Research Article

Cancer Immunology Research

Escalating Regulation of 5T4-Specific IFN-γ^+ CD4$^+$ T Cells Distinguishes Colorectal Cancer Patients from Healthy Controls and Provides a Target for *In Vivo* Therapy

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Abstract

The relationship between the adaptive CD4$^+$ T-cell response and human cancer is unclear. The oncofetal antigen 5T4 is expressed in many human carcinomas, including colorectal cancer cells, but has limited expression on normal tissues. We previously identified anti-5T4 CD4$^+$ T cells in a proportion of patients with colorectal cancer, and we extended this study to examine whether the quality or quantity of the T-cell response reflects tumor stage. An overlapping peptide library spanning 5T4 was used as a target to enumerate cognate IFN-γ^+ CD4$^+$ T cells [measured as spot-forming cells (SFC)/10^5 cultured T cells] in peripheral blood–derived lymphocytes following a 14-day *in vitro* culture period comparing patients preoperatively ($n = 27$) to healthy controls ($n = 17$). Robust 5T4-specific T-cell responses were present in 100% of healthy donors. There was a steady loss of T-cell responses with advancing tumors with a significant negative correlation from stage I to III ($P = 0.008$). The predictability of the decline meant <200 SFC/10^5 were only found in subjects with stage III colorectal cancer. The mechanism of loss of T-cell response is independent of HLA-DR type or patient age but does correspond to increases in Foxp3$^+$ regulatory T cells (Treg). Using low-dose cyclophosphamide to reduce the proportion of Tregs *in vivo* resulted in increased anti-5T4 T-cell responses in patients with colorectal cancer. The selective loss of 5T4-specific IFN-γ^+ CD4$^+$ T-cell responses implies a link between tumor stage and antitumor Th1 effector function; depleting Tregs can enhance such responses. *Cancer Immunol Res; 1(6); 416–25. ©2013 AACR.*

Introduction

Colorectal cancer is one of the most commonly diagnosed malignancies worldwide, accounting for more than 600,000 deaths per year (1). Where possible, a colectomy to remove the primary tumor is carried out; however, 40% to 50% of these patients relapse or die from metastatic disease. Patient outcome strongly correlates with histopathologic staging of the excised tumor; patients with a tumor–node–metastasis (TNM) stage I (Dukes' A) tumor confined to the bowel wall have a predicted 5-year survival rate of more than 90%, stage II (Dukes' B) tumor penetrated into serosal surface of bowel approximately 80%, stage III (Dukes' C) tumor spread to lymph nodes/adjacent organs approximately 50%, and stage IV (Dukes' D) tumor with distant metastases approximately 10%. The extent of lymphocyte infiltration into tumors has been shown to be an independent prognostic marker shown by increased intratumoral CD3$^+$ T cells (2). Infiltrating CD8$^+$ T cells are thought to eliminate tumor cells when receiving adequate CD4$^+$ T-cell help (3).

Tumor-associated antigens (TAA) comprise common proteins that are markedly upregulated in neoplastic cells or proteins that are expressed mainly or solely in neoplastic cells, i.e., tumor-specific antigens. The latter is an attractive group to target for therapy, as there should be limited cross-reactivity to healthy tissue. Expression of the trophoblast cell surface glycoprotein 5T4 is restricted to several human carcinomas, including colorectal cancer (4); this tumor-specific antigen lends itself as a candidate target for tumor-directed immune responses. Indeed, a recent report has shown the ability of 5T4-specific cytotoxic CD8$^+$ T cells to induce tumor cell death (5). We have previously identified *ex vivo* CD4$^+$ T-cell responses to 5T4 in approximately one third of patients with colorectal cancer awaiting surgical resection (6, 7).

CTLs require CD4$^+$ T-cell help for both effector function and development of a memory population (3, 8). The adoptive transfer of antitumor CD4$^+$ T cells leads to expanded antitumor cytotoxic CD8$^+$ T-cell responses (9). Most antitumor T cells described to date seem to recognize nonmutated self-antigens.

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This requires these cells to escape thymic deletion, and indeed T cells recognizing self-antigens can be detected in the peripheral blood of healthy individuals (10–12). Furthermore, $CD4^+$ T cells expressing high-affinity antigen receptors specific for self-antigens may be positively selected as $Foxp3^+$ regulatory T cells (Treg; ref. 13). Many questions remain over the role of $CD4^+$ T-cell responses to tumor antigens. Are they present in healthy subjects and if so, does gender or increasing age affect these responses? Do they impede tumor growth, or does progressive tumor growth impinge on these responses through mechanisms of tolerance, anergy, deletion, or regulation?

The role of naturally induced IFN-γ–producing $CD4^+$ T-cell responses in the pathogenesis of colorectal cancer is unclear. Furthermore, previous attempts to measure these responses without the addition of interleukin (IL)-2 were often disappointing. To address some of these fundamental questions, $CD4^+$ T-cell responses from patients awaiting surgical resection of a primary colorectal tumor were analyzed and compared with age-matched healthy donors for the breadth and magnitude of response to the TAA 5T4. To measure these T-cell responses, overlapping peptide pools covering the entire protein were used and T cells were cultured for 14 days with IL-2. In particular, we wished to examine how the range of epitopes recognized, and the magnitude of each epitope-specific T-cell response, compared with tumor stage (obtained later after resection). To our surprise, all healthy controls showed robust T-cell responses to multiple epitopes, and these responses were steadily diminished in patients with worsening tumor stage. *In vitro* depletion experiments and analyses after surgical resection suggested that Tregs were responsible for inhibiting measured responses. This led us to test the hypothesis in a proof-of-principle pilot study in patients with metastatic colorectal cancer that depleting Tregs *in vivo* would release antitumor T-cell responses and perhaps impinge on the natural history of these metastases. Collectively, these data offer considerable insight into the influence of Tregs and tumor burden on $CD4^+$ T-cell responses and progression of colorectal cancer and suggest a potential for future noninvasive screening strategies based on peripheral blood samples, as well as novel immunotherapeutic regimens.

Materials and Methods

Sample groups

Peripheral blood samples were obtained from 27 patients with colorectal cancer no more than 7 days before primary colorectal tumor resection (patient characteristics are detailed in Supplementary Table S1). Resected colon specimens from these patients were analyzed for tumor size, invasive status, and lymph node involvement. Immune responses were measured against tumors that were staged in two ways. First, the clinicopathologic classification using clinical data, radiologic imaging, macroscopic findings at surgical resection, and histology: stage I, confined to the wall of the colon (or rectum); stage II, penetrated serosal surface; stage III, invaded local structures or spread to lymph nodes; and stage IV, distant metastatic spread. Second, the microscopic histologic staging of the tumor: T1, penetrated muscularis mucosa into the submucosa; T2, penetrated into muscularis propria; T3, penetrated through muscularis propria but not through the serosal surface; T4, invaded through the serosal outmost layer of bowel.

Peripheral blood samples from 17 healthy age-matched donors were used as controls. Samples of placenta were obtained from women undergoing elective cesarian sections and small samples of inflamed colon from patients with Crohn disease or ulcerative colitis were obtained from resected colon specimens. In addition, 10 patients with metastatic colorectal cancer, who were enrolled as part of a phase II clinical trial (ISRCTN54669986) to deplete Tregs using metronomic low doses (50 mg twice daily) of cyclophosphamide, had their antitumor responses measured. Informed consent and HLA type (Welsh Transplantation and Immunogenetics Laboratory) were obtained from all participants. Local ethical approval was granted for the study by the Bro Taf Local Research Ethics Committee.

Antigens

Forty-one 20mer peptides overlapping by 10 amino acids covering the entire human 5T4 protein were synthesized by Fmoc chemistry to more than 95% purity (GL Biochem; Fig. 1A). The peptides were divided into 13 pools (Fig. 1B). Whole 5T4 protein was produced as previously described (14). Tuberculin purified protein derivative (PPD; Statens Serum Institut) and phytohemagglutinin (PHA; Sigma-Aldrich) were used as controls. All antigens were used at a final concentration of 10 μg/mL.

Lymphocyte purification and culture

Peripheral blood mononuclear cell (PBMC) purification and growth of short-term lines were conducted as described previously (15). Briefly, PBMC were isolated from heparinized blood by centrifugation over LymphoPrep (Axis-Shield). The cells were washed and resuspended in OpTmizer SFM (Life Technologies) supplemented with 5% batch-tested, pooled human AB serum, L-glutamine, and penicillin/streptomycin. Triplicate lines for each peptide pool [2×10^5 cells/well of 96-well plate (Nunc)] were cultured for 14 days and supplemented with 10 μL of CellKine media (Helvetica Healthcare) on day 3 and with fresh media containing 20 IU/mL IL-2 on days 6 and 9. The effect of Treg depletion from PBMC on antigen-specific IFN-γ production was assessed in parallel assays using magnetic separation with magnetic-activated cell sorting (MACS) CD25 microbeads II (Miltenyi-Biotec) to deplete $CD25^{hi}$ cells, as previously described (7).

ELISpot assays

Polymer-backed 96-well filtration plates (MAIP-S-4510; Millipore) were used for all enzyme-linked immunospot (ELISpot) assays. Antibodies were obtained from Mabtech, and the ELISpot assay was developed using the Alkaline Phosphatase Substrate Kit from Bio-Rad. The concentrations of antibodies used and washing steps were according to the manufacturer's instructions; all antibody incubations were in 50 μL/well. Cells were pooled from triplicate wells in identical culture conditions, washed, resuspended, and counted before being plated with or without the corresponding 5T4 peptide pool for direct comparison. The plate was incubated at 37°C, 5% CO_2 for 18

A

No.	Sequence	No.	Sequence	No.	Sequence
1	MPGGCSRGPAAGDGRLRLAR	15	LPSLRQLDLSHNPLADLSPF	29	LQGLPHIRVFLDNNPWVCDC
2	AGDGRLRLARLALVLLGWVS	16	HNPLADLSPFAFSGSNASVS	30	LDNNPWVCDCHMADMVTWLK
3	LALVLLGWVSSSSPTSSASS	17	AFSGSNASVSAPSPLVELIL	31	HMADMVTWLKETEVVQGKDR
4	SSSPTSSASSFSSSAPFLAS	18	APSPLVELILNHIVPPEDER	32	ETEVVQGKDRLTCAYPEKMR
5	FSSSAPFLASAVSAQPPLPD	19	NHIVPPEDERQNRSFEGMVV	33	LTCAYPEKMRNRVLLELNSA
6	AVSAQPPLPDQCPALCECSE	20	QNRSFEGMVVAALLAGRALQ	34	NRVLLELNSADLDCDPILPP
7	QCPALCECSEAARTVKCVNR	21	AALLAGRALQGLRRLELASN	35	DLDCDPILPPSLQTSYVFLG
8	AARTVKCVNRNLTEVPTDLP	22	GLRRLELASNHFLYLPRDVL	36	SLQTSYVFLGIVLALIGAIF
9	NLTEVPTDLPAYVRNLFLTG	23	HFLYLPRDVLAQLPSLRHLD	37	IVLALIGAIFLLVLYLNRKG
10	AYVRNLFLTGNQLAVLPAGA	24	AQLPSLRHLDLSNNSLVSLT	38	LLVLYLNRKGIKKWMHNIRD
11	NQLAVLPAGAFARRPPLAEL	25	LSNNSLVSLTYVSFRNLTHL	39	IKKWMHNIRDACRDHMEGYH
12	FARRPPLAELAALNLSGSRL	26	YVSFRNLTHLESLHLEDNAL	40	ACRDHMEGYHYRYEINADPR
13	AALNLSGSRLDEVRAGAFEH	27	ESLHLEDNALKVLHNGTLAE	41	YRYEINADPRLTNLSSNSDV
14	DEVRAGAFEHLPSLRQLDLS	28	KVLHNGTLAELQGLPHIRVF		

B

PP	1	2	3	4	5	6
7	1	2	3	4	5	6
8	7	8	9	10	11	12
9	13	14	15	16	17	18
10	19	20	21	22	23	24
11	25	26	27	28	29	30
12	31	32	33	34	35	36
13	37	38	39	40	41	

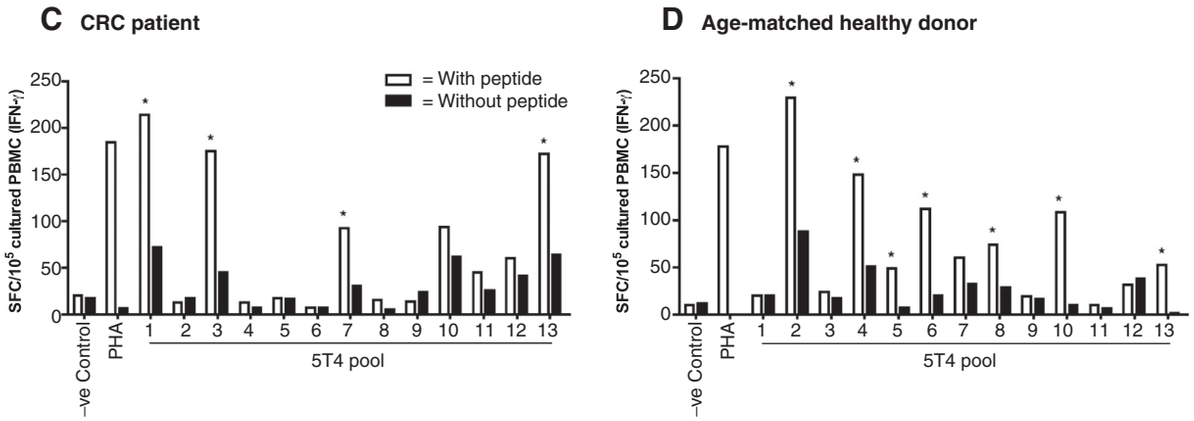

Figure 1. Culturing PBMC in 5T4 peptide pools for 14 days enriches for measurable 5T4-specific T-cell responses using IFN-γ ELISpot. A, 41 20mer peptides, overlapping by 10 amino acids and spanning the entire 5T4 protein, were used to determine epitope-specific T-cell responses. B, each one of the 41 peptides was placed in two peptide pools, containing between five and seven peptides as indicated, to allow for easier identification of peptide-specific responses. Freshly isolated PBMC from a representative colorectal cancer patient (C) or healthy donor (D) were cultured with peptide pools and IL-2 as detailed in Materials and Methods. After 14 days of culture, IFN-γ production to the 5T4 peptide pools was enumerated from T cells by ELISpot assay (see also Supplementary Fig. S1). *, Positive response, defined as a minimum of 20 SFC per 10^5 cultured cells, after subtraction of the background, and an increase of at least 50% above background. CRC, colorectal cancer.

to 24 hours. Cytokine-producing T cells were enumerated at the single-cell level by counting the number of spots per well using an automated ELISpot plate reader (AutoImmun Diagnostika GMBH). Positive responses were identified as having at least 20 spot-forming cells (SFC) per 10^5 cultured cells, after subtraction of the background, and an increase of at least 50% above background.

T-cell cloning

To generate 5T4-specific $CD4^+$ T-cell clones, T-cell lines were stimulated with the putative single 5T4 epitope and cultured for a further 14-day period. 5T4-positive cell lines were identified using the IFN-γ ELISpot assay. $CD4^+$ cells were negatively sorted using the MACS $CD4^+$ T Cell Isolation Kit II (Miltenyi-Biotec) as per the manufacturer's instructions. Isolated cells were resuspended in T-cell clone media comprising OpTmizer CTS (Invitrogen), 10% Human AB Serum, 20 U/mL IL-2, 2 μg/mL PHA, and 2×10^5 irradiated allogeneic PBMC, and cloned by limiting dilution in 96-well plates (Nunc). Plates were analyzed for clones 2 weeks later, and positive clones were identified using the IFN-γ ELISpot. 5T4 T-cell clones were expanded for further use.

Flow cytometry

Freshly isolated PBMC samples were resuspended in PBS at a concentration of 2 to 5×10^6 cells/mL in 96-well plates (Nunc). Cells were initially stained with the amine-reactive viability dye Live/Dead fixable Aqua (Life Technologies) for 15 minutes in the dark at room temperature. Subsequently, cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (PBS, 2% bovine serum albumin), then resuspended in 30 μL of FACS buffer for surface marker staining. The directly conjugated monoclonal antibodies (mAb; all from BioLegend), CD3-PerCPCy5.5, CD4-PE, CD8-FITC, and CD25-

BV421, were added to the cells and allowed to incubate for 20 minutes in the dark at 4°C. Following two more wash steps with FACS buffer, cells were permeabilized and fixed using a fixation/permeabilization kit (eBioscience), and incubated for 40 minutes at 4°C. Cells were then washed using 1× permeabilization buffer, and Fc receptors were blocked using rat serum for 15 minutes at 4°C. Foxp3-APC (eBioscience) was then added and allowed to incubate for 30 minutes in the dark at 4°C. The cells were then washed once with Perm buffer and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich). Fixed cells were stored in the dark at 4°C until acquisition on a BD FACSCanto II. Data were analyzed using FlowJo software version 9.4 (TreeStar Inc.) and gates were drawn based on fluorescence-minus-one (FMO) controls.

Immunohistochemistry

Fresh tissue samples obtained from surgery were immediately embedded in optimum cutting temperature (OCT) compound, frozen in liquid nitrogen, and stored in −80°C freezers until use. Five-micrometer-thick sections were cut, placed on slides, and fixed in acetone or 4% paraformaldehyde. Slides were incubated with 1.5 μg/mL primary anti-5T4 antibody (H8; Oxford Biomedica) overnight at 4°C, alongside a mouse immunoglobulin G1 (IgG1)–negative control antibody (BD Biosciences). Following wash steps, 3,3′–diaminobenzidine (DAB) solution was added for 10 minutes before counterstaining with hematoxylin. Slides were finally dehydrated through graded alcohols before viewing on a microscope.

Statistical analysis

GraphPad Prism Version 5 and Microsoft Office Excel 2007 were used for statistical analyses. Unpaired *t* tests were used to look at differences between normally distributed data; χ^2 and Fisher exact test were used to compare proportions of responders in patients and controls; linear regression was used to test the relationship between 5T4 responses and either Treg frequencies or tumor stages.

Results

5T4-specific T-cell responses are detectable in the memory pool of both colorectal cancer patients and healthy controls

Using whole protein antigen, we have previously detected *ex vivo* 5T4-specific T-cell responses in patients with colorectal cancer (6, 7), but not in healthy donors. The low frequency measured *ex vivo* renders detailed characterization of the response difficult. To facilitate the characterization of these responses, we established short-term T-cell cultures using PBMC from HLA-typed subjects, stimulated with pools of 20mer 5T4 peptides spanning the entire protein (Fig. 1A and B). Culturing PBMC led to expansion of antigen-specific T cells to more readily detectable levels, allowing the frequency of cognate Th1 cells in these short-term lines to be detected by IFN-γ release. Previously we have found that these cultured responses reflect a central memory population of T cells compared with an effector population identified by *ex vivo* assays (15). This change in methodology may account for why we now identify a population of 5T4-specific T cells in both patients (Fig. 1C) and healthy controls (Fig. 1D). The experiment using cells from one representative patient (Fig. 1C) showed that pools 1, 3, 7, and 13 were positive, suggesting that candidate epitopes were contained within peptides 1, 3, 37, and 39 (see matrix; Fig. 1B). These responses were typically robust, reaching frequencies of >200 T cells/10^5 cultured cells, i.e., >200 SFC/10^5, enabling further analysis as detailed below. We were surprised, considering the absence of *ex vivo* responses in healthy donors, to find equal/superior cultured responses in a healthy donor (Fig. 1D).

Selective reduction in 5T4-specific T-cell responses stratifies patients with more advanced cancer and is associated with metastatic recurrence at 12 months

The robustness of this approach enabled us to ask questions about the presence and frequency of 5T4-specific responses in healthy donors compared with patients with colorectal cancer with differing tumor stages. 5T4 ELISpot data from 17 healthy donors and 27 patients with colorectal cancer were used to calculate the total T-cell responses to all 5T4 peptides and the average response per 5T4 epitope. To distinguish between putative epitopes, distinct responses were defined as individual responses to nonoverlapping peptides; responses to overlapping peptides were defined as containing one putative epitope. If doubt remained, the lines were tested against individual peptides. The total response to the 5T4 peptide pools was significantly diminished in patients who were subsequently identified as having tumors that had penetrated the serosal surface of the bowel at operation and invaded local lymph nodes (i.e., stage III) as shown in Fig. 2A (stage I vs. stage III 548.1 ± 116.2 SFC/10^5 vs. 210.1 ± 72.73 SFC/10^5; $P = 0.017$) and concordantly between T1- and T3-graded patients with colorectal cancer (607.0 ± 163.4 SFC/10^5 vs. 258.4 ± 70.5 SFC/10^5; $P = 0.041$).

Very similar findings were obtained when comparing 5T4 responses on a response per-epitope basis (Fig. 2B). Healthy donors showed superior responses to patients with increasingly advanced tumors (healthy donors vs. stage III: 127.4 ± 13.1 SFC/10^5 vs. 48.5 ± 15.8 SFC/10^5, $P = 0.0006$; and healthy donors vs. T3: 127.4 ± 13.1 SFC/10^5 vs. 46.7 ± 13.4 SFC/10^5, $P = 0.0002$). Again, anti-5T4 T-cell responses decreased with tumor progression, significantly between stage I and III patients (130.1 ± 26.9 SFC/10^5 vs. 48.5 ± 15.8 SFC/10^5; $P = 0.011$); T1- and T3-graded patients (120.8 ± 6.3 SFC/10^5 vs. 46.7 ± 13.4 SFC/10^5; $P = 0.011$), and even between T2- and T3-graded patients (124.7 ± 38.3 SFC/10^5 vs. 46.7 ± 13.4 SFC/10^5; $P = 0.024$).

These data show a steady decrease in the responsiveness of T cells to 5T4 measured by IFN-γ production in patients with increasingly advanced colorectal tumors. Despite this, T-cell immunity to the recall antigen, PPD, was unaffected (Fig. 2C); thus, there does not seem to be nonspecific immunosuppression, as we and others have previously noted (6, 16). In the cohort of patients tested, 6 of 13 with local lymph node spread produced no detectable 5T4 T-cell response postculture, whereas every patient whose tumor was contained to the bowel wall produced a 5T4 response (106.7 ± 19.1 SFC/10^5 vs. 48.5 ± 15.8 SFC/10^5; $P = 0.027$; Fig. 2D). However, no overall

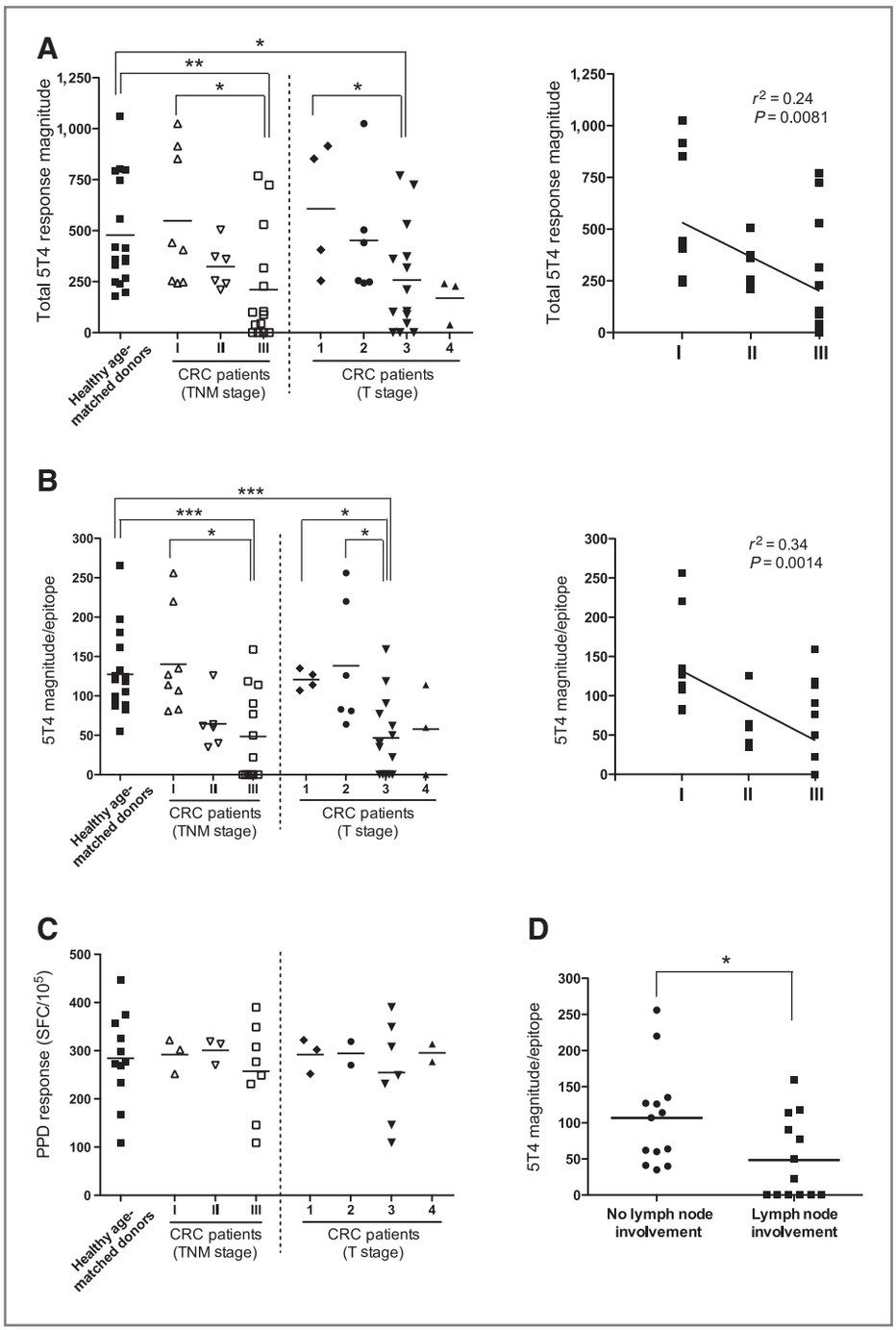

Figure 2. Anti-5T4 T-cell responses steadily decline in patients with colorectal cancer (CRC) with more advanced disease. A, cultured T-cell IFN-γ production to the 5T4 peptide pools was monitored after 14 days and defined in terms of the overall number of IFN-γ–producing 5T4-specific T cells to all peptide pools per 10^5 cultured PBMC (total 5T4 response magnitude) or B, the number of IFN-γ–producing 5T4-specific T cells to each putative 5T4 epitope per 10^5 cultured PBMC (5T4 magnitude/epitope). Only positively identified responses are included in the analysis, as defined previously. C, the response to the recall antigen tuberculin PPD was compared between healthy donors and patients with colorectal cancer after 14 days in culture. Data are expressed as the number of IFN-γ–producing cells (i.e., SFC) per 10^5 cultured PBMC. D, the 5T4 magnitude/epitope generated was compared between patients with colorectal cancer with and without histopathologically confirmed localized lymph node involvement. Significant differences are indicated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

difference was noted between pathologically confirmed stage III and TNM N1/N2–graded colorectal cancer tumors (data not shown), indicating that tumor spread to the apical lymph node does not result in a further reduction in 5T4-specific T-cell responsiveness.

Responses in all healthy controls (17 of 17) were robust, with a highly significant difference between healthy controls and patients with advanced cancer (healthy donors vs. stage III: 478.1 ± 64.3 SFC/10^5 vs. 210.1 ± 72.7 SFC/10^5, $P = 0.0097$; and healthy donors vs. T3: 478.1 ± 64.3 SFC/10^5 vs. 258.4 ± 70.5 SFC/10^5, $P = 0.028$). At a cutoff level of <200 SFCs/10^5, 0% (0 of 17) of healthy controls vs. 30% (8 of 27) of patients show such weak or absent responses ($P = 0.031$, Fisher exact test). Equally, with a cutoff level of <75 SFCs/10^5/5T4 epitope, 6% (1 of 17) of healthy controls versus 41% (11 of 27) of patients show poor responses ($P = 0.0003$, Fisher exact test).

Twelve-month outcome data were available from 13 of 14 patients with stage III tumors, which include 8 of these patients with low-level total 5T4 responses (<200 SFCs). Five of the patients had developed disease recurrence or metastatic disease, and of these patients, 80% (4 of 5) showed low-level (<200 SFCs) responses preoperatively (data not shown).

5T4 responses are CD4$^+$ T cells restricted by HLA-DR antigens

5T4 peptide-positive CD4$^+$ T-cell lines were further expanded and cloned as described in Materials and Methods; examples derived from a colorectal cancer patient (Supplementary Fig. S1A) and a healthy donor (Supplementary Fig. S1B) are shown. T-cell clones were CD4$^+$ and restricted by HLA-DR antigens [restriction mapping using matched/mismatched antigen-presenting cells (APC) showed HLA-DR1 in Supplementary Fig. S1A and HLA-DR4 in Supplementary Fig. S1B]. Addition of whole 5T4 protein-pulsed autologous irradiated APCs revealed the natural presentation of these 5T4 epitopes resulting in activation and IFN-γ production by these clones. As with other CD4$^+$ T-cell clones/lines we have tested (15, 17), they were also able to produce IL-10 after peptide stimulation, a possible result of repeat T-cell receptor (TCR) triggering of these cells, used as a mechanism to control excessive immune responses (18).

The influence of age and HLA-DR antigens on 5T4 T-cell responses

Increased age has been associated with a decline in T-cell function leading to the concept of immune senescence and susceptibility to infectious diseases and cancer (19). We have shown that healthy age-matched donors produce better responses than patients with colorectal cancer. We also found that patients aged 60 years or younger showed better 5T4 responses on a per-epitope basis than patients aged 80 years or older (Supplementary Fig. S2A: 120.1 ± 21.7 SFC/10^5 vs. 42.5 ± 18.2 SFC/10^5; $P = 0.03$). However, this finding was not mirrored in the total 5T4 responses (Supplementary Fig. S2B). There was no influence of age on the magnitude of response found in healthy controls (data not shown). Overall, age of subject has little effect on measured CD4$^+$ antitumor responses. Furthermore, these data did not show an effect of HLA-DR subtype or homo-/heterozygosity on the breadth or magnitude of CD4$^+$ T-cell responses measured (data not shown).

The influence of Tregs on 5T4 T-cell responses

Tregs have been shown to actively impinge on 5T4-specific antitumor T-cell responses (6, 7, 12). It is also well documented that patients with cancer have increased frequencies of Tregs, as denoted by Forkhead box P3 (Foxp3) expression (20). We have shown that surgical resection of colorectal cancer reduces the proportion of peripheral blood–derived Tregs (6). Five patients with low-level preoperative 5T4 responses were assessed 6 to 18 months after surgery. All 5 patients produced measurable increases in total IFN-γ production to 5T4 peptides (preoperative responses; 107.6 ± 40.6 SFC/10^5 vs. postoperative responses; 267.4 ± 71.4 SFCs/10^5 cultured cells; $P = 0.058$; Fig. 3A), and 4 of 5 patients had increased anti-5T4 T-cell responsiveness on a per-epitope basis (preoperative responses; 29.5 ± 13.0 SFC/10^5 vs. postoperative responses; 95.6 ± 22.2 SFC/10^5; $P = 0.038$; Fig. 3B).

Using blood samples from the same cohort of patients with colorectal cancer that were used to measure anti-5T4 T-cell responses, we analyzed the proportion of CD4$^+$ T cells that expressed Foxp3 by flow cytometry to determine whether increased numbers of Tregs correlated with the preoperative reduction in 5T4 responses. The proportion of Tregs in PBMC of 10 patients with metastatic colorectal cancer (stage IV) was also analyzed. Indeed, the proportion of Tregs was most significantly increased in the PBMC of patients with colorectal cancer with more advanced disease and concomitantly reduced T-cell responses (stage III vs. healthy donors: 10.8% ± 0.8% vs. 7.8%

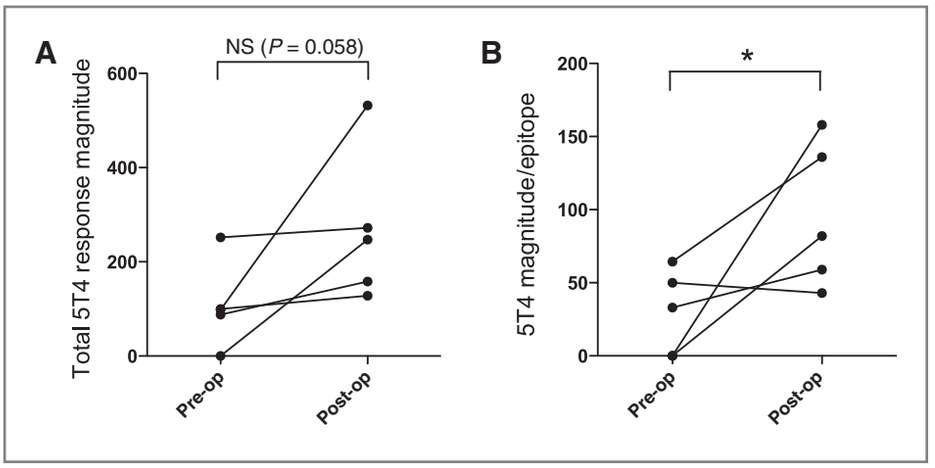

Figure 3. The effect of colorectal tumor resection on anti-5T4 T-cell responses. A, 5 patients with colorectal cancer (1 stage II and 4 stage III) with relatively poor preoperative 5T4-specific IFN-γ$^+$ T-cell responses (i.e., total 5T4 response magnitude <250 and 5T4 magnitude/epitope <75) were analyzed for total 5T4 responsiveness and B, average 5T4 responsiveness per epitope after surgery. Freshly isolated PBMC samples were stimulated with the 13 5T4 peptide pools as previously described, cultured for 14 days before analysis of positive 5T4-specific IFN-γ$^+$ responses. Significant differences indicate the results of a paired *t* test; $^*P < 0.05$.

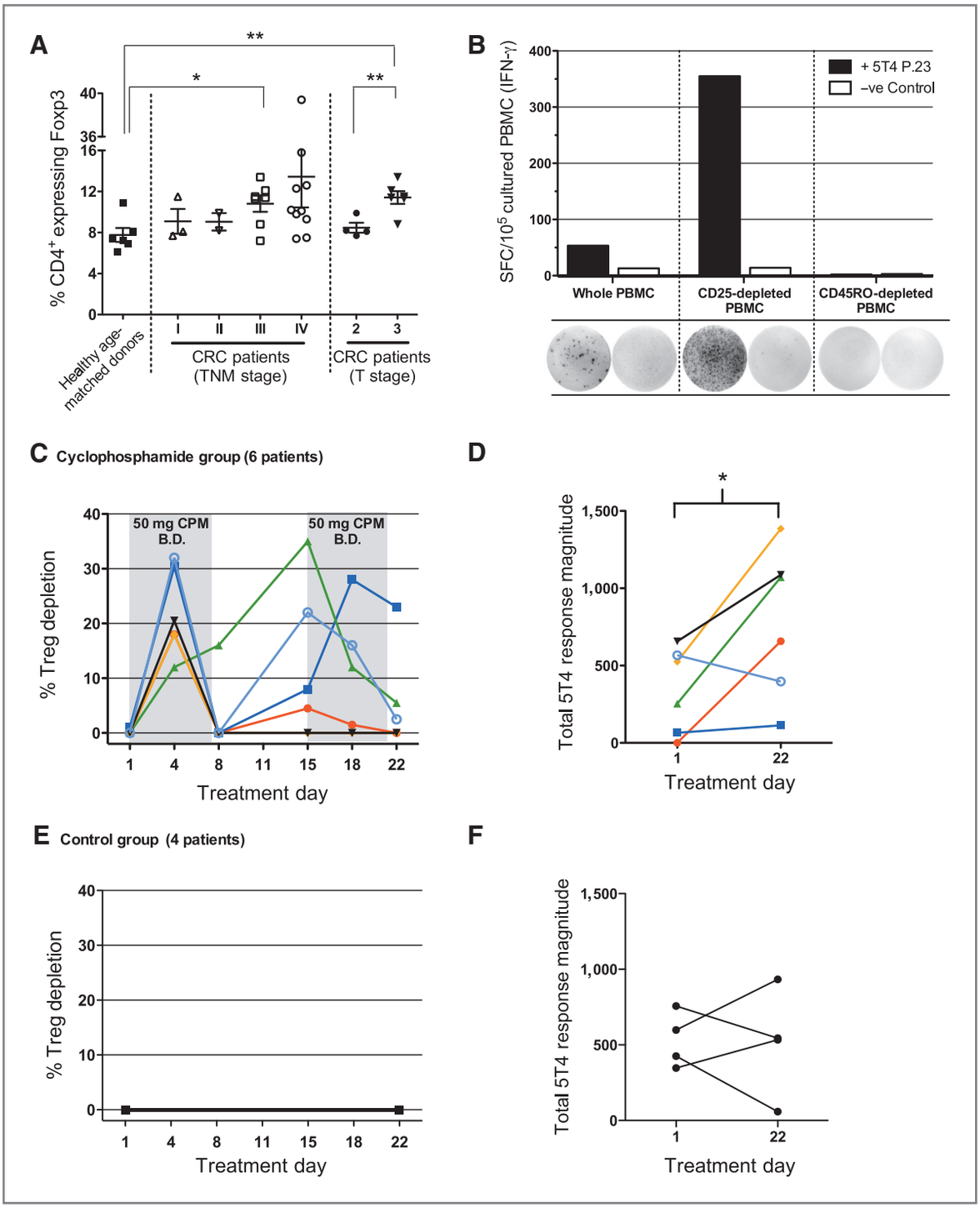

Figure 4. Increased Foxp3$^+$ Treg proportions among patients with colorectal cancer (CRC) can be reduced using low-dose cyclophosphamide (CPM), resulting in enhanced anti-5T4 T-cell responses. Freshly isolated PBMC from patients with colorectal cancer and healthy age-matched controls were stained with fluorescence-conjugated mAb to CD3, CD4, and Foxp3 and assessed for the proportion of live CD4$^+$ T cells that expressed intracellular Foxp3 by FACS. A, results from patients with colorectal cancer were correlated to histopathologic tumor score and significant differences are indicated; *, $P < 0.05$; **, $P < 0.01$. Individual 5T4 peptide-specific IFN-γ T-cell responses can be enhanced *in vitro* by depleting CD25$^+$ T cells before culture in identical conditions. These responses are completely abrogated by removal of CD45RO$^+$ memory T cells. B, an example of IFN-γ responses to 5T4 peptide 23 in one individual. C, 6 metastatic (stage IV) patients with colorectal cancer were given 50 mg twice daily (B.D.) of cyclophosphamide at indicated time points (gray bars) and Treg proportion among peripheral blood was analyzed throughout. D, corresponding measurements of total 5T4 response were taken before (day 1) and after (day 22) treatment, resulting in a significant increase in the overall anti-5T4 response in 6 patients taking cyclophosphamide; $^*P < 0.05$. E and F, the same analysis was conducted in another 4 metastatic patients with colorectal cancer who did not receive any treatment over the same time period.

± 0.68%; $P = 0.016$; and T3 vs. healthy donors: 11.4% ± 0.6% vs. 7.8% ± 0.7%; $P = 0.0026$; Fig. 4A). Furthermore, the proportion of Tregs increased with tumor progression in this cohort (T2 vs. T3: 8.48% ±0.49% vs. 11.42% ± 0.61%; $P = 0.0089$). Thus, Tregs could account for diminished responses in more advanced tumors. Indeed, responses could be markedly enhanced by the initial depletion of $CD25^{hi}$ T cells before culture (Fig. 4B), indicative of Treg involvement in suppressing 5T4-specific responses in certain cases, as suggested in previous *ex vivo* analyses (7, 12). In addition, these cultured responses were confined to the $CD45RO^+$ T cells (Fig. 4B), indicating that 5T4-specific responses are found within the antigen-experienced memory T-cell pool.

Targeting Tregs *in vivo* with cyclophosphamide restores 5T4-specific IFN-γ^+ T-cell responses and tumor control

The results above suggested that the loss of 5T4-specific IFN-γ^+ $CD4^+$ T cells was due to the effect of Tregs *in vivo*, and that this loss may contribute to poor tumor control. To test this hypothesis, we sought to determine whether modulation of Tregs *in vivo* might improve T-cell responses to 5T4 in patients with colorectal cancer. For this purpose, a pilot proof-of-principle study was carried out recruiting 10 cancer patients with metastatic colorectal cancer. Six patients were given metronomic low-dose cyclophosphamide, as previously described (21), in an attempt to reduce the proportion of $Foxp3^+$ Tregs within the $CD4^+$ T-cell population. All 6 patients exhibited a transient reduction in the proportion of peripheral blood–derived Tregs by day 22 (Fig. 4C), in marked contrast to none of the four controls not given cyclophosphamide (Fig. 4E). Five of 6 (83%) patients given cyclophosphamide produced a significantly enhanced anti-5T4 response at day 22 in terms of total number of IFN-γ–producing T cells to all 5T4 peptides (Fig. 4D; day 1 vs. day 22: 344.7 ± 113.3 SFC/10^5 vs. 785.3 ± 195.8 SFC/10^5; $P = 0.025$). There was no overall change in the T-cell responses of 4 patients who were not given cyclophosphamide (Fig. 4F). Thus, a partial reduction in Treg proportion seems to dramatically augment 5T4 T-cell reactivity, even when these responses are initially weak. Interestingly, in the one patient who had a relatively poor response to cyclophosphamide (i.e., no increase in the 5T4-specific responses) there was marked radiologic progression in the tumor metastases (Supplementary Fig. S3). None of the other patients in the cyclophosphamide group had clinical or radiologic evidence of tumor progression after 12 weeks (data not shown).

Discussion

Effective adaptive immunity is dependent on activation of helper $CD4^+$ T cells. In colorectal cancer, this process becomes restricted at the same time as there is growth and metastatic spread of cancerous tissue (6, 7), but understanding the relationship between cause and effect has proved challenging, in part due to the difficulties in identifying and accurately measuring tumor antigen–specific $CD4^+$ T-cell responses. The results in this article advance previous studies by both showing a robust method for measuring tumor antigen (i.e., 5T4)–specific T-cell responses, and showing that the magnitude of antitumor $CD4^+$ T-cell responses in the peripheral blood is inversely correlated to the stage of the colorectal cancer. The predictable loss of measurable magnitude and quality of tumor antigen–specific T-cell responses in patients and not in controls raises the possibility that it can be used as a biomarker to screen for and define patient populations for cancer therapies. Furthermore, the ability to measure these IFN-γ^+ $CD4^+$ T cells allowed us to identify the mechanism behind their loss, namely increasing encroachment by Tregs. Finally, this led us to explore the potential of Treg manipulation *in vivo* to generate increased tumor-specific T-cell responses. Although the numbers in the pilot study do not allow for a definitive conclusion, the data are compatible with the hypothesis that IFN-γ^+ $CD4^+$ T cells directly help control tumors *in situ*. Braumuller and colleagues also recently showed the importance of IFN-γ–producing $CD4^+$ T cells in controlling disease progression through driving cancer cell senescence (22).

We used the oncofetal antigen 5T4 as a candidate tumor-specific antigen. The use of 41 overlapping 20mer peptides allowed an unbiased approach to epitope mapping and negated the requirement for peptide-binding algorithm software, as 5T4 peptides with high HLA-binding affinities may not necessarily be those recognized *in vivo* due to thymic deletion. It was surprising that such robust responses to these peptides were found in healthy controls, although previous reports also described robust responses to some TAAs in cancer-free individuals (23). Collectively, these findings raised important questions about how and why these T cells are maintained at such a frequency in the $CD45RO^+$ memory pool. One possibility is transient upregulation of 5T4 in subjects with periods of inflammation of the colon (Supplementary Fig. S4). 5T4-specific responses, generated or maintained in this way, may actually participate in a continuing process of immunosurveillance of aberrant epithelial cells.

Cancer-bearing individuals have increased proportions of Tregs in the periphery (20, 24). We recently reported that the presence of colorectal cancer drives a population of Tregs that inhibit antitumor immune responses to TAAs [5T4 and carcinoembryonic antigen (CEA)], and although excision of the tumor led to normalization of Treg numbers, suppression of T-cell responses before resection was still associated with tumor recurrence at 12 months (6). Here, we have shown that decreased $CD4^+$ T-cell responses to 5T4 significantly correlated with a steadily worse histopathologic tumor grade (i.e., T1→T2→T3→T4), indicating that patients with more advanced tumors have a reduced capacity for T cell–mediated antitumor immunity. This was further substantiated by follow-up data showing that those patients with advanced stage III tumors, who had robust preoperative 5T4 T-cell responses, were less likely to develop metastatic disease 12 months after surgery (data not shown). In addition, resection of the colorectal tumor can itself help to enhance the 5T4 T-cell response. Given the role of 5T4 in facilitating metastatic spread (25, 26), it seems that 5T4 T-cell reactivity may reflect the ability of an individual to control cancerous disease from spreading.

Although inadequate or reduced anti-5T4 immune response is found in patients with colorectal cancer before surgery, as compared with responses in healthy controls, responses to

PPD remained unimpaired, confirming a tumor antigen–specific defect. In the cohort of patients with colorectal cancer we examined, a significant increase was noted in the proportion of peripheral blood–derived Tregs (i.e., $CD4^+$ T cells expressing the transcription factor Foxp3) as tumors became more advanced. Furthermore, removal of Tregs *in vitro* resulted in greater 5T4 responses to certain epitopes. This is mirrored *in vivo*, as using low-dose cyclophosphamide to reduce peripheral Treg proportion resulted in elevated anti-5T4 T-cell responses in 5 of 6 metastatic patients with colorectal cancer tested. We also found a decrease in 5T4 T-cell responses after culture if the initial proportion of Tregs was relatively higher. It is tempting to speculate that Tregs may be responsible for inhibiting the establishment of 5T4-specific effector T-cell activation and expansion over the short-term culture period, thus resulting in the diminished 5T4 responses identified in patients with more advanced tumors. Indeed, when we deplete $CD25^{hi}$ $CD4^+$ T cells (>90% $Foxp3^+$), 5T4 responses can be enhanced. Because Tregs are stimulated by cancer vaccines incorporating TAAs *in vivo* (27), this might inhibit effective cancer immunotherapy (28). Thus, targeting both effector T cells and Tregs could be crucial in maximizing the efficacy of antitumor immunotherapies.

We cannot rule out that the loss of Th1 responses may instead represent a skew in the cytokine profile of 5T4-specific T cells, perhaps to a detrimental Th2 or Th17 response, something that has been noted for T-cell responses to tumor antigens in patients with pancreatic cancer (16). Experiments incorporating antibodies to multiple cytokines (e.g., IL-4, IL-5, or IL-17 at the same time as IFN-γ) are currently under way to examine this possibility.

In summary, we provide evidence for the first time that loss of measurable antitumor $CD4^+$ T-cell responses in blood reflects both the presence and stage of colorectal cancer. These findings provide a basis for further studies to examine the usefulness of measuring T-cell responses as a disease biomarker, incorporating a panel of tumor antigens. It is possible that such a test might prove to be a noninvasive method of screening "healthy" populations for bowel cancer; the measurement of IFN-γ^+ T cells to specific disease-related antigens is already in clinical use for tuberculosis testing (29). These results also provide evidence for the key role Tregs play in controlling antitumor immune responses in colorectal cancer, and a rationale for planning further therapeutic approaches for boosting IFN-γ^+ T-cell responses in patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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