

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)

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Abstract Purpose: GOLFIG chemoimmunotherapy regimen proved to be a safe and very active chemoimmunotherapy regimen in advanced colon cancer patients. We have thus investigated the immunobiological feedback to the treatment and its possible correlation with the clinical outcome of these patients.

Experimental Design: This clinical and immunologic study involved 46 patients, 27 males and 19 females, enrolled in the GOLFIG-1 phase II trial who received gemcitabine (1,000 mg/m² on days 1 and 15), oxaliplatin (85 mg/m² on days 2 and 16), levofolinic acid (100 mg/m² on days 1, 2, 15, and 16), and 5-fluorouracil (400 mg/m² as a bolus, and 800 mg/m² as a 24-hour infusion on days 1, 2, 15, and 16) followed by s.c. granulocyte macrophage colony-stimulating factor (100 µg, on days 3-7) and interleukin 2 (0.5 × 10⁶ IU twice a day on days 8-14 and 17-29).

Results: The regimen was confirmed to be safe and very active in pretreated patients with metastatic colorectal cancer. A subgroup analysis of these patients revealed a prolonged time to progression and survival in six patients who developed late signs of autoimmunity. A multivariate analysis validated the occurrence of autoimmunity signs as an independent predictor of favorable outcome. A parallel immunologic study detected in the peripheral blood mononuclear cells of these patients a progressive increase in lymphocyte and eosinophil counts, amplification in central memory, a marked depletion of immunosuppressive regulatory T cells, and activation of colon cancer – specific cytotoxic T cells.

Conclusions: Our results suggest that immunity feedback to GOLFIG regimen and its antitumor activity are tightly correlated.

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Colorectal carcinoma is the second leading cause of cancer-related deaths (1, 2). The best therapeutic option for the advanced disease is represented by polychemotherapy with fluorouracil (5-FU) +/- levofolinic acid together with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) given alone, or in combination with bevacizumab or cetuximab, two monoclonal antibodies, respectively, directed against the vascular-endothelial growth factor and the epidermal growth factor receptor (3, 6). These combinations induce high response rates (45-50%) and prolong the time to disease progression (TTP; 9-12 months) and overall survival (OS) of these patients; however, regardless of the type and sequence of administration, their average OS is still no more than 20 to 22 months (3-6). In the attempt to improve these results, other therapeutic strategies are being investigated (5-10). A newest polychemotherapy regimen combining gemcitabine with oxaliplatin, levofolinic acid, and

5-FU (GOLF) showed synergistic cytotoxic and proapoptotic effects in human colon cancer cell lines *in vitro*, and also led to molecular and antigenic changes (11, 12), which could have promising immunotherapeutic implications. This regimen, in fact, showed the ability to convert the tumor cells into a powerful source of antigens, making them a potential vaccine source (12). This property has been related to the GOLF ability of inducing in colon cancer cells up-regulation of antigens such as carcinoembryonic antigen, thymidylate synthase, and peptide-rich heat shock proteins, at the same time as the appearance of a danger signal. The latter is capable of alerting both dendritic cells and antigen-specific CTL precursors, priming them to a more efficient response (13–16). The above findings provided the rationale to test a new chemoimmunotherapy strategy aimed at generating a drug-induced CTL response capable of improving the clinical results of GOLF chemotherapy in colon cancer patients.

This was attempted by designing the GOLFIG-1 regimen that combines the GOLF polychemotherapy, aimed to induce cytotoxic effects, tumor cell antigen remodeling and antigen release, and the cytokine-based IG-1 immunoadjuvant regimen based on a sequential combination of s.c. granulocyte macrophage colony-stimulating factor and metronomic ultra-low-dose aldesleukin (interleukin-2) and aimed to improve antigen presentation, thus generating an efficient antigen-specific CTL response. Either regimen has been tested in previous phase Ib-II trials whose results showed a significant antitumor activity of the GOLF regimen in colorectal carcinoma patients (17), a limited toxicity for both GOLF and IG-1 treatments (17, 18), and the biological effects of the IG-1 immunoadjuvant regimen of enhancing and activating peripheral dendritic cells and inducing an efficient antigen-specific T-cell response (18).

We have previously described preliminary biological and clinical results of GOLFIG regimen in a first cohort of 29 colon cancer patients (19). In the present study, we evaluated the immunobiological feedback to the treatment and its possible correlation with the clinical outcome of the patients enrolled in this study.

Patients and Methods

Study design. The study was authorized by the University Committee (equivalent to Human Subject Committee of Investigational Review Board) and by the Italian Ministry of Health and registered as GOLFIG-1 trial.

This phase II study was planned on the basis of the Simon's two-stage optimal design.

We selected a target activity of 40% to 50% response rate with a 0.05 α error and a 0.20 β error. The treatment should have been considered nonactive if it led to less than seven responses among the 16 consecutive patients (43%) in the first series, and less than 23 responses among the overall total of 46 patients (50%).

Patient characteristics. The inclusion criteria were as follows: a histologic diagnosis of colorectal carcinoma, an Eastern Cooperative Oncology Group performance status of 0 to 3, normal renal and hepatic functions, WBC count $\geq 2,500/\text{mm}^3$, hemoglobin levels ≥ 9 g/dL, platelet cell count $\geq 100,000/\text{mm}^3$, and normal cardiac function. The exclusion criteria were as follows: any major organ failure, central nervous system involvement, second malignancies, active infectious disease, major autoimmune diseases, and acquired immunosuppression (AIDS or major immunosuppressive agents).

Treatment schedule. All patients signed an informed consent and received biweekly chemotherapy with gemcitabine ($1,000 \text{ mg/m}^2$ on days 1 and 15), oxaliplatin (85 mg/m^2 on days 2 and 16), levofolinic acid (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-h infusion on days 1, 2, 15, and 16), followed by s.c. granulocyte macrophage colony-stimulating factor ($100 \mu\text{g}$, on days 3–7) and ultra-low-dose s.c. interleukin-2 (0.5×10^6 IU twice a day on days 8–14 and 17–29; ref. 19).

Treatment evaluation. Standard assessments (clinical history, physical examination, hematochemical analysis, carcinoembryonic antigen and CA19.9 assays, chest X-ray, and ultrasound scans) were made at baseline and repeated every 4 to 6 wk. High-definition, multislice computed tomography scans with contrast medium were recorded every 3 mo. All patients were evaluated for TIP, OS, overall response, and toxicity, assessed according to the standard WHO criteria. An independent radiology evaluation was carried out to confirm the objective responses.

Patient monitoring, CTL assay, and cell cultures. Peripheral blood samples for patient monitoring were drawn at baseline and at the beginning of each treatment cycle and managed as described previously (19). The CTL lines and dendritic cells were generated from the peripheral blood mononuclear cells (PBMC) of these patients and characterized as previously described (12). Colon carcinoma WiDr cells were cultured as described in previous studies (12).

Flow cytometry. The procedure for single-color, double-color, and triple-color flow cytometry analysis has been described previously (12, 18, 19). Anti-CD3, anti-CD4, anti-CD8, anti-CD56, and anti-CD27 monoclonal antibodies were purchased from Exbio Praha; anti-FoxP3, anti-CD25, anti-CD62L, and anti-CD45Ro monoclonal antibodies were purchased from Ancell Corporation Bayport; polyclonal antibody to CCR7 was purchased from Alexis Biochemicals; anti-HLA class I was from Scra (W6/32); and MOPC-21 was from Cappel/Organon Technica Corp. The samples were analyzed immediately using a Becton Dickinson FACScan equipped with a blue laser with an excitation level of 15 nW at 488 nm. The data gathered from 10,000 alive cells were used to evaluate the results.

Pathology study. Portions of tumor mass 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 H&E and histologically examined by an expert pathologist.

Immunohistochemistry. Immunohistochemical staining was done on 3- μm -thick sections of each block using the streptavidin-biotin method. After being dewaxed and rehydrated, sections were incubated with 3% H_2O_2 in TBS 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 min at 98°C ; Bio-Optica) and incubated with antihuman monoclonal antibody CD4 (clone 4B12; 1:50; Menarini). For CD8^+ T cells, to improve the detection of the antigen, we first used a pretreatment with a microwave oven in citrate buffer (0.01 mol/L; pH 6.0) at 750 W for 5 min, three cycles, and then incubated with antihuman monoclonal antibody CD8 (clone CD8-144B; 1:50; Dako). For FoxP3 ($\text{CD4}^+\text{CD25}^+$ T cells), we did a pretreatment with a microwave oven in EDTA (0.05 mol/L; pH 8.0) at 750 W for 5 min at three cycles and then incubated with antihuman monoclonal antibody FoxP3 (clone 22510; 1:50; 60 min; Abcam). The epitopes were detected with the Ultravision Detection System and revealed with the diaminobenzidine for 5 min (Dako); sections were counterstained with hematoxylin. Negative controls were obtained by replacing the specific antibody with nonimmune serum immunoglobulins at the same concentration as the primary antibody. Chemokine receptor CCR7 was detected by immunofluorescence. Tissue sections were dewaxed and rehydrated, incubated in TBS (pH 7.6) for 1 h at 37°C , pretreated with trypsin at 37°C for 30 min, again placed in TBS at 4°C for 30 min, and finally incubated with antibody CCR7 (1:20; Alexis Biochemicals) for 30 min. Immunostaining was examined with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy).

Quantitative evaluation of T lymphocyte subtypes. Sections stained for CD4, CD8, FoxP3, and (CCR7) were scored in coded slides by one observer (C.G.). The number of positive cells for each marker was recorded in five randomly chosen HGPf in the stroma adjacent to neoplastic glands. Positive cells were scored as follows: 0 = 1 to 10; 1 = 11 to 15; 2 = 16 to 30; 3 = 31 to 50; 4 = 51 to 70; 5 = >70.

Cytotoxic assays. Chromium-51 release assays and inhibition of HLA-restricted CTL activity by anti-HLA monoclonal antibodies were done as described in previous studies (12). To be used as targets, WiDr target cells were transfected with the *HLA-A2.1* gene (A2-WiDr) before being tested in CTL assays (12). Specific lysis was calculated as follows:

$$\% \text{ Specific lysis} = \frac{\text{Observed release (cpm)} - \text{spontaneous release (cpm)}}{\text{Total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined from the wells to which 100 μ L of medium were added instead of effector cells. Total releasable radioactivity was obtained after treating the target with 2.5% Triton X-100.

Statistical analysis. Survival plots were constructed using the Kaplan-Meier method, and the survival data were analyzed by using the GraphPad Instat 3.2 statistical software.

Results

Patient characteristics. Forty-six patients, 27 males and 19 females, were enrolled in the study between October 2001 and December 2005. Their median age was 62 years (range 28-83 years). Their primary tumor site was colon and rectum in 39 and 7 patients, respectively. At the treatment baseline, 43 had undergone previous surgery, 32 had liver metastases, 12 had never received chemotherapy, 24 had received one line of chemotherapy, and 10 had received two lines of chemotherapy with either FOLFOX or FOLFIRI regimens. Among those patients who had received one chemotherapy line, 9 had received FU/FA or capecitabine, 4 received FOLFIRI, and 11 received FOLFOX polychemotherapy. Six of these patients had received chemotherapy in combination with cetuximab, and three received chemotherapy in combination with bevacizumab.

Toxicity. The regimen proved to be quite safe and the occurrence of adverse events were similar to those described in the previous report (19). Fever during interleukin-2 administration was confirmed as the most frequent adverse event as it was present in 60% of the patients. Moderate (grade 1-2) asthenia and fatigue were observed in 25% of the cases. Grade 1 to 2 hematologic toxicity was a common event with anemia, neutropenia, and thrombocytopenia, occurring in 6 (13%), 5 (10.8%), and 5 (10.8%) cases, respectively. Reversible episodes of grade 3 to 4 anemia and thrombocytopenia also occurred in 2 (4.3%) and 5 (10.8%) patients, respectively. Grade 1 to 2 nausea/vomiting episodes occurred in 2 patients (6.5%), whereas grade 1 to 2 diarrhea and mucositis episodes were recorded in 9 cases (17.1%). These episodes occurred 7 to 10 days after the chemotherapy and lasted a few days and were not suggestive of an immune-mediated event. Grade 3 to 4 diarrhea and mucositis were conversely observed in two patients who subsequently showed a marked deficit in the dihydropyrimidine dehydrogenase, an enzyme that is involved

in fluoropyrimidine catabolism. These patients continued the treatment using a 75% reduced dose of 5-FU.

Five patients showed hypersensitivity to oxaliplatin with hypotension, erythema, dyspnea, and fever occurring between the 7th and 11th treatment cycle. Granulocyte macrophage colony-stimulating factor-related bone pain and oxaliplatin-related neurologic toxicity were also recorded at lesser extents.

Clinical signs of autoimmunity were observed in six cases. One of the patients developed cutaneous lesions; the histologic analysis of this patient suggested a diagnosis of discoid lupus erythematosus. Before being enrolled in the trial, this patient had undergone preoperative radiotherapy and left colectomy. The other 5 patients showed mono/oligo-articular arthritis accompanied by fever and increase of inflammatory markers, localized at the knees, and/or elbows, shoulders, wrists, and/or hands. Clinical diagnosis was formulated when patients presented clear signs and symptoms of arthritis, including pain, swelling, and severe malfunctions of involved joints. In these patients, ultrasound scan and magnetic resonance showed the presence of synovial effusion, synovitis, and tenosynovitis. These patients had received a previous chemotherapy line and before starting the GOLFIG regimen had never suffered of demonstrable autoimmunity and/or other rheumatic or cutaneous pathologies. Autoimmune symptoms appeared 4 to 9 months after the beginning of the treatment, were self-limiting, and were controlled with nonsteroidal anti-inflammatory drugs.

In these patients, anti-dsDNA, anti-nuclear antibodies, and anti-cyclic citrullinated peptide antibodies were always in the reference range, although a significant increase in circulating immune complexes was observed in two of them. During the acute manifestation of arthritis, there was a significant increase of nonspecific inflammatory markers (C-reactive protein,

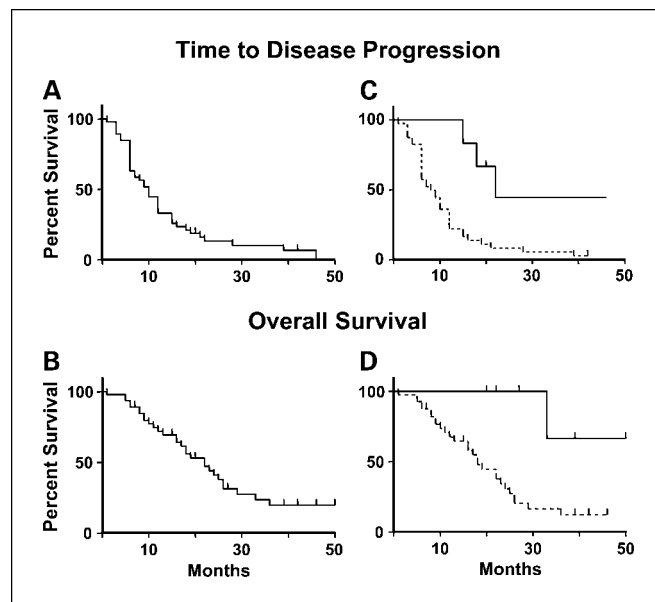


Fig. 1. Actuarial Kaplan-Meier survival curves for TTP (12.26 mo; 95% CI, 9.2-15.2; A) and overall survival (18.76 mo; 95% CI, 15.2-22.3; B) of the patients enrolled in the GOLFIG-1 trial. TTP (C) and OS (D) of the patients who developed autoimmunity signs (---) and those who did not (—). The patients experiencing autoimmunity had a much longer TTP (23.83 vs 10.52 mo, $P = 0.0039$) and OS (31.83 vs 16.8 mo, $P = 0.0080$).

Table 1. Cyclooxygenase regression model (with 95% CI) according to different prognostic variables

Prognostic variable	P	Risk ratio (95% CI)
Risk of disease progression		
Autoimmunity	0.0046*	0.1646 (0.0472-0.5738)
Age	0.1059	0.9764 (0.9485-1.0051)
Liver metastases	0.3401	0.7044 (0.3429-1.4470)
Previous chemotherapy	0.7919	0.8986 (0.4060-1.9886)
Performance status	0.0877	1.2872 (0.9634-1.7200)
Sex	0.6697	1.1621 (0.5827-2.3176)
Risk of death		
Autoimmunity	0.0256*	0.0884 (0.0105-0.7440)
Age	0.6415	0.9916 (0.9570-1.0274)
Liver metastases	0.8232	0.8970 (0.3457-2.3275)
Previous chemotherapy	0.7046	0.8201 (0.2941-2.2867)
Performance status	0.0042*	1.7248 (1.1881-2.5040)
Sex	0.7519	0.8776 (0.3905-1.9721)

NOTE: Autoimmunity and performance status were the most predictive variables of prolonged TTP and survival.

*Statistically significant values.

erythrocyte sedimentation rate, and fibrinogen) whose level were significantly greater when compared with the ones recorded in those patients who did not show this kind of symptoms. An immunocytofluorimetric analysis showed in these patients a significant decrease in regulatory T cells whose average percentage in their PBMCs resulted 12.5% (± 3.75) at baseline and 2.5% (± 4.25) after 12 cycles of treatment.

Treatment response. This phase II study was planned on the basis of the Simon's two-stage optimal design and the

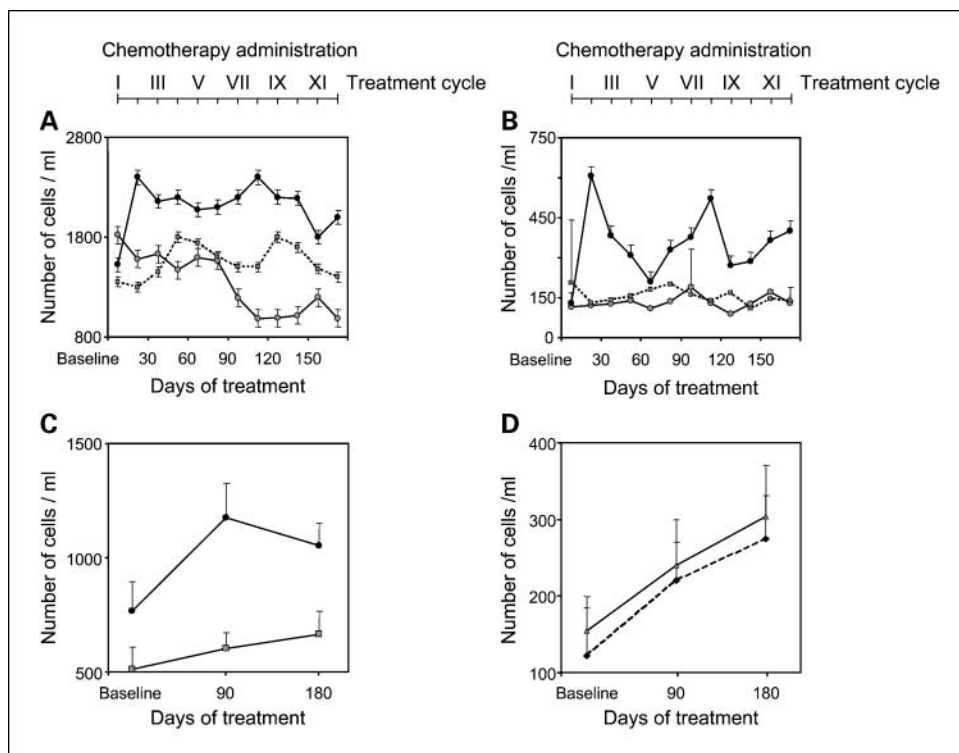
progression from the step I to the step II was granted by the finding of 13 of 16 responses in the first step series of patients. The overall response (see below) fulfilled the target activity of 43%.

At the formal end of the study, 10 patients achieved a complete response, 16 achieved a partial response, and 16 showed stable disease. The overall response rate (complete response + partial response) was 56.5% [95% confidence interval (95% CI), 42.1-69.8%], and the disease control rate (complete response + partial response + stable disease) was 91.3% (95% CI, 79.6-96.4%). Complete responses were observed in five chemotherapy-naïve patients with liver, lung, and peritoneum metastases, and in six patients with liver and peritoneum metastases who had received previous lines of chemotherapy. Four patients experienced disease progression, all of whom had received at least one previous line of chemotherapy, had a poor performance status (Eastern Cooperative Oncology Group ≥ 2), and showed a bulky metastatic liver involvement. The mean TTP was 12.26 (95% CI, 9.2-15.2) and the mean OS was 18.76 months (95% CI, 15.2-22.3; Fig. 1A and B). Previous patient treatment did not result as a negative prognostic variable (data not shown).

Those patients who showed clinical signs of autoimmunity showed a TTP and OS significantly longer than those recorded in the patients who did not develop autoimmunity signs ($P = 0.0039$ and $P = 0.0080$, respectively; Fig. 1C and D). A multivariate analysis confirmed the occurrence of autoimmunity as the most significant predictive variable for both prolonged TTP and OS (Table 1).

Biological evaluation. A progressive increase in peripheral lymphocyte counts was detected during the GOLFIG treatment. These results were statistically different ($P < 0.05$) from those recorded in a sample of patients administered with FOLFOX-4

Fig. 2. The curves compare lymphocyte (A) and eosinophil counts (B) in patients receiving the GOLFIG regimen (—●—) in a group of 20 patients with metastatic colorectal carcinoma receiving the standard FOLFOX-4 chemotherapy (---□---) or in a group of 10 patients receiving the GOLFIG chemotherapy without cytokine administration (---■---). Higher lymphocyte and eosinophil counts in patients receiving chemoimmunotherapy ($P < 0.05$) were shown. C and D, a progressive increase in the number of T lymphocytes expressing a CD3⁺CCR7⁺ (—●—), CD3⁺CD45Ro⁺ (---□---), CD8⁺CD27⁺ (---■---), or CD3⁺CD62L⁺ (---▲---) phenotype in the PBMCs of 20 patients receiving GOLFIG chemoimmunotherapy.



and GOLF regimens. Lymphocyte counts gradually decreased in the patients receiving FOLFOX regimen, whereas they remained stable in those receiving GOLF polychemotherapy (Fig. 2A). We did not find any significant correlation between lymphocyte increase and either TTP or OS although a positive trend was observed in the outcome of those patients who showed >50% lymphocyte increase during the treatment (data not shown).

We also found an increase in eosinophil cell number (Fig. 2B) and monocytes (data not shown) in the patients administered with GOLFIG regimen, which again was not observed in those patients receiving either FOLFOX or GOLF chemotherapy.

All of the control patients who underwent FOLFOX chemotherapy had a histologic diagnosis of colon carcinoma and showed metastatic disease (mainly liver and lung), whereas five of them were in second-line chemotherapy. All of the patients who underwent GOLF chemotherapy had a histologic diagnosis of colorectal carcinoma and metastatic disease. They all received a previous line of chemotherapy, except for three patients who received two lines.

Analysis of peripheral lymphocyte subsets. The results of an immunocytofluorimetric study suggested that the lymphocyte increase mainly regarded the T-cell compartment (either CD3⁺CD4⁺ and CD3⁺CD8⁺) and led to a complete restoration of CD3⁺CD4⁺/CD3⁺CD8⁺ cell ratio (19), a functional marker that strongly reflects the immunocompetent status of advanced cancer patients. A significant increase in the number of T cells expressing CD197 (CCR-7), CD27, and CD45Ro, a particular T-lymphocyte subset recognized as responsible for central memory (20–22), was also observed. This event was accompanied by a progressive increase in T cells expressing CD62L, an adhesion molecule present on activated T lymphocytes (Fig. 2C and D; ref. 23).

Effects of GOLFIG chemoimmunotherapy on immunoregulatory T-cell subset. Preclinical results suggest that gemcitabine, 5-FU, and oxaliplatin may empower the cytotoxic activity of antigen-specific CTLs *in vitro* and *in vivo* models by distressing T-cell response-associated T-regulatory (T_{reg}) lymphocytes (24) with a CD4⁺CD25⁺ CD45Ro⁺FoxP3⁺-immunosuppressive phenotype (25–28). In line with those results, we found in our patients a rapid T_{reg} decline that remained stable for several months and became similar to the values normally observed in healthy individuals (Fig. 3). This effect was not observed in patients administered with FOLFOX chemotherapy.

These results therefore suggest that our therapeutic combination is capable of inducing a decline in blood cells with a T_{reg} phenotype. Possible effects of the function of these cells will be evaluated in future studies.

Immunohistochemical study. An immunohistochemical analysis of lymphocyte infiltration in the tumor tissue was done in four patients who achieved a partial response after 12 cycles of treatment and underwent liver or lung metastasectomy. Two patients who underwent surgery after standard FOLFOX chemotherapy were used as control. In these six patients, we also examined pretreatment samples of the same tumor tissue that had been previously taken for diagnostic purpose. A moderate reduction of FoxP3⁺ T cells and a marked increase of CCR7⁺ lymphocytes were detected in the tumor samples of those patients who had completed the GOLFIG regimen. These events were not observed in the tumor tissue of those patients who had received FOLFOX chemotherapy and

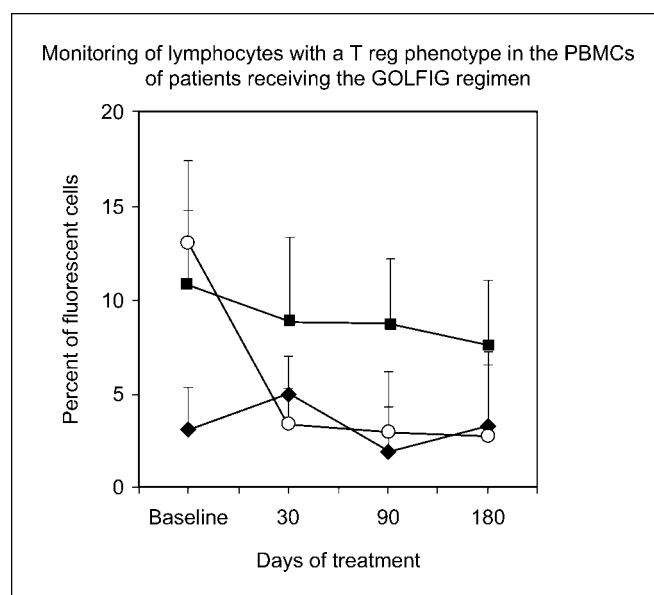


Fig. 3. Curves showing the percentage of lymphocytes expressing a CD4⁺CD25⁺FoxP3⁺ phenotype in the PBMCs of 20 patients during treatment with the GOLFIG regimen (○). PBMCs derived from 10 patients treated with FOLFOX regimen (■) and four healthy donors (◆) were used as controls. There was a significant and stable decrement in the number of T_{reg} cells in the patients after treatment ($P < 0.05$), with the levels becoming similar to those observed in the PBMCs of healthy donors.

did not show a significant change in FoxP3⁺ cells and CCR7⁺ lymphocytes (Fig. 4).

Colon cancer-specific CTL assays. Antigen-specific CTLs are the ultimate effectors of active specific anticancer immunotherapy. To test a possible effect of GOLFIG regimen on antigen-specific CTL activity, we characterized colon cancer antigen-specific CTL lines derived from the PBMCs of six patients with a HLA-A2.1 haplotype, isolated at baseline and after two cycles of chemoimmunotherapy. CTL lines were generated by sensitizing *in vitro* PBMCs with autologous dendritic cells loaded with cell lysate of colon carcinoma cell lines, untreated (chemokine receptor CTLs) or previously treated with GOLF chemotherapy (GOLF CTLs; ref. 12). Cytotoxic assays showed that the CTL lines derived from the posttreatment PBMCs had much more cytotoxic activity *in vitro* against class I HLA matching colon cancer target cells [transfected with the HLA-A(*)02.01 gene; A2-WiDR] than those derived from baseline PBMCs ($P < 0.05$; Fig. 5A-B). In particular, a more efficient killing activity was detected in the CTL lines sensitized with GOLF-treated tumor cell lysate (Fig. 5C and D). Involvement of natural immunity was excluded by the finding that no difference among pretreatment- and posttreatment-derived CTLs was observed when their cytolytic activity was tested against HLA-A2.1-negative target cells like WiDr or K562 leukemia cells (Fig. 5 and data not shown).

Discussion

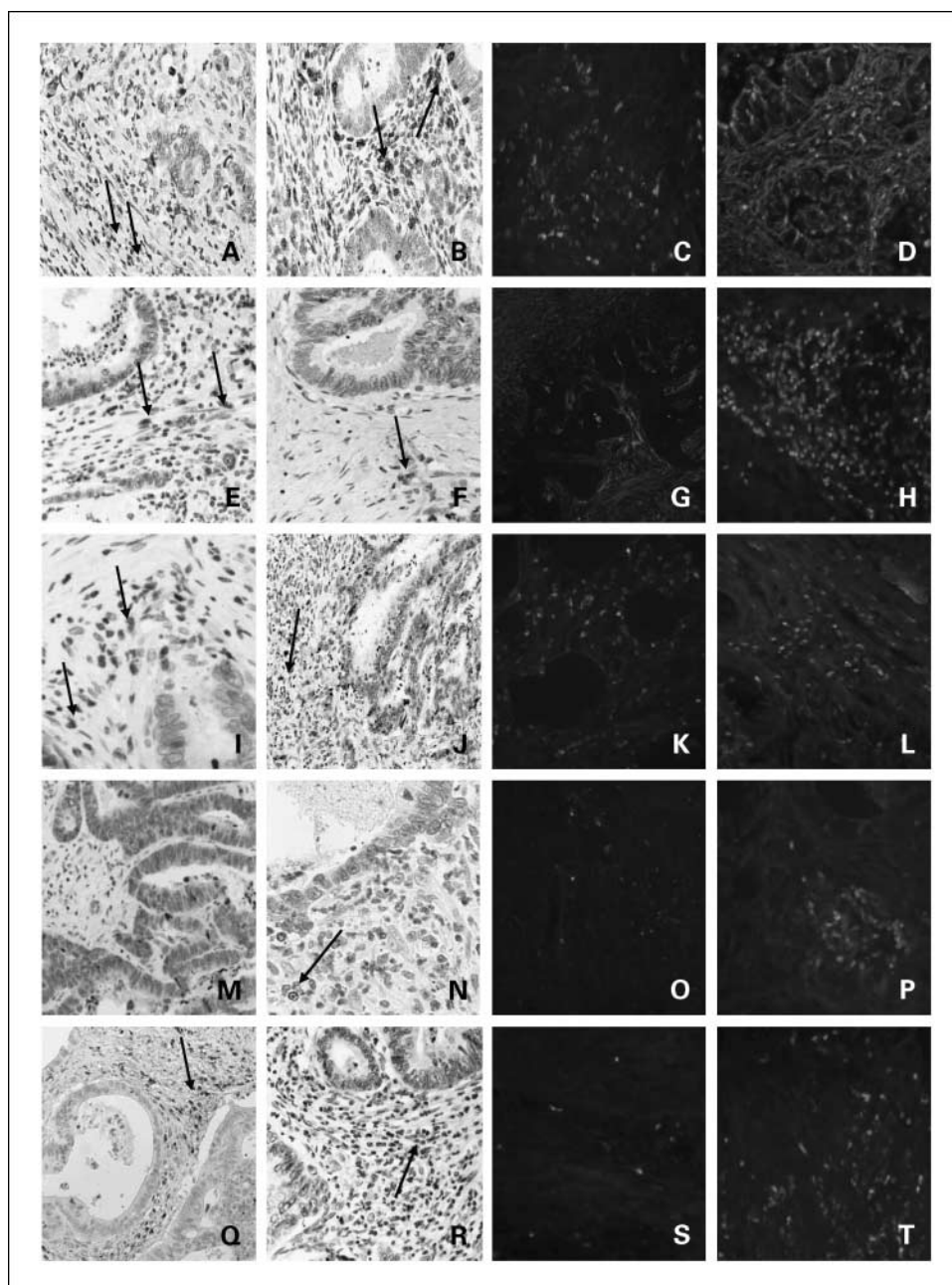
In this article, at the formal completion of the GOLFIG-1 trial, we report the results of an immunobiological investigation aimed to evaluate the immunobiological feedback to the treatment and its possible correlation with the clinical outcome of these patients. According to the prospective design, our

results confirm the reasonable safety and tolerability and the promising antitumor activity of the GOLFIG-1 regimen described in the previous report (19).

We additionally report the results of a multivariate analysis that validated the occurrence of autoimmunity as the most relevant independent predictive variable of prolonged TTP and OS. The same analysis recognized a poor performance status at baseline as an independent predictive variable for poor outcome in terms of OS whereas it did not assign any predictive value to the chemo-naïve status. These results seem to be of particular interest considering that the most common multi-drug combinations are poorly effective in colorectal carcinoma patients when used in second or third line of treatment (15-30% of overall response rate and 3-4 months of TTP), and that the majority of them contained 5-FU and oxaliplatin,

which are also included in the GOLFIG chemotherapy (5–10). This finding suggests that the antitumor effect of GOLFIG is not simply related to the additive cytotoxic effect of the drugs and it supports the hypothesis of a possible immunologic involvement. In this context, the immunologic results of our study could explain the occurrence of both immune response and autoimmunity. In fact, we detected an increased number of peripheral lymphocytes and eosinophils and an increased number of activated and central memory T lymphocytes. These events occurred in synchrony with a progressive depletion of immunosuppressive T_{reg} subsets. Previously, we detected a treatment-related increase in the frequency of colon cancer antigen (carcinoembryonic antigen and thymidylate synthase peptide)-specific CTL precursors in patients with the HLA-A2.1 haplotype (19). In this study, we found that CTL lines

Fig. 4. Immunohistochemical analysis using anti-FoxP3 (A, B, E, F, I, J, M, N, Q, and R) and anti-CCR7 (C, D, G, H, K, L, O, P, S, and T) polyclonal antibodies in tumor specimens of colon cancer patients before (first and third column) and after (second and fourth column) 12 cycles of chemotherapy. A to D, tumor samples of a representative control patient who underwent FOLFOX chemotherapy; E to T, tumor samples of patients who underwent GOLFIG regimen. The pictures show a significant reduction of FoxP3⁺ lymphocytes (brown) and a significant increase of CCR7⁺ lymphocytes (green) in the tumor specimens derived from patients who underwent GOLFIG chemioimmunotherapy compared with those derived from patients who underwent FOLFOX chemotherapy ($P < 0.05$). Score 0 of FoxP3⁺ lymphocytes was shown in F, J, M, and N; score 1 of CCR7⁺ lymphocytes was shown in C, G, O, and S; score 2 was shown in A, D, E, I, and K; score 3 was shown in L and R; score 4 was shown in B, H, P, and T; and score 5 was shown Q. Original magnifications, $\times 100$ (A-D, F-H, J-L, and O-S) and $\times 200$ (E, I, M, N, P, and T).



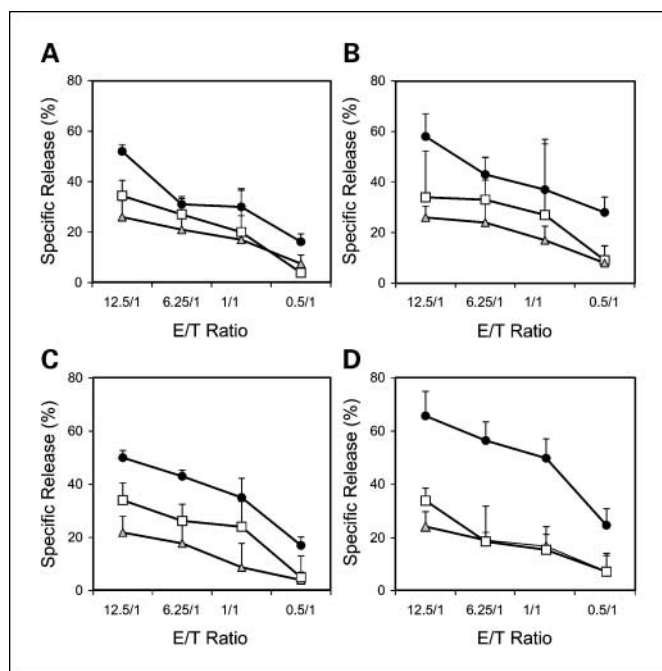


Fig. 5. The killing activity of CTL lines derived from six colon cancer patients expressing a HLA-A(*)02.01 haplotype. CTL lines, *in vitro* sensitized with a lysate of colon cancer cells (see Materials and Methods) untreated (A and C) or previously treated with GOLFIG polychemotherapy (B and D), were tested against class I-HLA matching colon carcinoma WiDr target cells transfected with HLA-A(*)02.01 gene (A2-WiDr; ●). HLA-A(*)02.01 negative WiDr (▲) and A2-WiDr exposed to anti-HLA-A(*)02.01, A2.69 monoclonal antibody (□) were used as negative controls. These curves show that CTL lines derived from the PBMCs of GOLFIG-treated patients (B and D) had a much greater colon cancer-specific cytolytic activity than those derived from baseline PBMCs ($P < 0.04$; A and C). The CTL lines derived from the PBMCs of GOLFIG-treated patients and *in vitro* sensitized with the lysate of GOLFIG-treated colon cancer cells showed the greatest colon cancer-specific cytolytic activity ($P < 0.05$; D). Points, specific release (%) at different effector-to-target ratios; bars, SD. Each curve represents a mean of six different donors. The experiment was repeated twice with similar results.

generated from the PBMCs of the same patients after they had received the GOLFIG regimen also showed an augmented antitumor *ex vivo* activity against class I HLA matching colon cancer cells (A2-WiDr). This empowering effect of GOLFIG regimen on the CTLs is in line with the above-reported increase in the frequency of T-cell precursors and central memory-T lymphocytes. This particular subset has been recognized as the one responsible in driving the immune response into the tumor site and lymphoid organs where the immune attack takes place (20–23). These T cells in fact express CCR7, a receptor able to bind different ligands produced by activated dendritic cells and other inflammatory cells (21, 22). Once engaged by its ligands during the immune attack, CCR7 primes an intracellular process in the T cells that guides their chemotactic homing to lymph nodes, tumor/infected tissues, and target cells. These T cells also express CD62L, an adhesion molecule that plays a primary role in T-cell homing by mediating initial leukocyte interaction with activated vascular endothelium (23). An increase in the number of T cells expressing these receptors means a greater amount of mobilized effector cells available to sustain an antigen-specific T cell-mediated immune response.

The increase of central memory T cells and the empowered CTL activity are also in line with the progressive decline in T_{reg} cells, a lymphocyte subset that has the specific task of

attenuating a potentially dangerous overreactive immune response (24–33). T_{reg} cells are strictly dependent on interleukin-2 levels in the blood, and their number is increased as consequence of a specific immune response, multiple vaccinations, or diseases that like cancer are often accompanied by a chronic state of inflammation and immune attack (32–34). We hypothesized that the T_{reg} depletion observed in these patients may be due to a specific cytotoxic activity of 5-FU, gemcitabine, and oxaliplatin on this kind of lymphocytes especially when they are proliferating in response to the GOLFIG-induced CTL response. Mature CTLs proved to be much more resistant to these drugs, a phenomenon that is considered mainly a consequence of the fact that their proliferative activity only occurs close to the antigen presentation and progressively declines a few days later. This hypothesis has been challenged in pre-clinical models where the decline/inactivation and enhanced cytolytic activity of T_{reg} cells was observed in virus- and tumor antigen-specific T-cell lines exposed to gemcitabine, oxaliplatin, and/or 5-FU late after (14 days) each *in vitro* stimulation with antigen-pulsed dendritic cells (24). Analogous results were obtained in HLA-A(*)02.01 transgenic mice inoculated with autologous tumor cells, in which the immunologic and therapeutic effect of a thymidylate synthase-directed cancer vaccine (TS/PP) was significantly augmented when used in combination with GOLFIG polychemotherapy only when the latter was administered late after mouse vaccination. This effect was completely lost when chemotherapy and antigen stimulation or vaccination were administered simultaneously (24).

The occurrence of autoimmunity and its possible correlation with a good outcome in patients receiving the GOLFIG regimen are also in line with the hypothesis of a chemotherapy-induced distress of T_{reg} cells and with an overstimulation of T cell-mediated immune response. In this context, the pathogenesis of either arthritis, discoid lupus erythematosus (which is a chronic cutaneous manifestation of systemic lupus erythematosus), and other immune diseases have been related to a specific T_{reg} malfunctioning (25–35).

A tight correlation between treatment efficacy and long survival with autoimmunity in cancer patients was also reported by other authors in different studies testing completely different immunologic agents (32–43). All these results suggest that autoantigens are critical partners in driving the autoimmune response, and those unique changes in antigen expression and conformation in the immunizing tumor and the target tissue may play a role in antigen selection and ongoing damage (39).

In conclusion, the results of this study suggest that the antitumor activity and immunobiological effects of GOLFIG chemioimmunotherapy regimen are tightly correlated and that it is an attractive strategy to challenge in randomized trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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