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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Tumor Infiltration by T Lymphocytes Expressing Chemokine Receptor 7 (CCR7) Is Predictive of Favorable Outcome in Patients with Advanced Colorectal Carcinoma

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Abstract

Purpose: An efficient adaptive immunity is critical for a longer survival in cancer. We investigated the prognostic value of tumor infiltration by $CD8^+$ T cells expressing the chemokine-receptor-7 (T_{ccr7}) and the correlation between tumor infiltration by T_{ccr7} and regulatory $CD4^+FoxP3^+$ T cells (T_{reg}) in 76 metastatic colorectal cancer (mCRC) patients enrolled in a phase III trial.

Experimental Design: T_{ccr7} and T_{reg} cell infiltration in tumor samples was quantified by immunohistochemistry. The correlation among T_{ccr7}, T_{reg} tumor infiltration, and patients' outcome was evaluated.

Results: High T_{ccr7} tumor infiltration was predictive of prolonged OS [high vs. low T_{ccr7} score: median 38 months (95% CI: 24.5–51.4) vs. 20 months (95% CI: 11.4–28.5); HR = 0.48 (95% CI: 0.24–0.96); $P = 0.03$] and prolonged progression-free survival [PFS; high vs. low T_{ccr7} score: median 12 months (95% CI: 7.7–16.2) vs. 7 months (95% CI: 5.2–8.7); HR = 0.54 (95% CI: 0.28–1.01); $P = 0.01$] after front-line chemotherapy. Regression analysis did not show correlation between T_{ccr7} and T_{reg} infiltration levels. However, the cluster of patients showing concomitant high infiltration by both T_{ccr7} and T_{reg} disclosed a favorable outcome [double high vs. double low tumor infiltration score: median OS = 35 months (95% CI: 20.8–49.1) vs. 17 months (95% CI: 4.6–29.3); HR = 0.32 (95% CI: 0.12–0.87); $P = 0.02$ and median PFS = 11 months (95% CI: 9.4–12.5) vs. 5 months (95% CI: 2.2–7.7); HR = 0.43 (95% CI: 0.17–1.06); $P = 0.01$].

Conclusions: High T_{ccr7} tumor infiltration score is a favorable prognostic factor for mCRC. Our findings underline the relevance of microenvironment-related immunologic events for patient outcome. *Clin Cancer Res; 18(3); 850–7. ©2011 AACR.*

Introduction

Colorectal carcinoma (CRC) is the third most common cause of cancer worldwide (1). At the present, the best therapeutic option for advanced disease is represented by chemotherapy regimens containing fluorouracil (5-FU) ± levofolinic acid (LF) together with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX), given alone or in combination with bevacizumab, a monoclonal antibody (mAb) to the vascular-endothelial growth factor, or cetuximab and panitumumab, mAbs directed against the epidermal growth factor-receptor (2). For patients with advanced disease there are no effective parameters able to support the choice of specific treatments, which, currently, are selected on the basis of risk factors, performance status, and more recently on the presence in the tumor tissue of activating k-ras mutations, which makes ineffective the addition of cetuximab or panitumumab to chemotherapy (3).

Recently, it has been proposed that adaptive antitumor immune response and autoimmunity in cancer patients, occurring spontaneously or as a consequence of specific treatments, may be a critical feature for good outcome and prolonged survival (4). On the basis of preclinical findings (5), we designed a novel therapeutic regimen that includes a sequential combination of 5-FU–based polychemotherapy (gemcitabine, oxaliplatin, levofolinic acid, and 5-FU; ref. 6), followed by the immune-adjuvant cytokine recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF), and ultra low-dose metronomic human recombinant interleukin-2 (aldesleukine rIL-2). rGM-CSF was included

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Translational Relevance

Our report describes the results of a preplanned side study aimed to evaluate in a prospective setting the role of different tumor-infiltrating T-lymphocyte subsets at baseline in advanced colorectal cancer patients enrolled in the GOLFIG-2 phase III trial. This study was designed with the major aim to compare the antitumor activity of GOLFIG (gemcitabine + oxaliplatin, levo-folinic acid, and 5-FU followed by granulocyte macrophage colony-stimulating factor and recombinant inteleukin-2) chemo-immune-burst versus the FOLFOX-4 (oxaliplatin, levofolinic acid, and 5-FU) regimen, based on the promising results of a previous phase II study. We show that high tumor infiltration by cytotoxic (CD8$^+$) T cells expressing the chemokine receptor-7 (CCR7; T_{ccr7}) has a favorable prognostic value in these patients. We also report a correlation among tumor infiltration by both T_{ccr7}s and T_{reg}s (CD4$^+$FOXP3$^+$) lymphocytes and patients' outcome. These results provide novel information on the immunologic microenvironment of colorectal cancer and, therefore, offer the rationale for novel therapeutic approaches, which might take benefit from the antitumor immune response in the microenvironmental *milieu*, and put forward a new perspective to enhance the activity of colorectal cancer treatment.

in the aim to activate peripheral dendritic cells (DC) and rIL-2 to endure an antitumor antigen-specific T-cell–mediated immune response. This chemo-immune-burst regimen, named GOLFIG, resulted safe and active in largely pretreated metastatic CRC (mCRC) patients in a previous phase II trial (7, 8). In that study, we registered in 19% of patients the occurrence of autoimmunity witch resulted the most efficient predictor of good outcome in a multivariate analysis (8). Moreover, we observed significant changes in specific T-lymphocyte subsets both in the primary tumor and in the peripheral blood. Along the treatment, it was observed a progressive increase of tumor antigen [Carcinoembryonic antigen (CEA) and thymidylate synthase (TS)]-specific cytotoxic T-cell precursors and central memory T-lymphocytes (CD8$^+$CD45RA$^-$CCR7$^+$) together with a progressive reduction of immune-suppressive regulatory T cells (CD4$^+$CD25^{hi+}FoxP3$^+$; T_{reg}; refs. 7, 8). These intriguing results led us to design a phase III trial, which is presently ongoing in mCRC patients (GOLFIG-2 trial). This study was aimed to evaluate the efficacy of front line GOLFIG regimen in comparison with the standard FOLFOX-4 chemotherapy.

We also preplanned an immune biological investigation, aimed to evaluate in a prospective setting the role of different tumor-infiltrating T-lymphocyte subsets at baseline in mCRC patients enrolled in the GOLFIG-2 trial. In this context, a first analysis revealed a highly favorable outcome in those patients whose primary tumor at diagnosis was associated with high infiltration by lymphocytes expressing T_{reg} immunophenotype. A high T_{reg} tumor infiltration score was strongly predictive of prolonged overall survival (OS) and progression-free survival (PFS) in either FOLFOX-4 or GOLFIG treatment arms (9). Considering that this lymphocyte subset represents an inhibitory feed-back response to a preexisting immune stimulation (10–15), we formulated the hypothesis that T_{reg} infiltration in the tumor tissue might be an indirect and powerful indicator of local antitumor immune response. On these bases, we have subsequently investigated whether T_{reg} infiltration is also correlated to the presence of other activated immune effectors. In particular, we evaluated in the same patient population whether the level of tumor infiltration by CD8$^+$ T cells expressing CCR7 could predict patient outcome and might correlate with the extent of T_{reg} tumor infiltration.

In this study, we focused therefore on the population of lymphocytes expressing a CD8$^+$CCR7$^+$ phenotype, including naive (CD8$^+$CD45RA$^+$CCR7$^+$) and central memory (CD8$^+$CD45RA$^-$CCR7$^+$CD27$^+$CD62L$^+$; T_{cm}) subsets, which represent a fresh source of activated immune effectors. These cells under specific microenvironmental conditions may either differentiate in antigen-specific long-term memory or highly cytotoxic T-cell effectors (16, 17). We decided to investigate lymphocytes bearing CCR7 (T_{ccr7}), taking in account that it is a homing receptor for chemokine ligand 19 and 21 (CCL19, CCL21; ref. 18), which are able to drive these effector lymphocytes in lymph nodes and in sites were the immune attack takes place (19). The manuscript has been drafted according to REMARK criteria (20).

Patients and Methods

Patient characteristics

The inclusion criteria were: written informed consent, histologically confirmed diagnosis of CRC, no previous chemotherapy for advanced disease, measurable disease (according to WHO tumor response criteria), an ECOG performance status ≤ 2, normal renal and hepatic function, white blood cell count 2,500/mm^3 or more, hemoglobin levels 9 g/dL or more, platelet cell count 100,000/mm^3 or more and normal cardiac function. The exclusion criteria were: any major organ failure, central nervous system involvement, second malignancies, active infectious disease, major autoimmune diseases, and acquired immune suppression.

Treatment schedules and patients' evaluation

All patients were randomized to receive FOLFOX-4 or GOLFIG treatment. The FOLFOX-4 arm received biweekly chemotherapy with oxaliplatin (85 mg/m^2 on day 1), LF (100 mg/m^2 on days 1–2) and 5-FU (400 mg/m^2 as a bolus and 600 mg/m^2 as a 22 hour infusion on days 1–2); the GOLFIG arm received biweekly chemotherapy with gemcitabine (1,000 mg/m^2 on days 1 and 15), oxaliplatin (85 mg/m^2 on days 2 and 16), LF (100 mg/m^2 on days 1,2,15 and 16), and 5-FU (400 mg/m^2 as a bolus and 800 mg/m^2 as a 24-hour infusion on days 1, 2, 15, and 16), followed by

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subcutaneous rGM-CSF (100 μg, on days 3–7) and ultra low-dose subcutaneous rIL-2 (0.5×10^6 IUs twice a day on days 8–14 and 17–29; ref. 7). Standard assessments (clinical history, physical examination, blood chemistry, evaluation of serum CEA, and CA19.9 concentration) were carried out at baseline and repeated every 2 weeks, chest *x*-ray and ultrasound scans every 4 weeks. High-definition, multislice computed tomography scans with contrast medium were recorded every 3 months. Both treatments were continued until disease progression, occurrence of unacceptable toxicity, clinical judgment, or withdrawal of consent. All patients were evaluated for PFS (calculated from trial enrolment to disease progression or death) and OS (calculated from trial enrolment to death).

Specimen characteristics

Pathology study. Tumor tissues derived from biopsy or radical surgery were fixed in 10% buffered neutral formalin and paraffin embedded for histology and immunohistochemistry. Sections of each specimen were stained with hematoxilin and eosin and histologically examined by an expert pathologist.

Assay methods

Immunohistochemistry. Immunohistochemical staining was carried out on 3-μm thick sections of each block by the streptavidin-biotin method. The cores were taken randomly from within the tumor block face and at least 3 different samples for each patient were evaluated.

After being dewaxed and rehydrated, sections were incubated with 3% H_2O_2 in TBS solution to inhibit endogenous peroxidase and processed with different methods for each antibody. To show $CD4^+$ T cells, the sections were unmasked with Wcap buffer (pH 6.0 for 40 minutes at 98°C; Bio-Optica) and were incubated with anti-human monoclonal antibody CD4 (clone 4B12; 1:50; Menarini). For $CD8^+$ T cells, pretreatment with a microwave oven in citrate buffer (0.01 mol/L, pH 6.0) at 750 W for 5 minutes was carried out for 3 cycles, and the sections were incubated with anti-human monoclonal antibody CD8 (clone CD8–144B; 1:50; Dako); the epitopes were detected with the Ultravision Detection System and revealed with the diaminobenzidine for 5 minutes (Dako). For detection of $FoxP3^+$T cells, we used EDTA (0.05 mol/L, pH 8.0) pretreatment in a microwave oven at 750 W for 5 minutes. After 3 cycles, the sections were incubated with anti-human FoxP3 mAb (clone 22510; 1:50; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; R&D Systems).

The primary antibody enhancer was associated with an AP Polymer (Ultravision LP Detection System AP Polymer, LAB Vision) and revealed with fuchsin (DakoCytomation). Negative controls were obtained by replacing the specific antibody with nonimmune serum immune-globulins at the same concentration of the primary antibody. Immune staining was examined with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy). Blind reanalysis was carried out to confirm the results.

Quantitative evaluation of T-lymphocyte subtypes

Sections stained for $CD4^+$-, $CD8^+$-, $CCR7^+$-, or $FoxP3^+$-lymphocytes were scored in coded slides by 1 observer. The number of positive cells for each marker was recorded in 5 randomly chosen HGPFs in the stroma adjacent to neoplastic glands. Cut-off value to discriminate between high and low lymphocyte infiltration was determined as 20 positive cells both for $CCR7^+$- or $FoxP3^+$-lymphocytes (median value of both groups). Actuarial survival curves and Cox analysis were constructed on the above described survival endpoints (PFS and OS). Regression analysis was also carried out to evaluate the potential correlation among the specific lymphocyte infiltration scores (as a continuous variable) and the survival endpoints.

GOLFIG-2 study design

The current study has been designed as a preplanned biological analysis in the framework of the GOLFIG-2 prospective clinical trial. The phase III trial was authorized by the Institutional Ethical Committee and by the Italian Ministry of Health. The role of the steering Committee was to provide executive oversight and supervision for the conduct of the trial, through review of trial enrolment, protocol and clinical conduct, and blinded safety data. All patients were in first line chemotherapy for mCRC and signed an informed consent.

The rationale for the trial was to compare efficacy of GOLFIG chemoimmunotherapy with FOLFOX-4 chemotherapy as frontline treatment for mCRC patients. Time period for enrolment was from 2005 July to 2010 November. Median follow-up was 18 months. The established observation period from the end of enrolment was estimated of 3 years, due to the prognosis in advanced colorectal disease. Primary end point was a 2 months advantage in term of PFS in the GOLFIG arm, and secondary endpoints were OS and response rate (RR).

Statistical analysis

We carried out a preplanned immuno-histochemistry analysis on specimens collected from 76 patients consecutively enrolled in GOLFIG-2 trial and randomized to receive FOLFOX-4 chemotherapy or GOLFIG chemoimmunotherapy. In particular, we investigated the possible prognostic value of tumor infiltration by several lymphocyte subsets [$CD4^+$, $CD8^+$ (CTLs), T_{ccr7} ($CD8^+CCR7^+$), and T_{reg} ($CD4^+FoxP3^+$)] on the surgical pathology sample. At this aim, OS and PFS were evaluated with the Kaplan-Meyer, whereas log-rank test was carried out to evaluate statistical differences between the groups with high and low T_{ccr7} tumor infiltration score method (SPSS 17 statistic software). A post-hoc multivariate analysis trough COX's regression analysis (NCSS statistical software), with several prognostic factors at trial entry (performance status; sex; age; tumor grading; presence or absence of liver metastases) was also carried out to assess the independent prognostic value of each variable. Regression analysis (by the GraphPad Instat 3.2 statistic software) was carried out to evaluate whether the above mentioned survival parameters were significantly

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correlated to T_{ccr7} tumor infiltration extent as a continuous variable. Unpaired 2-tailed *t* student test (by the GraphPad Instat 3.2 statistic software) was carried out to compare survival means between different groups.

Results

Demography and study design

Seventy-six patients with mCRC, 48 males and 28 females, with a median age of 65 years were included in this study. These patients had been consecutively enrolled in the GOLFIG phase III trial and randomized to receive upfront treatment according to FOLFOX-4 chemotherapy (44 patients) or GOLFIG (32 patients) chemoimmunotherapy.

An immunohistochemistry study, performed to evaluate the extent of tumor infiltration by T_{ccr7} and T_{reg} was carried out on tumor samples obtained from these patients before any systemic treatment. This analysis included fewer patients in the GOLFIG treatment arm because for many of them there were not adequate histologic samples available for the immunohistochemistry study.

For the correlative studies, the high and low lymphocyte expression levels were calculated on the basis of the median value of the number of lymphocytes expressing the specific marker (CD8 for CTLs and CCR7 for T_{ccr7}) detected in each high power slide field (Fig. 1A–E). The characterization of FoxP3$^+$ T cells as active T_{reg} with immunosuppressive status was defined in a previous study (9). A double color immunohistochemistry characterization carried out on 10 samples showed CCR7$^+$ expression mainly on CD8$^+$ T cells (Fig. 1F).

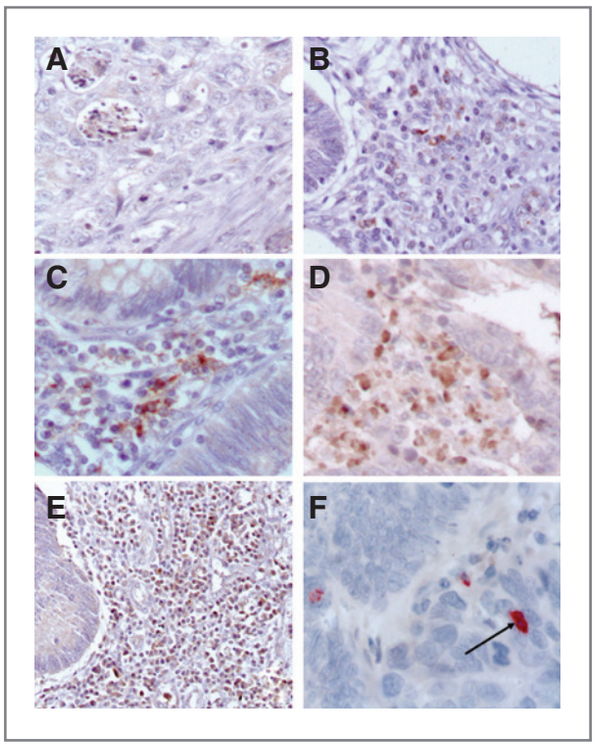

Figure 1. Identification by immunohistochemistry of CCR7+ lymphocytes in the primary tumor. Panel A shows no lymphocyte infiltration; panels B–E, respectively, show increasing CCR7+ infiltration (magnification 50–200×). Panel F shows CCR7$^+$CD8$^+$ lymphocytes (arrow) in a double color immunohistochemistry analysis. This analysis shows that in the primary tumor CCR7 is mainly expressed on CD8$^+$ T cells. CCR7$^+$CD8$^+$ cells show an intense membranous and cytoplasm stain in comparison with CCR7$^+$CD8$^-$ or CCR7$^-$CD8$^+$ lymphocytes which show an irregular and weak red stain (magnification 400×).

High expression of CCR7-positive TILs is predictive of good outcome in patients with advanced CRC

Our analysis indicated that a high tumor T_{ccr7} infiltration score at the diagnosis correlated with a prolonged OS [high vs. low T_{ccr7} score: median 38 months (95% CI: 24.5–51.4) vs. 20 months (95% CI: 11.4–28.5); HR = 0.48 (95% CI: 0.24–0.96); $P = 0.03$] and prolonged PFS [high vs. low T_{ccr7} score: median 12 months (95% CI: 7.7–16.2) vs. 7 months (95% CI: 5.2–8.7); HR = 0.54 (95% CI: 0.28–1.01); $P = 0.01$] after front-line chemotherapy. The latter result provides clear evidence that T_{ccr7} has a prognostic value in mCRC patients (Fig. 2A–B).

A further multivariate COX analysis also indicated a favorable prognostic value for T_{ccr7} infiltration score in terms of prolonged OS ($P = 0.05$). Moreover, a linear regression analysis confirmed the statistical correlation between PFS and tumor infiltration by T_{ccr7} cells as a continuous variable ($r^2 = 0.11$; $P = 0.02$; Fig. 3A), paralleled by a *t* test, carried out by grouping the patients considering $T>_{ccr7}$ values as a dicotomic high/low variable and comparing the mean PFS values (low CCR7 = 7.7 months, high CCR7 = 17.3 months, $P = 0.02$; Fig. 3B).

Correlation between T_{reg} and T_{ccr7} infiltration in tumor tissues

We next investigated for a possible correlation in term of tumor infiltration levels among different T-lymphocyte subsets. We were unable to show any correlation among T_{ccr7} infiltration and: (i) total lymphocyte density; (ii) CD8$^+$/CD4$^+$ T-cell tumor infiltration; (iii) tumor grading; and (iv) tumor stage at the diagnosis (data not shown).

Regression analysis failed to show any significant correlation between T_{ccr7} and T_{reg} tumor infiltration extent. However, we identified 4 different clusters of patients, whose primary tumor presented infiltration by each one of these T-cell subsets according to the following scenario: (i) low T_{reg} and low T_{ccr7} (LL); (ii) high T_{ccr7} and low T_{reg} (HL); (iii) low T_{ccr7} and high T_{reg} (LH); high T_{reg} and high T_{ccr7} (HH)], which were associated with different outcomes. In fact, we detected the longest survival in those patients who showed a combined high tumor T_{reg} and T_{ccr7} infiltration and the worst outcome in those who presented a combined tumor infiltration by either lymphocyte subsets [double high vs. double low tumor infiltration score: median OS = 35 months (95% CI: 20.8–49.1) vs. 17 months

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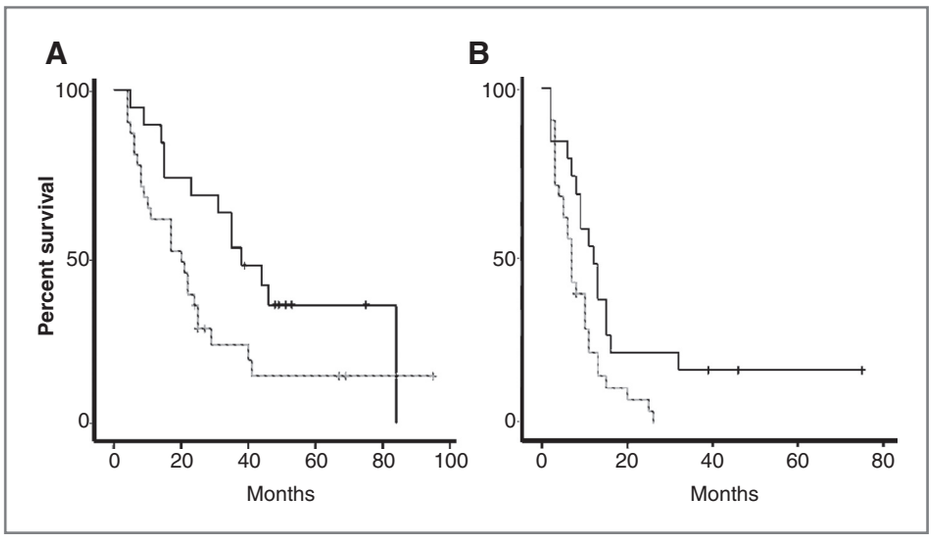

Figure 2. Actuarial Kaplan-Meyer's survival curves of 76 colorectal cancer patients who had undergone FOLFOX-4 or GOLFIG treatment whose tumor was scored by immune histochemistry for T_{ccr7} infiltration. Panels compare OS (A) and PFS (B) in patients with high (——) and low (— — —) T_{ccr7} tumor infiltration score.

(95% CI: 4.6–29.3); HR = 0.32 (95% CI: 0.12–0.87); P = 0.02, with more than 20% patients surviving more than 75 months, and median PFS = 11 months (95% CI: 9.4–12.5) vs. 5 months (95% CI: 2.2–7.7); HR = 0.43 (95% CI: 0.17–1.06); P = 0.01; Fig. 4A–B].

These analyses were carried out on the whole patient population and did not take in account the actual patient treatment; it was, in fact, not possible to achieve any significant results in either the FOLFOX-4 or GOLFIG arm due to the small sample and this important investigation point was considered beyond the scope of the present report.

Discussion

The results of the present study suggest that a greater infiltration by $CD8^+CCR7^+$ T cells in the primary tumor at the diagnosis is predictive of better outcome in advanced mCRC patients. We were unable to show a direct statistical correlation between T_{ccr7} and T_{reg} tumor infiltration extent; however, a concomitant high expression of both T-lymphocyte subsets in the tumor helps to identify a cluster of good prognosis patients. In contrast, those patients, whose tumor tissue presents a combined low infiltration score for both lymphocyte populations, have a very poor outcome. These findings withstand the hypothesis that primary tumor infiltration by different T-cell subsets may reflect the state of immune-competence of the patients and produce a reliable prognostic factor. A limit of the present study is the lack of a confirmatory data set. To this end, we are planning confirmatory analysis within the whole trial patient population and, in additional series, to provide additional evidence on the prognostic role of T_{ccr7} lymphocytes in colorectal cancer as shown in this hypothesis-generating study.

Zitvogel and colleagues (4) has already highlighted the important role played by the immune-surveillance in conditioning cancer patient survival. These authors hypothesized a possible synergistic interaction between administration of cytotoxic drugs and host immune response. They have in fact speculated that chemotherapy is able to reduce the immunosuppressive tumor burden (4, 21), induce antigen remodeling (immune-editing), and promote immunologic danger signals (5, 22). Moreover, cytotoxic drug treatment may also damage immunosuppressive cell

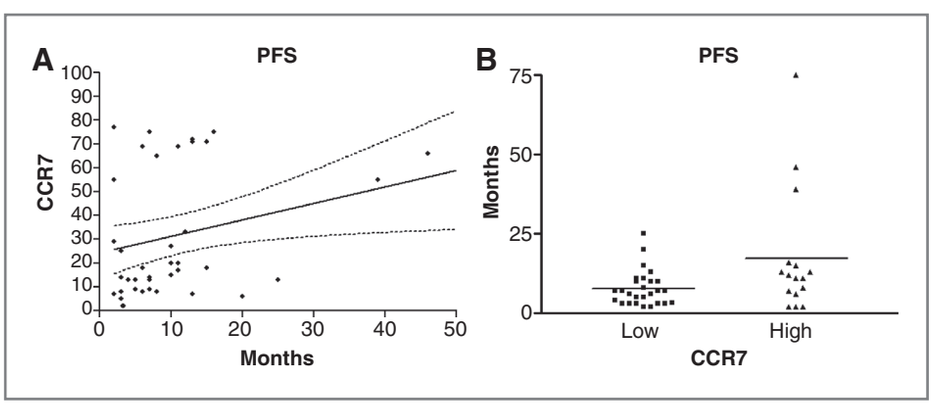

Figure 3. Regression curve (with 95% CI) of CCR7 expression as a continuous variable in the tumor tissue and PFS (A). *t* test comparing mean PFS values between high versus low CCR7 expression groups (B).

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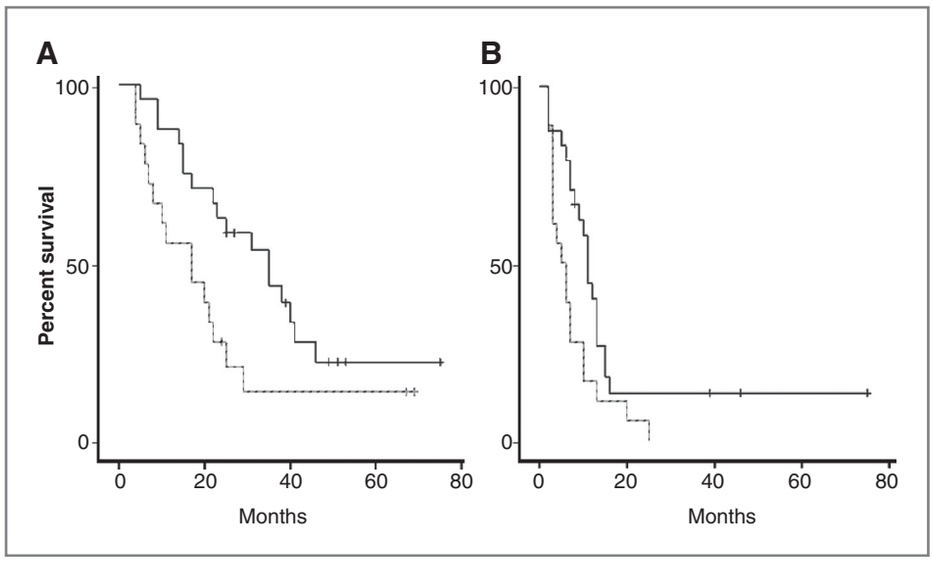

Figure 4. Actuarial Kaplan-Meyer's survival curves of patients whose primary tumor presented a high infiltration score of both T_{ccr7} and T_{reg} subsets (——) compared with those presenting a double low infiltration score (— — —) of either lymphocyte populations. Double T-cell population infiltration score was respectively compared in term of: (A) OS and (B) PFS.

populations, such as inhibitory myeloid cells or *T_{reg}* which have been often found mostly overexpressed in cancer patients (4).

Several studies in mCRC patients have also investigated the prognostic role of the basal inflammatory status and lymphocyte density in the primary tumor with conflicting results (23–26). A reliable prognostic value has been proposed for tumor infiltration by professional antigen presenting cells such as macrophages and DCs in patients who underwent surgery for CRC (27).

A more controversial scenario concerns the prognostic role of tumor infiltration by T_{reg} cells defined by a $CD4^+CD25^{hi+}FoxP3^+$ immune-phenotype in CRC patients. This lymphocyte subset, which has a powerful immunosuppressive activity, is often overexpressed during viral infection, chronic inflammatory diseases, and cancer, in the attempt to prevent a dangerous overresponse and autoimmunity (28–31). Recently, it has been shown that tumor infiltration by immune-regulatory T cells defined by a T_{reg} immune-phenotype is a favorable prognostic factor in early and mCRC. Salama and colleagues, in fact, showed, on a very large series of early CRC patients, that T_{reg} infiltration in the primary tumor is predictive of longer survival. They also showed that the protective value was completely lost when the T_{reg} presence was detected in the draining nodes (23).

Similarly, our group has shown that high T_{reg} tumor infiltration in mCRC patients enrolled in the GOLFIG-2 phase III trial is predictive of favorable outcome in term of OS and PFS (9). The findings from this early report on T_{reg} and the current study on T_{ccr7} infiltration, and specifically the data on double positive (high T_{reg} and high $CCR7^+$) versus double negative (low T_{reg} and low $CCR7^+$) depict a scenario in which different lymphocyte populations may represent marker of protective anticancer response which has an important impact on patient outcome.

T_{ccr7} lymphocytes population includes 2 different populations of $CD8^+CCR7^+$ T immune-effectors which can be distinguished in naive and central memory T cells depending on CD45RA expression. These lymphocytes are recalled by specific chemokines in the tumor site, where eventually differentiate in antigen specific T cells with long-term memory cells or effector cells able to kill the specific tumor targets with high efficiency (16). CCR7 is a receptor able to bind different ligands (CCL-19 and 21) produced by activated DCs and other inflammatory cells (32, 33). Once engaged by its ligands during the immune attack, CCR7 regulates the homeostatic recirculation through body cavities and primes an intracellular process in the T cells that guides their chemotactic homing to lymph nodes, tumor/infected tissues and target cells (34). These lymphocytes also acquire the expression of CD62L, an adhesion molecule that plays a primary role in T-cell homing by mediating leukocyte interaction with activated vascular endothelium in high endothelial venules (16, 33, 34).

An increase in the number of T cells expressing these receptors indicates a greater amount of freshly mobilized lymphocytes available to differentiate in immune-effector that sustain a more prolonged antigen-specific T-cell–mediated immune response.

The results of a large pathology study, supported by microarray transcriptional profiling of early colorectal cancer samples, revealed that the presence of memory T lymphocyte infiltration and activated cytotoxic T-cell gene signature is strongly predictive of prolonged survival and reduced risk of relapse, while no prognostic role for inflammatory microenvironment was shown (35). The same authors confirmed in these patients with early CRC an adverse prognostic value for high vascular, lymphatic, and perineural tumor cell infiltration score (VeLiPi), reporting that it was inversely correlated with memory T-cell tumor infiltration and memory molecular profiling signature.

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We can also take in consideration that $CD3^+CD8^+$ (including T_{ccr7}) T lymphocytes, in comparison with T_{reg}, are much more resistant to chemotherapy, as we showed in a previous study (22). These lymphocytes are, in fact, sensitive to the cytotoxic effects of chemotherapy only when they are in active proliferation, which takes place in response to antigen presentation by DCs and by other antigen presenting cells in the primary lymphoid organs only. On this basis, T_{ccr7} lymphocytes are mostly refractory to the drugs after their expansion and release from the primary lymphoid organs. On the other hand, T_{reg} proliferation may occur *in situ* where it is nourished by selective cytokine/chemokine production in the tumor microenvironment. Several studies have already shown that T_{reg} are sensitive to cytotoxic drugs such as cyclophosphamide (36, 37), gemcitabine, 5-FU, or oxaliplatin (22).

In conclusions, the results of this study indicate that T_{ccr7} infiltration is a favorable prognostic factor for mCRC patients and suggest that high T_{reg} infiltration represents a feed-back to a preexisting immune response. We believe that our findings on the immunologic tumor microenvironment hearten additional investigation on this specific topic to gain relevant information for the design of more efficient therapeutic strategies for mCRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–917.
2. Meyerhardt JA, Mayer RJ. Systemic therapy for colorectal cancer. New Engl J Med 2005;352:476–87.
3. Karapetis CS, Khambata-Ford S, Jonker DJ, O' Callaghan CJ, TU D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.
4. Zitvogel L, Apetoh L, Ghiringhelli F, André F, Tesniere A, Kroemer G. The anticancer immune response: indispensable for therapeutic success? J Clin Invest 2008;118:1991–2001.
5. Correale P, Cusi MG, Del Vecchio MT, Aquino A, Prete S, Tsang KY, et al. Dendritic cell-mediated cross-presentation of antigens derived from colon carcinoma cells exposed to a highly cytotoxic multidrug regimen with gemcitabine, oxaliplatin, 5-fluorouracil, and leucovorin, elicits a powerful human antigen-specific CTL response with antitumour activity in vitro. J Immunol 2005;175:820–8.
6. Correale P, Messinese S, Caraglia M, Marsili S, Piccolomini A, Petrioli M, et al. A novel biweekly multi-drug regimen of gemcitabine, oxaliplatin, 5-fluorouracil (5-FU), and folinic acid (FA) in pre-treated patients with advanced colorectal carcinoma. Br J Cancer 2004;90:1710–4.
7. Correale P, Cusi MG, Tsang KY, Del Vecchio MT, Marsili S, La Placa M, et al. Chemo-immunotherapy of metastatic colorectal carcinoma with Gemcitabine + FOLFOX 4 followed by subcutaneous (sc) Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin-2 (IL-2) induces strong immunological and antitumour activity in metastatic colon cancer patients. J ClinOncol 2005;23:8950–8.
8. Correale P, Tagliaferri P, Fioravanti A, Del Vecchio MT, Remondo C, Montagnani F, et al. Immunity feedback and clinical outcome in colon cancer patients undergoing chemoimmunotherapy with gemcitabine + FOLFOX followed by subcutaneous granulocyte macrophage colony-stimulating factor and aldesleukin (GOLFIG-1 Trial). Clin Cancer Res 2008;14:4192–9.
9. Correale P, Rotundo MS, Del Vecchio MT, Remondo C, Migali C, Ginanneschi C, et al. Regulatory (FoxP3+) T-cell tumour infiltration is a favourable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy. J Immunother 2010;33:435–41.
10. Dubois B, Chapat L, Kaiserlian D. CD4+CD25+ T cells as key regulators of immune responses. Eur J Dermatol 2003;13:111–6.
11. Khazaie K, Von Boehmer H. The impact of CD4+CD25+ Treg on tumour specific CD8+ T cell cytotoxicity and cancer. Semin Cancer Biol 2006;16:124–36.
12. Nomura T, Sakaguchi S. Naturally arising CD25+CD4+ regulatory T cells in tumour immunity. Curr Top Microbiol Immunol 2005;293:287–302.
13. Beyer M, Schultze JL. Regulatory T cells in cancer. Blood 2006;108:804–11.
14. Rudensky AY, Campbell DJ. *In vivo* sites and cellular mechanism of T reg cell-mediated suppression. J Exp Med 2006;203:489–92.
15. Vojdani A, Erde J. Regulatory T cells, a potent immunoregulatory target for CAM researchers: the ultimate antagonist (I). Evid Based Complement Alternat Med 2006;3:25–30.
16. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708–12.
17. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–63.
18. Burgstahler R, Kempkes B, Steube K, Lipp M. Expression of the chemokine receptor BLR2/EBI1 is specifically transactivated by Epstein-Barr virus nuclear antigen 2. Biochem Biophys Res Commun 1995;215:737—43.
19. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 1999;99:23–33.
20. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumourMARKer prognostic studies (REMARK). Nat Clin Pract Oncol 2005;2:416–22.
21. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008;8:59–73.
22. Correale P, Del Vecchio MT, La Placa M, Montagnani F, Di Genova G, Savellini GG, et al. Chemotherapeutic drugs may be used to enhance the killing efficacy of human tumour antigen peptide-specific CTLs. J Immunother 2008;31:132–47.
23. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, et al. Tumour-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J ClinOncol 2009;27:186–92.
24. Clarke SL, Betts GJ, Plant A, Wright KL, El-Shanawany TM, Harrop R, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942–9.
25. Miracco C, Mormouras VM, Biagioli M, Rubegni P, Mannucci S, Monciatti I, et al. Utility of tumour infiltrating CD25+FOXP3+ regulatory

Published OnlineFirst December 5, 2011; DOI: 10.1158/1078-0432.CCR-10-3186

T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. Oncol Rep 2007;18:1115–22.

26. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, et al. Intratumoural balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol 2007;25:2586–93.
27. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C. Tumour-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumour-associated antigens and survival. J Transl Med 2007;5:62.
28. Knoechel B, Lohr J, Kahn E, Bluestone JA, Abbas AK. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. J Exp Med 2005;202:1375–86.
29. Lu LF, Rudensky A. Molecular orchestration of differentiation and function of regulatory T cells. Genes Dev 2009;23:1270–82.
30. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151–64.
31. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775–87.
32. Radstake T, Van der Voort R, Ten B, De Waal Malefijt M, Looman M, Figdor C, et al. Increased expression of CCL18, CCL19, and CCL17 by dendritic cells from patients with rheumatoid arthritis, and regulation by Fc gamma receptors. Ann Rheum Dis 2005;64:359–67.
33. Ebert LM, Schaerli P, Moser B. Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues. MolImmunol 2005;42:799–809.
34. Höpken UE, Winter S, Achtman AH, Krüger K, Lipp M. CCR7 regulates lymphocyte egress and recirculation through body cavities. J Leukoc Biol 2010;87:671–82.
35. Pagès F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J ClinOncol 2009;27:5944–51.
36. Ghiringhelli F, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido C, et al. CD4+CD25+ regulatory T cells suppress tumour immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumours to be curative. Eur J Immunol 2004;34:336–44.
37. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005;105:2862–8.

Published OnlineFirst December 5, 2011; DOI: 10.1158/1078-0432.CCR-10-3186

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