The association of clinical outcome and peripheral T-cell subsets in metastatic colorectal cancer patients receiving first-line FOLFIRI plus bevacizumab therapy

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ABSTRACT

The first-line standard of care for patients with metastatic colorectal cancer (mCRC) is FOLFIRI (irinotecan, levo-leucovorin, 5-fluorouracil (5-FU)) plus bevacizumab. With the renewed interest in cancer immunotherapy with agents such as vaccines, checkpoint inhibitors and immune modulators, the possibility exists for the use of one or more of these immunotherapeutics in the first-line setting and thus in combination with the FOLFIRI and bevacizumab regimen. Studies were undertaken to study the effects of FOLFIRI and bevacizumab therapy on peripheral T-cell subsets, and to determine if there are any associations between these subsets and response to therapy. Peripheral blood mononuclear cell subsets of patients with mCRC (n = 23) were analyzed prior to and during therapy. While there were differences among patients, the majority of patients showed either a minimal change or an increase in CD4+ T cell to regulatory T cell (Treg) ratios during therapy, as well as either minimal change or a decrease in Treg suppressive activity during therapy. There was also an association (p = 0.036) between a decrease in Treg frequency during FOLFIRI therapy and overall survival, and an association (p = 0.037) between the frequency of Tregs prior to therapy and progression-free survival. Responders to the chemotherapy by RECIST criteria also had a greater decrease in Tregs during therapy vs. pre-therapy (p = 0.0064) as compared to non-responders. While the number of mCRC patients undergoing chemotherapy in this study is relatively small, it provides the rationale for the use of immunotherapeutics in this first-line metastatic setting.

ABBREVIATIONS: ALP, alkaline phosphatase; CEA, carcinoembryonic antigen; CI, confidence interval; CRC, colorectal cancer; FDA, Food and Drug Administration; 5-FU, 5-fluorouracil; FOLFIRI, irinotecan, levo-leucovorin, 5-fluorouracil; LDH, lactate dehydrogenase; MAb, monoclonal antibody; mCRC, metastatic colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MVA, Modified Vaccinia Ankara strain; OS, overall survival; PANVAC, rV-CEA-MUC1-TRICOM (B7.1, ICAM-1, LFA-3); PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein-1; PD-L1, programmed cell death protein-1 ligand; PFS, progression-free survival; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease; Tregs, regulatory T cells

Introduction

Several immunotherapeutic agents have recently been approved by the Food and Drug Administration (FDA) for a range of human cancers, including melanoma, non-small cell and squamous cell lung cancer, and renal cancer. While colorectal cancer (CRC) remains a leading cause of cancer death, to date no immunotherapeutics have been approved for CRC therapy. This is paradoxical in a way, since the elegant studies of Galon and Fridman1,4 and others5–7 have shown that the presence of an immune infiltrate in primary CRC tumors is an excellent positive prognostic indicator.

A minority (15%) of sporadic CRC tumors possess microsatellite instability (MSI), whereas almost all cases of hereditary non-polyposis CRC do.6 The combination of MSI positivity and a lymphocytic infiltrate has been shown to be a favorable prognostic indicator.6 These MSI positive tumors have been shown to respond clinically to monoclonal antibodies (MAbs) directed against the programmed cell death protein-1 (PD-1)-programmed cell death protein-1 ligand (PD-L1) axis. The vast majority of metastatic CRC (mCRC) tumors, however, have shown only limited responses to these checkpoint inhibitors. One contributing factor could be that metastatic lesions, which have escaped first-, second- or third-line

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SUPPLEMENTAL INFORMATION

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chemotherapy are at this point “non-inflamed,” i.e., devoid or having minimal immune infiltrate.

The standard of care for first-line therapy in patients with mCRC is the regimen consisting of FOLFIRI (irinotecan, levo-leucovorin, 5-fluorouracil (5-FU)) and bevacizumab. One potential therapeutic approach would be to employ immunotherapeutics such as checkpoint inhibitors or vaccines early in the metastatic process, i.e., in the first-line metastatic setting in combination with FOLFIRI. In this case, it would be necessary to interrogate whether the FOLFIRI regimen with bevacizumab would be deleterious to the CRC patients’ immune system, be immune inert or enhance certain immune components.

In the studies reported here, we have analyzed various immune subsets in the peripheral blood mononuclear cells (PBMCs) of 23 mCRC patients both prior to and during first-line FOLFIRI therapy. While there were differences among patients, the majority of patients showed either a minimal change or an increase in CD4+ T cell to regulatory T-cell (Treg) ratios during therapy, as well as either minimal change or a decrease in Treg suppressive activity during therapy. There was also an association (p = 0.036) between the decrease in Treg frequency during FOLFIRI therapy and overall survival (OS), and an association (p = 0.037) between the frequency of Tregs prior to therapy and progression-free survival (PFS). While the number of patients (n = 23) is relatively small, these studies provide the rationale for the combined use of immunotherapeutic agents such as vaccines, immune modulators such as immunocytokines, and/or checkpoint inhibitor MABs with FOLFIRI plus bevacizumab in first-line therapy in patients with mCRC.

Results

Clinical outcome

Twenty-three patients with first-line metastatic colon or rectal cancer (Table 1) were enrolled in the study. All underwent FOLFIRI therapy, consisting of irinotecan (180 mg/m² day 1), levo-leucovorin (200 mg/m² day 1), 5-FU (400 mg/m² bolus day 1 and 2400 mg/m² continuous infusion over 48 h), and bevacizumab (5 mg/kg day 1). This treatment schedule was repeated every 2 weeks. Peripheral blood samples were collected from each patient prior to the start of cycle I, and after 30 d, i.e. prior to initiation of the third cycle. As shown in Table 2, cytometric analysis of PBMCs revealed no statistical differences between the PBMCs collected at baseline and post 30 d of therapy in terms of PBMC amount, percent of CD4+ cells relative to total PBMCs, total number of CD4+ T cells, percent of CD8+ cells relative to total PBMCs, total number of CD8+ T cells, percent of Tregs relative to total PBMCs, the change in CD4+ :Treg ratio, or the ratios between CD4+ effectors and Tregs, CD8+ effectors and Tregs, or CD4+ and CD8+ effectors. A waterfall plot of the change in the ratio between CD4+ effector T cells and regulatory T cells (CD4+ :Treg ratio) in the course of FOLFIRI therapy is shown in Fig. 1A. Increases in the CD4+:Treg ratio > 25% were seen in 10/23 (43%) patients, while decreases in this ratio of > 25% were seen in only 3/23 (13%) patients. Ten of 23 patients had minimal changes (an increase or decrease < 25%) in the CD4+ :Treg ratio. To assess any factors that could contribute to the variability between patients, the change in CD4+ :Treg ratio was divided at the median, and the two cohorts were compared (Table S1). There were significant differences regarding age (p = 0.0353, patients who had a greater than the median change in the CD4+:Treg ratio were older, median age 74 y vs. median age 65 y, respectively); moreover, there were also significant changes in the number of liver metastases (p = 0.0475, patients who had a greater increase in the CD4+ :Treg ratio had fewer metastases, 1.6 vs. 2.3, respectively).

Sufficient PBMCs from 13/23 patients were available to conduct a functional study of the suppressive activity of Tregs at baseline and after 30 d of therapy. A waterfall plot of the changes in suppressive activity is shown in Fig. 1B. Six of 13 (46%) patients showed more than a 10% decrease in suppressive activity, while 4/13 (31%) showed a ≥10% increase.

### Table 1. Patient demographics and clinical characteristics.

| Parameter     | Median (% or IQR) |
|---------------|-------------------|
| Age (years)   | 71 (55–83)        |
| Gender        |                   |
| Male          | 11 (48%)          |
| Female        | 12 (52%)          |
| Primary tumor |                   |
| Colon         | 19 (83%)          |
| Rectal        | 4 (17%)           |
| Grade         |                   |
| 1             | 0 (0%)            |
| 2             | 16 (70%)          |
| 3             | 7 (30%)           |
| ECOG          |                   |
| 0             | 16 (70%)          |
| 1             | 7 (30%)           |
| 3             | 0 (0%)            |
| Mucinous/Non-mucinous |       |
| Mucinous      | 7 (30%)           |
| Non-mucinous  | 16 (70%)          |
| Metastases    |                   |
| Liver only    | 8 (35%)           |
| Other tissues | 9 (35%)           |
| Liver and other tissues | 7 (30%) |
| CEA           | 6.7 (3.1–22.5)    |
| CA19.9        | 23 (5.3–108.5)    |
| ALP           | 210 (130–268)     |
| LDH           | 172 (133–293)     |

The median and frequency (%) or interquartile range (IQR) for all parameters are shown. ECOG, Eastern Cooperative Oncology Group, NC; CEA, carcinoembryonic antigen; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

### Table 2. Evaluation of immune cell subsets in colorectal cancer patients prior to and after 30 d of standard-of-care anticancer therapy.

| Parameter                | Baseline | Post 30 d | N  |
|--------------------------|----------|-----------|----|
| Number of PBMCs (x10^3/μL) | 2.1 (1.9–2.6) | 2.1 (1.5–2.7) | 23 |
| CD4+ T cells (%)         | 47 (31–53) | 47 (39–52) | 23 |
| Number of CD4+ T cells (x10^3/μL) | 0.6–1.1 | 1.0 (0.7–1.3) | 23 |
| CD8+ T cells (%)         | 24 (15–30) | 22 (19–26) | 23 |
| Number of CD8+ T cells (x10^3/μL) | 0.4–0.8 | 0.5 (0.3–0.6) | 23 |
| Treg of CD4+ T cells (%) | 5.8 (4.7–8.0) | 5.7 (4.6–6.7) | 23 |
| Treg of PBMCs (%)        | 2.6 (2.1–3.3) | 2.6 (1.5–3.2) | 23 |
| Number of Tregs (x10^3/μL) | 0.06 (0.04–0.07) | 0.06 (0.03–0.07) | 23 |
| CD4+ T cell:Treg Ratio   | 17.0 (12.4–21.3) | 17.6 (14.9–21.7) | 23 |
| CD8+ T cell:Treg Ratio   | 9.9 (6.1–15.0) | 8.7 (5.7–16.0) | 23 |
| CD4+CD8+ T cell Ratio    | 1.9 (1.1–3.1) | 2.0 (1.4–3.2) | 23 |
| Treg suppression (%)     | 44 (34.5–51) | 39 (28.0–58.0) | 13 |

Twenty-three first-line metastatic colorectal cancer patients were enrolled and treated with FOLFIRI therapy. PBMCs were collected at baseline and post 30 d of therapy, and subjected to flow cytometry analysis as described in Methods section. Values are the median (interquartile range). Wilcoxon test between baseline and post 30 d showed no statistical differences for any of the immune cell subsets studied, or for the Treg suppression assay.

PBMCs, peripheral blood mononuclear cells; Treg, regulatory T cells.
We also evaluated the change in the ratio between CD8$^+$ effector T cells and regulatory T cells (CD8$^+$:Treg ratio). As seen in Fig. 2, there were similar numbers of patients showing an increase or decrease in the CD8$^+$:Treg ratio. To assess any factors that could contribute to the variability between patients, the change in CD8$^+$:Treg ratio was divided at the median, and the two cohorts were compared (Table S1). There was a significant difference regarding the time from progressive disease to death ($p = 0.0312$, patients who had a greater than the median change in the CD8$^+$:Treg ratio had a longer time from progressive disease to death, 15.6 vs. 6.8 mo, respectively).

The clinical characteristics and outcome of the individual patients enrolled on the study are shown in Table 3. Primary tumor biopsy samples were available from 15 patients. Analysis of the levels of CD3$^+$, CD8$^+$ and CD4$^+$ are shown in Table 3. Only two of these patients were MSI positive. We evaluated whether there was any association between the frequency of Tregs prior to therapy and progression-free survival (PFS) as seen in Fig. 3. The median PFS for patients with <2.5% Tregs of PBMCs at baseline was 23.3 months, and for patients with >2.5% Tregs of PBMCs at baseline, PFS was 10.7 months ($p = 0.037$, n = 23, 3 patients were censored) (Fig. 3). We also evaluated if there was any clinical benefit associated with the change in Treg frequency during FOLFIRI therapy. As seen in Fig. 4A, the median OS for patients with any decrease in Tregs after 30 d treatment was 43.3 mo, and for patients with any increase in Tregs after treatment 23.3 mo ($p = 0.036$, n = 23). It should be noted that the median decrease in Treg frequency was −26% (Fig. 4B), and the median increase was +23% (Fig. 4C).

We also correlated the levels of Tregs with antitumor response to therapy by RECIST (Response Evaluation Criteria in Solid Tumors) criteria. We found that 12/23 patients displayed a partial response (PR) by RECIST, 7/23 patients had stable disease (SD), and 4/23 had progressive disease (Table 3). It is interesting to note that there was a trend in that of the 12 responders by RECIST criteria on the study, 67% (8/12) displayed Treg levels <2.5% at baseline, whereas only 18% (2/11) of the non-responders by RECIST criteria (including those patients with stable disease) had equivalent
### Table 3. Clinical characteristics of patients enrolled on the study

| Pt | Age | Gender | Primary Tumor | Mucinous/Non-mucinous | ECOG Grade | Metastases | CEA Pre | CA19.9 Pre | Best Response by RECIST | PFS (mo) | OS (mo) | MSI status | CD3⁺ | CD8⁺ | CD4⁺ |
|----|-----|--------|---------------|------------------------|------------|------------|---------|------------|-------------------------|----------|--------|-------------|------|------|------|
| 1  | 73  | F      | colon         | Non Muc               | 1          | 2          | 3       | 94.6       | PR                      | 55.3     | 55.3   | MSS moderate | mild | mild | moderate |
| 2  | 80  | F      | colon         | Non Muc               | 0          | 2          | 1       | 6.6        | 20.8                    | 11.4     | 38.3   | MSS moderate | mild | moderate | mild |
| 3  | 70  | F      | colon         | Muc                    | 0          | 2          | 2       | 14.7       | 40.1                    | 8.6      | 11.4   | MSS moderate | mild | mild | moderate |
| 4  | 81  | M      | colon         | Non Muc               | 1          | 2          | 3       | 3.1        | 8.1                     | 1.8      | 14.1   | mild | mild | mild |
| 5  | 74  | M      | colon         | Muc                    | 0          | 3          | 1       | 15.5       | 57.9                    | 9.7      | 25.7   | mild | mild | mild |
| 6  | 63  | F      | colon         | Non Muc               | 0          | 3          | 3       | 6.7        | 23.2                    | 42.7     | 42.7   | MSS severe | moderate | mild |
| 7  | 79  | F      | colon         | Non Muc               | 0          | 2          | 3       | 1          | 3.8                     | 21.3     | 43.9   | MSS mild | mild | mild |
| 8  | 56  | M      | colon         | Non Muc               | 0          | 3          | 1       | 17.0       | 108.5                   | 7.8      | 13.5   | MSS severe | mild | mild |
| 9  | 68  | F      | colon         | Muc                    | 1          | 2          | 1       | 22.5       | 410.1                   | 4.7      | 27.2   | mild | mild | mild |
| 10 | 55  | F      | colon         | Non Muc               | 0          | 2          | 1       | 1389       | 10000                   | 5.9      | 8.1    | mild | mild | mild |
| 11 | 83  | F      | colon         | Muc                    | 1          | 3          | 1       | 0.8        | 0.6                     | 13.2     | 40.9   | MSS moderate | mild | mild |
| 12 | 57  | F      | rectal        | Non Muc               | 0          | 2          | 2       | 26.5       | 1473                    | 10.7     | 10.7   | mild | mild | mild |
| 13 | 41  | M      | rectal        | Non Muc               | 0          | 2          | 3       | 3.3        | 8.8                     | 3.9      | 9      | MSI mild | absent | mild |
| 14 | 71  | M      | rectal        | Non Muc               | 0          | 2          | 1       | 2.7        | 5.3                     | 15.5     | 43.3   | MSS moderate | mild | mild |
| 15 | 66  | F      | rectal        | Non Muc               | 0          | 3          | 3       | 6.6        | 22.9                    | 37.9     | 28.9   | MSS severe | moderate | mild |
| 16 | 77  | M      | colon         | Non Muc               | 0          | 2          | 2       | 5.2        | 9.8                     | 34.1     | 37.2   | mild | mild | mild |
| 17 | 62  | F      | colon         | Muc                    | 0          | 3          | 3       | 26.8       | 2412                    | 22.0     | 55.9   | MSS moderate | mild | mild |
| 18 | 66  | M      | colon         | Non Muc               | 0          | 2          | 1       | 1.8        | 4.7                     | 30.1     | 51.6   | MSS moderate | mild | mild |
| 19 | 72  | M      | colon         | Muc                    | 1          | 2          | 2       | 1.7        | 4.5                     | 23.3     | 23.3   | MSS moderate | mild | mild |
| 20 | 75  | M      | colon         | Non Muc               | 0          | 3          | 2       | 12.6       | 34.5                    | 9.9      | 31     | mild | mild | mild |
| 21 | 69  | F      | colon         | Muc                    | 0          | 2          | 3       | 6.9        | 25.5                    | 12.4     | 47.2   | mild | mild | mild |
| 22 | 76  | F      | colon         | Non Muc               | 1          | 2          | 2       | 6.2        | 13.4                    | 12.7     | 18.7   | mild | mild | mild |
| 23 | 76  | M      | colon         | Non Muc               | 1          | 2          | 2       | 125        | 0.1                     | 3.9      | 6.9    | mild | mild | mild |

ECOG, Eastern Cooperative Oncology Group; NCI, National Cancer Institute; CEA, carcinoembryonic antigen; RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival; MSI, microsatellite instability; MSS, microsatellite stability.

**Figure 3.** Association between the frequency of regulatory T cells (Tregs) in peripheral blood mononuclear cells (PBMCs) at baseline and progression-free survival (PFS) after FOLFIRI therapy in patients with colorectal cancer. Patients were categorized based on their frequency of Tregs (< or ≥ 25% of PBMCs) before therapy. A Kaplan–Meier curve is shown for these two groups and the association with PFS. The median PFS for patients with <2.5% Tregs pre-treatment was 23.3 months, and for patients with >2.5% Tregs pre-treatment 10.7 months (p = 0.037, n = 23, 3 patients were censored).
low levels of Tregs ($p = 0.019$). In addition, 75% of the responders displayed a decreased frequency of Tregs in peripheral blood after 30 d of therapy, whereas only 18% of the 11 non-responders by RECIST criteria on the trial had a decrease in Tregs ($p = 0.0064$ with Pearson test, $\chi^2 = 7.4$). There were no other significant differences between responders and non-responders regarding age, gender, CEA, CA19.9, alkaline phosphatase (ALP), lactate dehydrogenase
Metastases were categorized as 1. Liver vs. 2. liver vs. 3. Other sites. Tregs, regulatory T cells; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; IDO (indoleamine-2,3-dioxygenase) inhibitor. Reduction of immune inhibitory factors such as IL8 and TGF-β could also be employed in combination therapies.

The majority of immunotherapy trials in CRC have been carried out in patients with advanced disease who have already received multiple regimens of chemotherapy. Previous studies have revealed that there is an inverse correlation between the number of prior chemotherapy regimens and the ability of mCRC patients to generate immune responses to vaccine. Prior clinical studies have shown that administration of docetaxel to patients with metastatic prostate cancer, as well as that of cisplatin plus vinorelbine to non-small cell lung cancer patients, showed a significant increase in the ratio between effector T cells and Tregs and a reduction in the immunosuppressive activity of Tregs. Treatment of breast cancer patients with tamoxifen had minimal effect on Tregs, while sunitinib exerted differential effects on Tregs among patients with metastatic renal cancer. Preclinical studies have shown that Tregs are more susceptible to certain chemotherapy regimens compared to CD4+ effector cells.

One obvious strategy would be to deliver an immunotherapeutic early in the metastatic process. This would require administering the immunotherapeutic treatment first as a single agent. The study reported here was undertaken to determine if this first-line chemotherapy regimen would have an adverse, neutral or enhancing effect on the patients’ immune system. We show here for the first time that for the majority of patients, the FOLFIRI regimen either had minimal effect or a positive effect on the effector CD4+ T-cell phenotype of the tumor cell is modified to make it more susceptible to immune-mediated attack. It would also be of value to further study in preclinical models the mechanisms involved in the use of FOLFOX or FOLFIRI bevacizumab in combination with various immunotherapy regimens.

While the number (n = 23) of mCRC patients undergoing first-line chemotherapy in this study is relatively small, it provides the rationale for the use of immunotherapeutics in this setting.

### Discussion

While no immune therapy has been approved by the FDA for patients with CRC, there has been some evidence of immune-mediated interventions in Phase II clinical studies. For example, the PANVAC vaccine, consisting of rV-CEA-MUC1-TRICOM (B7.1, ICAM-1, LFA-3) as a primary vaccination and multiple boosts with rF-CEA-MUC1-TRICOM, has been administered to patients with CRC metastatic to the liver or lung post-metastasectomy. Survival was approximately 95% at 2 y and approximately 90% at 4 y. A contemporary control group displayed identical time to progression as the vaccinated group, but OS was approximately 50% at 4 y, which is comparable to other reported studies in this population. An adenovirus-CEA vaccine and an MVA-5T4 (TroVax) vaccine also reported some evidence of clinical benefit in patients with advanced mCRC. While very few RECIST responses have been observed in non-MSI mCRC patients treated with MAbs directed against the checkpoint inhibitors anti-PD1/PDL1, several patients have shown long-term stable disease post-therapy. New immunotherapy approaches for metastatic CRC are clearly warranted. One approach would be to inflame the tumor microenvironment with a vaccine directed against CRC antigens or neo-epitopes followed by the use of checkpoint inhibitor antibodies. Other approaches to inflame the tumor microenvironment or decrease suppressive cell subsets include the use of radiation, chemotherapy, immunocytokines or an IDO (indoleamine-2,3-dioxygenase) inhibitor. Reduction of

### Materials and methods

**Patients and anticancer treatments**

Twenty-three patients with first-line metastatic colon or rectal cancer were enrolled in the study at the University of Rome.
Tor Vergata, Rome, Italy. All underwent FOLFIRI therapy, consisting of irinotecan (180 mg/m² day 1), levo-leucovorin (200 mg/m² day 1), 5-FU (400 mg/m² bolus day 1 and 2400 mg/m² continuous infusion over 48 h) and bevacizumab (5 mg/kg day 1). This treatment schedule was repeated every 2 weeks. Peripheral blood samples were collected prior to the start of cycle I, and after 30 d. All patients signed an informed consent form. The procedures were conducted in accordance with the Helsinki Declaration of 1975 in the study at Tor Vergata University Clinical Center, Rome, Italy.

**Assessment of response**

Radiologic assessment was performed with a contrast enhanced thorax-abdomen pelvis CT scan or MRI at baseline, within 30 d before chemotherapy began, and every 3 mo thereafter, during treatment. Radiologic response and disease progression were defined according to RECIST 1.0 criteria. Disease response was defined as a decrease of at least 30% in the sum of the longest diameter of target lesions, as compared to the baseline. Early changes in immune cells, after 1 mo of therapy, were correlated with the best radiologic response in accordance with RECIST criteria.

**Collection of PBMC**

PBMCs were isolated by Ficoll (MP Biomedicals, Santa Ana, CA) density gradient separation, washed twice and cryopreserved in 90% heat-inactivated human AB serum and 10% DMSO in liquid nitrogen at a concentration of 1 x 10⁷ cells/mL until assayed.

**Flow cytometry analysis**

Cryopreserved PBMCs were analyzed by four-color flow cytometry for phenotypic characterization of Tregs as described by Vergati et al.²² Cells were resuspended in staining buffer (PBS containing 3% fetal bovine serum) and stained for 30 min at 4°C with FITC-conjugated anti-CD4+ (BD PharMingen, San Jose, CA), phycoerythrin-conjugated anti-CD25 (BD) and PerCP Cy5.5-conjugated anti-CD127 (eBioscience, San Diego, CA). FoxP3 intracellular staining was done on the cells stained with anti-CD4+, anti-CD25 and anti-CD127. Cells were fixed and permeabilized using a fix/perm buffer (eBioscience) according to the manufacturer’s instructions, then labeled with Allophycocyanin-conjugated anti-FoxP3 (eBioscience) or its isotype control as a negative control. Flow cytometry was performed on a FACScanCalibur (BD Biosciences): 5 x 10⁴ events were acquired and data were analyzed using CellQuest software (BD Biosciences). To determine the percentage of Tregs, lymphocytes were gated by plotting forward vs. side scatter. The CD4+ population was gated first, followed by the CD25+CD127neg population and finally the FoxP3+ population was gated into the CD4+/CD25+/CD127neg population. Tregs are thus defined as the CD4+/CD25+/CD127neg/FoxP3+ population.

**CD4⁺CD25high T-cell enrichment**

CD4⁺CD25high T cells were enriched using a CD4⁺CD25⁺ Treg isolation kit (Miltenyi Biotec, San Diego, CA), with modifications to the manufacturer’s instructions. After CD4⁺ T cells were negatively enriched, positive selection for CD25high T cells was done on the negatively selected CD4⁺ T cells. In order to achieve a consistently high CD25high purity rate, the amount of CD25 antibody microbeads was decreased by 70% and the incubation time was decreased by 15%. The CD25high fraction was collected by eluting twice the cells through a magnetic separation (LS) column to further enrich for CD4⁺CD25high T cells.

**Treg suppression assay**

RPMI 1640 medium supplemented with 10% AB serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin (Mediatech, Manassas, VA), and 2 mmol/L of L-glutamine (Mediatech) was used for T-cell culture. Responder CD4⁺CD25neg T cells were labeled with 2 μM CFSE (Sigma, St. Louis, MO). In suppression assays, to assess CD4⁺CD25high T cells’ suppressive capacity, 1 x 10⁴ CFSE-labeled responder CD4⁺CD25⁺ T cells were cultured alone or cocultured with 1 x 10⁴ CD4⁺CD25high T cells in the presence of Mitomycin-treated T-cell-depleted PBMC as antigen-presenting cell, and were stimulated with 0.5 μg/mL plate-bound anti-CD3 antibody (OKT3; eBioscience) in 96-well round-bottom plates.²¹ Proliferation of CFSE-labeled cells was assessed by flow cytometry after 4 d of culture. The percent suppression was calculated using the following formula: [(percent of proliferating effector cells (CD4⁺CD25neg) when cultured alone minus the percent of proliferating CFSE-diluting effector cells in the presence of suppressor cells (CD4⁺CD25hi)) at a 1:1 ratio)/percent of proliferating responder cells when cultured alone] x 100.

**Immunohistochemistry**

Tissue sections of formalin-fixed, paraffin-embedded, colon carcinomas were tested for CD3+, CD4+, CD8+, MLH1 and MSH6 expression using the Ventana BenchMark XT automated staining platform with the ultraView Universal DAB Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany).²³ For each slide, three to five random fields were evaluated by two pathologists in an independent blinded manner, with an interobserver variability <5%. Tumoral lymphocyte infiltration was characterized by evaluation of CD3+, CD4+, CD8+ immunostainings performed in a traditional microscope-based manner at 40x magnification. For each slide, three to five random fields were evaluated and graded arbitrarily, considering density of each T-cell subset (cells/mm²) according to the observed mean values as follows: CD3⁺ (slight: <67 cells/mm², moderate between 67 and 670 cells/mm², and severe >670 cells/mm²), CD4⁺ (slight: <30 cells/mm², moderate between 30 and 300 cells/mm², and severe >300 cells/mm²) and CD8⁺ (slight: <35 cells/mm², moderate between 35 and 350 cells/mm², and severe >350 cells/mm²). DNA mismatch repair (MMR) deficiency leading to MSI²⁴ was analyzed by immunohistochemistry,²⁵ and tumors with MSI-high were
defined as those demonstrating an MMR protein expression less than 10%. Staining of nuclei of adjacent normal crypts, stromal cells and lymphocytes was used as internal positive controls.

**Statistical analysis**

Statistical analysis was performed using the non-parametric Wilcoxon test or Friedman test with Dunn’s multiple comparison for paired samples (GraphPad Software, La Jolla, CA). The probabilities of survival and progression-free survival as a function of time were established with the Kaplan–Meier method, with 95% confidence intervals (CIs) around the median established with a reflected CI approach. Multiple regression analysis was performed in a forward stepwise fashion. A p value < 0.01 was considered statistically significant, and a p value < 0.05 was considered a trend.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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