression analysis, adjusted by tumor local invasion (pT). HR values are for recurrence risk
Figure Captions

Figure 1. Hierarchical recursive analysis of factors predicting postsurgical recurrences in 413 patients with stage II-III CRC. Classification and regression tree (CART) modeled (Salford Systems, San Diego, CA, USA) by entering patient demographics, tumor pathological features (including MS status), as well as densities of FoxP3\(^+\) and CD3\(^+\) TILs. TNM staging (stage III vs. stage II) ranked as the hierarchically highest prognostic node \((P < 0.001, \text{ Chi-square test})\). Within stage III, only nodal status (N2 vs. N1) was a further discriminating factor \((P < 0.001)\). In contrast, the software identified cutoff values of TIL densities efficiently predicting recurrences in stage II CRC. In this subset, low densities of FoxP3\(^+\) TILs ranked first in the decisional branching \((P < 0.001)\), whereas low densities of CD3\(^+\) TILs further discriminated the outcome of CRC with low density of FoxP3\(^+\) TILs \((P < 0.001)\). MS status was not recognized as a discriminating predictor.

Figure 2. ROC curves for densities of FoxP3\(^+\) and CD3\(^+\) TILs as predictors of postsurgical cancer recurrence in patients with stage II MSS CRC. FoxP3\(^+\) TILs: AUC, 0.77 (bootstrap standard error, 0.05; 95\%CI, 0.67-0.86); at cutoff 0.23 IRA\%, sensitivity, 0.79; specificity, 0.71. CD3\(^+\) TILs: AUC, 0.71 (bootstrap standard error, 0.05; 95\%CI, 0.61-0.81); at cutoff 1.86 IRA\%, sensitivity, 0.82; specificity, 0.58.

Figure 3. Kaplan-Meier curves for the duration of disease-free survival in patients with stage II-III CRC. A. MSS stage II trial set cancers stratified by combined analysis of FoxP3\(^+\) and CD3\(^+\) TIL densities (low vs. high, by cutoff at ROC curves). The outcome for patients with MSS CRCs harboring low densities of FoxP3\(^+\) cells and low densities of CD3\(^+\) cells was significantly worse than that of patients with cancers with high densities of both cell types \((P < 0.001)\) or discordant TIL densities (low-high or high-low; \(P = 0.001)\). B. MSS stage III cancers stratified by combined analysis of FoxP3\(^+\) and CD3\(^+\) TIL densities. The outcome for patients with MSS CRCs harboring low densities of both FoxP3\(^+\) and CD3\(^+\) TILs was similar to that of patients with high densities of
both cell types (p=0.26) or discordant TIL densities (p=0.42). **C. MSI stage II/III trial set cancers,** stratified by combined analysis of FoxP3\(^+\) and CD3\(^+\) TIL densities. The outcome for patients with MSI CRCs harboring low densities of both FoxP3\(^+\) and CD3\(^+\) TILs was like that of patients with high densities of both cells types (p=0.26) or discordant TIL densities (P = 0.22). **D. MSS stage II validation set cancers stratified by combined analysis of FoxP3\(^+\) and CD3\(^+\) TIL densities.** The outcome for patients with MSS CRCs harboring low densities of both FoxP3\(^+\) and CD3\(^+\) TILs was significantly worse than that of cancers with high densities of both cell types (P < 0.001) or discordant TIL densities (P = 0.002).
Figure 1

All cases
- Relapse: n (\%) 311 (75.3), 102 (24.7)

TNM Stage
- OR, 2.97 C.I. 95%, 1.85-4.77

Stage II
- Relapse: n (\%) 179 (84.8), 32 (15.2)

FoxP3+ TILs
- OR, 5.20; C.I. 95%, 2.26-11.9

LOW (≤0.23% IRA)
- Relapse: n (\%) 59 (72.0), 23 (28.0)

HIGH (>0.23% IRA)
- Relapse: n (\%) 120 (93.0), 9 (7.0)

CD3+ TILs
- OR, 4.46; C.I. 95%, 1.58-12.6

LOW (≤1.86% IRA)
- Relapse: n (\%) 20 (55.6), 19 (44.4)

HIGH (>1.86% IRA)
- Relapse: n (\%) 39 (84.8), 7 (15.2)

Stage III
- Relapse: n (\%) 132 (65.3), 70 (34.7)

Nodal Status
- OR, 3.56; C.I. 95%, 1.93-6.58

N1
- Relapse: n (\%) 99 (75.6), 32 (24.4)

N2
- Relapse: n (\%) 33 (46.5), 38 (53.5)
Figure 2

The graph shows the receiver operating characteristic (ROC) curves for different immune cell populations. The curves are labeled as follows:

- **FoxP3^+**, AUC = 0.77 (0.67-0.86)
- **FoxP3^+ 0.23 IRA%**
- **CD3^+**, AUC = 0.71 (0.61-0.81)
- **CD3^+ 1.86 IRA%**

Sensitivity is plotted on the y-axis, and 1 - Specificity is plotted on the x-axis.
Combined low densities of FoxP3+ and CD3+ tumor-infiltrating lymphocytes identify stage II colorectal cancer at high risk of progression

Tommaso Cavalleri, Paolo Bianchi, Gianluca Basso, et al.

_Cancer Immunol Res_ Published OnlineFirst February 25, 2019.