Tumour infiltration by T-lymphocytes expressing chemokine receptor 7 (CCR7) is predictive of favourable outcome in patients with advanced colorectal carcinoma.

Pierpaolo Correale, Maria Saveria Rotundo, Cirino Botta, Maria Teresa Del Vecchio, Chiara Ginanneschi, Antonella Licchetta, Raffaele Conca, Serena Apollinari, Fabio De Luca, Pierfrancesco Tassone and Piersandro Tagliaferri.

Medical Oncology Unit, Oncology Department, Azienda Ospedaliera Universitaria Senese, Siena; Pathology Section, Human Pathology and Oncology, Siena University School of Medicine, Italy; Medical Oncology Unit and Translational Medical Oncology Unit and Referral Center for Innovative Treatments, Campus Salvatore Venuta, “Magna Graecia” University and “Tommaso Campanella” Cancer Center, Catanzaro, Italy; Temple University’s College of Science & Technology, Philadelphia, PA, USA

Running Title: Prognostic value of tumour infiltrating CCR7+ T-lymphocytes.

Key words: colorectal carcinoma, tumour-infiltrating T-lymphocytes, CCR7.

Corresponding Author:
Prof. Piersandro Tagliaferri, “Campus Salvatore Venuta”, “Magna Graecia” University and Tommaso Campanella Cancer Center, Catanzaro, 88100 - Italy; tel. +3909613694324; fax +3909613647341; email: tagliaferri@unicz.it
Dr. Pierpaolo Correale, Medical Oncology Unit, Siena University “Santa Maria alle Scotte” Hospital, Viale Bracci 11, 53100, Siena, Italy. +390577586369; email: correale@unisi.it.

**Financial support:** This study has been supported by a grant from the Italian Ministry of Scientific Research [MIUR]. Unit Leader: Prof. Maria Teresa Del Vecchio; Project Coordinator: Prof. Angelo Aquino.
STATEMENT OF TRANSLATIONAL RELEVANCE

Our report describes the results of a preplanned side study aimed to evaluate in a prospective setting the role of different tumour-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 anti-tumour activity of GOLFIG (gemcitabine + oxaliplatin, levo-folinic acid and 5-FU followed by GM-CSF and recombinant interleukin-2) chemo-immune-burst versus the FOLFOX-4 (oxaliplatin, levo-folinic acid and 5-FU) regimen, based on the promising results of a previous phase II study. We demonstrate that high tumour infiltration by cytotoxic (CD8⁺) T cells expressing the chemokine receptor-7 (CCR7) (T_{CCR7}) has a favourable prognostic value in these patients. We also report a correlation among tumour infiltration by both T_{CCR7}s and T_{reg} (CD4⁺FOXP3⁺) lymphocytes and patients’ outcome.

These results provide novel informations on the immunologic microenvironment of colorectal cancer and, therefore, offer the rationale for novel therapeutic approaches, which might take benefit from the antitumour immune response in the micro-environmental milieu, and put forward a new perspective to enhance the activity of colorectal cancer treatment.
ABSTRACT

Purpose. An efficient adaptive immunity is critical for a longer survival in cancer. We investigated the prognostic value of tumour infiltration by CD8⁺ T cells expressing the chemokine-receptor-7 (Tccr7) and the correlation between tumour infiltration by Tccr7 and regulatory CD4⁺FoxP3⁺ T cells (Treg) in 76 metastatic colorectal cancer (mCRC) patients enrolled in a phase III trial.

Experimental design. Tccr7 and Treg cell infiltration in tumour samples was quantified by immuno-histochemistry. The correlation among Tccr7, Treg tumour infiltration and patients’ outcome was evaluated.

Results. High Tccr7 tumour infiltration was predictive of prolonged OS [high versus low Tccr7 score: median 38 months (95% CI; 24.5-51.4) versus 20 months (95% CI; 11.4-28.5); HR= 0.48 (95% CI; 0.24-0.96); P= 0.03] and prolonged PFS [high versus low Tccr7 score: median 12 months (95% CI; 7.7-16.2) versus 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 demonstrate correlation between Tccr7 and Treg infiltration levels. However, the cluster of patients showing concomitant high infiltration by both Tccr7 and Treg disclosed a favourable outcome [double high versus double low tumour infiltration score: median OS= 35 months (95% CI; 20.8-49.1) versus 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) versus 5 months (95% CI; 2.2-7.7); HR= 0.43 (95% CI; 0.17-1.06); P= 0.01].

Conclusions. High Tccr7 tumour infiltration score is a favourable prognostic factor for mCRC. Our findings underline the relevance of microenvironment-related immunological events for patient outcome.
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) +/- levofoinic 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 tumour 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 anti-tumour 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 poly-chemotherapy (gemcitabine, oxaliplatin, levofoinic acid and 5-FU) [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 in the aim to activate peripheral dendritic cells (DCs) and rIL-2 in order to endure an anti-tumour 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 which 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 tumour as well as in the peripheral blood. Along the treatment, it was observed a progressive increase of tumour antigen [Carcino-embryonic 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+CD25hi+FoxP3+) (T_{reg}) [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 pre-planned an immune biological investigation, aimed to evaluate in a prospective setting the role of different tumour-infiltrating T-lymphocyte subsets at baseline in mCRC patients enrolled in the GOLFIG-2 trial. In this context, a first analysis revealed a highly favourable outcome in those patients whose primary tumour at diagnosis was associated with high infiltration by lymphocytes expressing T_{reg} immunophenotype. A high T_{reg} tumour 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 tumour tissue might be an indirect and powerful indicator of local anti-tumour 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 tumour
infiltration by CD8\(^+\) T cells expressing CCR7 could predict patient outcome and might correlate with the extent of T\(_\text{reg}\) tumour infiltration.

In the present study, we focused therefore on the population of lymphocytes expressing a CD8\(^+\)CCR7\(^+\) phenotype, including naïve (CD8\(^+\)CD45RA\(^+\)CCR7\(^+\)) and central memory (CD8\(^+\)CD45RA\(^-\)CCR7\(^+\)CD27\(^+\)CD62L\(^+\)) (T\(_\text{cm}\)) subsets, which represent a fresh source of activated immune-effectors. These cells under specific micro-environmental 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\(_\text{ccr7}\)), taking in account that it is a homing receptor for chemokine ligand 19 and 21 (CCL19, CCL21) [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 tumour response criteria), an ECOG performance status ≤ 2, normal renal and hepatic function, white blood cell count ≥ 2,500/mm³, hemoglobin levels ≥ 9 g/dl, platelet cell count ≥ 100,000/mm³ 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/m² on day 1), LF (100 mg/m² on days 1-2) and 5-FU (400 mg/m² as a bolus and 600 mg/m² as a 22 hour infusion on days 1-2); the GOLFIG arm received biweekly chemotherapy with gemcitabine (1000 mg/m² on days 1 and 15), oxaliplatin (85 mg/m² on days 2 and 16), LF (100 mg/m² on days 1,2,15 and 16) and 5-FU (400 mg/m² as a bolus and 800 mg/m² as a 24 hour infusion on days 1,2,15,16), followed by subcutaneous rGM-CSF (100 µg, on days 3-7) and ultra-low dose subcutaneous rIL-2 (0.5 X 10⁶ IUs twice a day on days 8-14 and 17-29) [7]. Standard assessments (clinical history, physical examination, blood chemistry, evaluation of serum CEA and CA19.9 concentration) were performed at baseline and repeated every 2 weeks, chest x-ray and ultrasound scans every 4 weeks. High-definition, multi-slice computed tomography scans with contrast medium were recorded every three 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**

Tumour tissues derived from biopsy or radical surgery were fixed in 10% buffered neutral formalin and paraffin embedded for histology and immuno-histochemistry. Sections of each specimen were stained with haematoxilin and eosin and histologically examined by an expert pathologist.

**Assay methods**

**Immunohistochemistry**

Immunohistochemical staining was performed on 3 µm-thick sections of each block by the streptavidin-biotin method. The cores were taken randomly from within the tumour block face and at least three different samples for each patient were evaluated. After being de-waxed and rehydrated, sections were incubated with 3% H₂O₂ in Tris Buffered Saline 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’ at 98°C; Bio-Optica, Milan, Italy) and were incubated with anti-human monoclonal antibody CD4 (clone 4B12; 1:50; Menarini, Florence, Italy). For CD8⁺ T cells, pre-treatment with a microwave oven in citrate buffer (0.01 M, pH 6.0) at 750W for 5 min was performed for three cycles, and the sections were incubated with anti-human monoclonal antibody CD8 (clone CD8-144B; 1:50; Dako, Milan, Italy); the epitopes were
detected with the Ultravision Detection System and revealed with the di-amino-benzidined for 5 min (Dako, Milan, Italy). For detection of FoxP3+ T cells, we used EDTA (0.05 M, pH 8.0) pre-treatment in a microwave oven at 750 W for 5 minutes. After three cycles, the sections were incubated with anti-human FoxP3 mAb (clone 22510; 1:50; 60'; Abcam, Cambridge, UK) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60’ 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 non immune serum immune-globulins at the same concentration of the primary antibody. Immune staining was examined with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany). Blind reanalysis was carried out to confirm the results.

Quantitative evaluation of T lymphocytes subtypes

Sections stained for CD4+-, CD8+-, CCR7+- or FoxP3+-lymphocytes were scored in coded slides by one observer. The number of positive cells for each marker were recorded in five 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 performed 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 pre-planned 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 chemo-immunotherapy with FOLFOX-4 chemotherapy as frontline treatment for mCRC patients. Time period for enrollment was from 2005 July to 2010 November. Median follow-up was 18 months. The established observation period from the end of enrollment was estimated of 3 years, due to the prognosis in advanced colorectal disease. Primary end point was a two months advantage in term of PFS in the GOLFIG arm, and secondary endpoints were OS and response rate (RR).

Statistical analysis

We performed a pre-planned immuno-histochemistry analysis on specimens collected from 76 patients consecutively enrolled in GOLFIG-2 trial and randomized to receive FOLFOX-4 chemotherapy or GOLFIG chemo-immunotherapy. In particular, we investigated the possible prognostic value of tumour infiltration by several lymphocyte subsets [CD4⁺, CD8⁺ (CTLs), T_{crr7} (CD8⁺CCR7⁺) and T_{reg} (CD4⁺FoxP3⁺)] on the surgical pathology sample. At this aim, OS and PFS were evaluated with the Kaplan-Meyer, while Log-Rank test was performed to evaluate statistical differences between the groups with high and low T_{crr7} tumour infiltration score method (SPSS 17 statistic software). A post-hoc multivariate
analysis through COX’s regression analysis (NCSS statistical software), with several prognostic factors at trial entry (performance status; sex; age; tumour grading; presence or absence of liver metastases) was also performed to assess the independent prognostic value of each variable. Regression analysis (by the GraphPad Instat 3.2 statistic software) was carried out in order to evaluate whether the above mentioned survival parameters were significantly correlated to $T_{ccr7}$ tumour infiltration extent as a continuous variable. Unpaired two-tailed t-student test (by the GraphPad Instat 3.2 statistic software) was performed in order 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) chemo-immunotherapy. An immuno-histochemistry study, carried out in order to evaluate the extent of tumour infiltration by T_{ccr7} and T_{reg} was performed on tumour samples obtained from these patients before any systemic treatment. This analysis included less patients in the GOLFIG treatment arm because for many of them there was not adequate histological samples available for the immuno-histochemistry 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 immuno-suppressive status was defined in a previous study [9]. A double colour immuno-histochemistry characterization performed on ten samples showed CCR7^{+} expression mainly on CD8^{+} T cells (Fig. 1f).

High expression of CCR7 positive TILs is predictive of good outcome in patients with advanced colorectal carcinoma

Our analysis indicated that a high tumour T_{ccr7} infiltration score at the diagnosis correlated with a prolonged OS [high versus low T_{ccr7} score: median 38 months (95% CI; 24.5-51.4) versus 20 months (95% CI; 11.4-28.5); HR= 0.48 (95% CI; 0.24-0.96); P= 0.03] and
prolonged PFS [high versus low T
crr7 score: median 12 months (95% CI; 7.7-16.2) versus 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
crr7 has a prognostic value in mCRC patients (Fig. 2A-B).

A further multivariate COX analysis also indicated a favourable prognostic value for T
crr7 infiltration score in terms of prolonged OS (P = 0.05). Moreover, a linear regression analysis confirmed the statistical correlation between PFS and tumour infiltration by T
crr7 cells as a continuous variable (r^2 = 0.11; P = 0.02) (Fig. 3A), paralleled by a t-test, performed by grouping the patients considering T
crr7 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 Treg and T
crr7 infiltration in tumour tissues

We next investigated for a possible correlation in term of tumour infiltration levels among different T-lymphocyte subsets. We were unable to demonstrate any correlation among T
crr7 infiltration and: i) total lymphocyte density; ii) CD8^+CD4^+ T cell tumour infiltration; iii) tumour grading; and iii) tumour stage at the diagnosis (data not shown).

Regression analysis failed to demonstrate any significant correlation between T
crr7 and Treg tumour infiltration extent. However, we identified four different clusters of patients, whose primary tumour presented infiltration by each one of these T cell subsets according to the following scenario: 1) low Treg and low T
crr7 (LL); 2) high T
crr7 and low Treg (HL); 3) low T
crr7 and high Treg (LH); high Treg and high T
crr7 (HH)], which were associated with different outcomes. In fact, we detected the longest survival in those patients who showed a combined high tumour Treg and T
crr7 infiltration and the worst outcome in those who
presented a combined tumour infiltration by either lymphocyte subsets [double high versus double low tumour infiltration score]: median OS= 35 months (95% CI; 20.8-49.1) versus 17 months (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) versus 5 months (95% CI; 2.2-7.7); HR= 0.43 (95% CI; 0.17-1.06); \( P = 0.01 \) \( \text{(Fig. 4A-B)} \).

These analyses were performed 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 tumour at the diagnosis is predictive of better outcome in advanced mCRC patients. We were unable to demonstrate a direct statistical correlation between T_{ccr7} and T_{reg} tumour infiltration extent; however a concomitant high expression of both T lymphocyte subsets in the tumour helps to identify a cluster of good prognosis patients. In contrast, those patients, whose tumour tissue presents a combined low infiltration score for both lymphocyte populations, have a very poor outcome. These findings withstand the hypothesis that primary tumour 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 et al [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 immuno-suppressive tumour burden [4, 21], induce antigen re-modelling (immune-editing) and promote immunological danger signals [5, 22]. Moreover, cytotoxic drug treatment may also damage immunosuppressive cell 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 tumour with conflicting results [23-26]. A reliable prognostic value has been proposed for tumour infiltration by professional antigen presenting cells such as macrophages and dendritic cells in patients who underwent surgery for CRC [27].

A more controversial scenario concerns the prognostic role of tumour 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 over-expressed during viral infection, chronic inflammatory diseases and cancer, in the attempt to prevent a dangerous over-response and autoimmunity [28-31]. Recently, it has been shown that tumour infiltration by immune-regulatory T cells defined by a T_{reg} immune-phenotype is a favourable prognostic factor in early and mCRC. Salama et al, in fact, showed, on a very large series of early CRC patients, that T_{reg} infiltration in the primary tumour 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} tumour infiltration in mCRC patients enrolled in the GOLFIG-2 phase III trial is predictive of favourable 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 two different populations of CD8^+CCR7^+ T immune-effectors which can be distinguished in naïve and central memory T cells.
depending on CD45RA expression. These lymphocytes are recalled by specific chemokines in the tumour site, where eventually differentiate in antigen specific T cells with long-term memory cells or effector cells able to kill the specific tumour targets with high efficiency [16]. CCR7 is a receptor able to bind different ligands (CCL-19 and 21) produced by activated dendritic cells 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, tumour/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 (HEV) [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 micro-array 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 demonstrated [35]. The same authors confirmed in these patients with early CRC an adverse prognostic value for high vascular, lymphatic and peri-neural tumour cell infiltration score (VeLiPi), reporting that it was inversely correlated with memory T cell tumour infiltration and memory molecular profiling signature.

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 these 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 \textit{in situ} where it is nourished by selective cytokine/chemokine production in the tumour 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 favourable prognostic factor for mCRC patients and suggest that high T_{reg} infiltration represents a feed-back to a pre-existing immune-response. We believe that our findings on the immunological tumour microenvironment hearten additional investigation on this specific topic in order to gain relevant information for the design of more efficient therapeutic strategies for mCRC.
REFERENCES


Figure Legends

Figure 1. Identification by immuno-histochemistry of CCR7+ lymphocytes in the primary tumour. Panel (a) shows no lymphocyte infiltration; panels (b), (c), (d) and (e), respectively, show increasing CCR7+ infiltration (magnification 50-200x).

Panel (f) shows CCR7+CD8+ lymphocytes (arrows) in a double color immunohistochemistry analysis. This analysis demonstrates that in the primary tumour 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- lymphocyte which show an irregular and weak red stain (magnification 400x).

Figure 2. Actuarial Kaplan Meyer’s survival curves of 76 colorectal cancer patients who had undergone FOLFOX-4 or GOLFIG treatment whose tumour was scored by immune histo-chemistry for T_{ccr7} infiltration. Panels compare overall survival (A) and progression free survival (B) in patients with high (_____ ) and low ( _ _ _ _ ) T_{ccr7} tumour infiltration score.

Figure 3. Regression curve (with 95% CI) of CCR7 expression as a continuous variable in the tumour tissue and progression free survival (A). T-test comparing mean PFS values between high vs low CCR7 expression groups (B).

Figure 4. Actuarial Kaplan Meyer’s survival curves of patients whose primary tumour 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.
Fig. 2
Tumour infiltration by T-lymphocytes expressing chemokine receptor 7 (CCR7) is predictive of favourable outcome in patients with advanced colorectal carcinoma.

Pierpaolo Correale, Maria Saveria Rotundo, Cirino Botta, et al.

_Clin Cancer Res_ Published OnlineFirst December 5, 2011.

**Updated version**

Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-3186

**Author Manuscript**

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.