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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Keywords: colorectal cancer, CD4+ T cells, 5T4, epitopes, regulatory T cells.

Running Title: Anti-5T4 T cell responses in advanced cancer

Word Count: 4,663

Total Figures: 4

This work was funded by Cancer Research Wales. The authors declare no conflicts of interest.

Abbreviations Used: CRC, colorectal cancer; Foxp3, Forkhead Box P3; Treg, regulatory T cell; IFN-γ; Interferon-γ; ELISpot, enzyme-linked immunoSpot; PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell sorting; TAA, tumor-associated antigen, PPD, purified protein derivative; SFC, spot-forming cell.
Abstract

The relationship between the adaptive CD4+ T cell response and human cancer is unclear. The oncofetal antigen 5T4 is expressed on many human carcinomas, including colorectal cancer (CRC) cells, but has limited expression on normal tissues. We previously identified anti-5T4 CD4+ T cells in a proportion of CRC patients, 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 12-day in vitro culture period comparing patients pre-operatively (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 was only found in subjects with stage III CRC. 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 (Tregs). Using low-dose cyclophosphamide to reduce the proportion of Tregs in vivo resulted in increased anti-5T4 T cell responses in CRC patients. 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.
Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide, accounting for >500,000 deaths per year (1). Where possible, a colectomy to remove the primary tumor is performed, however 40-50% of these patients relapse or die from metastatic disease. Patient outcome strongly correlates with histopathological staging of the excised tumor; patients with a TNM stage I (Dukes’ A) tumor confined to bowel wall have a predicted 5 year survival >90%, stage II (Dukes’ B) tumor penetrated serosal surface of bowel ~80%; stage III (Dukes’ C) tumor spread to lymph nodes / adjacent organs ~50%; and stage IV (Dukes’ D) tumor with distant metastases ~10%. The extent of lymphocyte infiltration into tumors has been shown to be an independent prognostic marker demonstrated by increased intratumoral CD3+ T cells (2). Infiltrating CD8+ T cells are thought to eliminate tumor cells if receiving adequate CD4+ T cell help (3).

Tumor-associated antigens comprise common proteins that are markedly up-regulated 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 CRC (4); this tumor-specific antigen lends itself as a candidate target for tumor-directed immune responses. Indeed a recent report has demonstrated 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 CRC patients awaiting surgical resection (6, 7).
Cytotoxic T cells require CD4+ T cell help for both effector function and development of a memory population (3, 8). The adoptive transfer of anti-tumor CD4+ T cells leads to expanded anti-tumor cytotoxic CD8+ T cell responses (9). Most antitumor T cells described to date appear to recognize non-mutated self-antigens. 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 (Tregs) (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 CRC is unclear. Furthermore, previous attempts to measure these responses without the addition of 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 to age-matched healthy donors for the breadth and magnitude of response to the tumor-associated antigen 5T4. To measure these T cell responses, overlapping peptide pools covering the entire protein were used and T cells were cultured for 12-14 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 to tumor
stage (obtained later after resection). To our surprise, all healthy controls demonstrated 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 post 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 CRC 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 CRC and suggest a potential for future non-invasive screening strategies based on peripheral blood samples, as well as novel immunotherapeutic regimes.
Materials and Methods

Sample Groups

Peripheral blood samples were obtained from 27 CRC patients no more than 7 days prior to primary colorectal tumor resection (patient characteristics are detailed in Supplementary Table 1). 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. Firstly the clinicopathological classification using clinical data, radiological 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; stage IV: distant metastatic spread. Secondly the microscopic histological 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 Caesarian sections and small samples of inflamed colon from patients with Crohn's disease or ulcerative colitis were obtained from resected colon specimens. In addition, ten patients with metastatic colorectal cancer, who were enrolled as part of a phase II clinical trial (ISRCTN54669986) to deplete regulatory T cells using metronomic low-doses (50mg B.D.) of cyclophosphamide, had their antitumor responses measured. Informed
consent and HLA type (Welsh Transplantation and Immunogenetics Laboratory, Pontyclun, UK) 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 >95% purity (GL Biochem, Shanghai, China) (Figure 1A). The peptides were divided into 13 pools (Figure 1B). Whole 5T4 protein was produced as previously described (14). Tuberculin Purified Protein Derivative (PPD) (Statens Serum Institut, Denmark) and phtohaemagglutinin (PHA) (Sigma) were used as controls. All antigens were used at a final concentration of 10 μg/ml.

**Lymphocyte Purification and Culture**

PBMC purification and growth of short-term lines were performed as described (15). Briefly, PBMCs were isolated from heparinized blood by centrifugation over Lymphoprep (Axis-Shield, Oslo, Norway). The cells were washed and re-suspended in OpTmizer SFM (Invitrogen) supplemented with 5% batch-tested, pooled human AB serum, L-glutamine and penicillin / streptomycin. Triplicate lines for each peptide pool (2 x 10^5 cells per well of 96 well plate (Nunc)) were cultured for 12-14 days supplemented with 10 μl CellKine media (Helvetica Healthcare) on day 3, 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 MACS CD25 microbeads II (Miltenyi-Biotec, Germany) to deplete CD25<sup>hi</sup> cells, as previously described (7).

**ELISpot Assays**

Polymer-backed 96-well filtration plates (MAIP-S-4510) (Millipore, Moslheim, France) were used for all ELISpot assays. Antibodies were obtained from Mabtech (Natka, Sweden) and the ELISpot assay was developed using the alkaline phosphatase substrate kit from Bio-Rad (Hercules, California). 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, re-suspended 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<sub>2</sub> for 18-24 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<sup>5</sup> cultured cells, after subtraction of the background, and an increase of at least 50% above background.

**T cell Cloning**

To generate 5T4-specific CD4<sup>+</sup> T cell clones, T cell lines were stimulated with the putative single 5T4 epitope and cultured for a further 12-day period. 5T4-positive cell lines were identified using the IFN-γ ELISpot
assay. CD4+ cells were negatively sorted using MACS CD4+ T cell isolation kit II (Miltenyi-Biotec, Germany) as per 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 phytohaemagglutinin and 2x10^5 irradiated allogeneic PBMC, and cloned by limiting dilution in 96-well plates (Nunc). Plates were analyzed for clones two weeks later and positive clones 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 2-5 x 10^6 cells/ml in 96-well plates (Nunc). Cells were initially stained with the amine-reactive viability dye Live/Dead fixable Aqua (Invitrogen) for 15 minutes in the dark at room temperature. Subsequently, cells were washed twice in FACS buffer (PBS, 2% BSA), then resuspended in 30 μl FACS buffer for surface marker staining. The directly conjugated mAbs (all from BioLegend) CD3-PerCPCy5.5, CD4-PE, CD8-FITC, 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 1X 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 FMO controls.

**Immunohistochemistry**

Fresh tissue samples obtained from surgery were immediately embedded in OCT compound, frozen in liquid nitrogen and stored in -80°C freezers until use. 5 μm thick sections were cut, placed on slides and fixed in acetone or 4% PFA. Slides were incubated with 1.5 μg/ml primary anti-5T4 antibody (H8; Oxford Biomedica) overnight at 4°C, alongside a mouse IgG1 negative control antibody (BD). Following wash steps, DAB solution was added for 10 minutes, before counterstaining with haematoxylin. 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; χ² and Fisher’s exact test were use 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 CRC patients and healthy controls

Using whole protein antigen, we have previously detected *ex vivo* 5T4-specific T cell responses in patients with CRC (6, 7), but not in healthy donors. The low frequency measured *ex vivo* renders detailed characterization of the response difficult. In order to facilitate the characterization of these responses we establish short-term T cell cultures using PBMCs from HLA-typed subjects, stimulated with pools of 20mer 5T4 peptides spanning the entire protein (Figures 1A and 1B). 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 to 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 (Figure 1C) as well as healthy controls (Figure 1D). The experiment using cells from one representative patient (Figure 1C) demonstrated pools 1, 3, 7 and 13 were positive suggesting candidate epitopes are contained within peptides 1, 3, 37 and 39 (see matrix, Figure 1B). These responses were typically robust reaching frequencies of > 200 T cells / 10^5 cultured cells i.e. > 200 spot forming cells (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 (Figure 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 regarding the presence and frequency of 5T4-specific responses in healthy donors compared to CRC patients with differing tumor stages. 5T4 ELISpot data from 17 healthy donors and 27 CRC patients 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 non-overlapping 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 which had penetrated the serosal surface of the bowel at operation and invaded local lymph nodes (i.e. stage III) as shown in Figure 2A (stage I vs. stage III 548.1 ± 116.2 SFC/10⁵ vs. 210.1 ± 72.73 SFC/10⁵; P = 0.017) and concordantly between T1- and T3-graded CRC patients (607.0 ± 163.4 SFC/10⁵ vs. 258.4 ± 70.5 SFC/10⁵; P = 0.041).

Very similar findings were obtained when comparing 5T4 responses on a response per epitope basis (Figure 2B). Healthy donors demonstrated superior responses to patients with increasingly advanced tumors (HD vs.
stage III: 127.4 ± 13.1 SFC/10^5 vs. 48.5 ± 15.8 SFC/10^5, P = 0.0006; and HD 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 stage 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 demonstrate 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 (Figure 2C), thus there does not appear to be non-specific 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 post-culture, 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) (Figure 2D). However, no overall difference was noted between pathologically confirmed stage III and TNM N1 / N2 graded CRC 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 (17/17) were robust, with a highly significant difference between healthy controls and patients with advanced cancer (HD vs. stage III: 478.1 ± 64.3 SFC/10^5 vs. 210.1 ± 72.7 SFC/10^5, P = 0.0097 and HD vs. T3: 478.1 ± 64.3 SFC/10^5 vs. 258.4 ± 70.5 SFC/10^5, P = 0.028). At a cut off level of <200 SFCs/10^5, 0% (0/17) of healthy controls vs.
30% (8/27) of patients demonstrate such weak or absent responses (P = 0.031 Fishers’ exact test). Equally, with a cut off level of <75 SFCs/10^5/ 5T4 epitope, 6% (1/17) of healthy controls vs. 41% (11/27) of patients demonstrate poor responses (P = 0.0003 Fishers’ exact test).

Twelve-month outcome data were available on 13 of 14 patients with stage III tumors, which include these 8 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% (4/5) demonstrated low level (<200 SFCs) responses pre-operatively (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 CRC patient (Supplementary Figure 1A) and a healthy donor (Supplementary Figure 1B) are shown. T cell clones were CD4^+ and restricted by HLA-DR antigens (restriction mapping using matched/mismatched APCs showed HLA-DR1 in Supplementary Figure 1A; HLA-DR4 in Supplementary Figure 1B). 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 TCR triggering of these cells, employed 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 CRC patients. We also found that patients ≤ 60 years demonstrated better 5T4 responses on a per epitope basis than patients ≥ 80 years old (Supplementary Figure 2A: 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 Figure 2B). 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 demonstrate 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 Foxp3 expression (20). We have shown that surgical resection of CRC reduces the proportion of peripheral blood-derived Tregs (6). Five patients with low-level pre-operative 5T4 responses were assessed 6-18 months post-surgery. All 5 patients produced measureable increases in total IFN-γ production to 5T4 peptides
(pre-op responses; 107.6 ± 40.6 SFC/10^5 vs. post-op responses; 267.4 ± 71.4 SFCs / 10^5 cultured cells, P = 0.058) (Figure 3A), and 4/5 patients had increased anti-5T4 T cell responsiveness on a per-epitope basis (pre-op responses; 29.5 ± 13.0 SFC/10^5 vs. post-op responses; 95.6 ± 22.2 SFC/10^5, P = 0.038) (Figure 3B).

Using blood samples from the same cohort of CRC patients 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 pre-operative reduction in 5T4 responses. The proportion of Tregs in PBMC of ten patients with metastatic colorectal cancer (stage IV) was also analyzed. Indeed, the proportion of Tregs was most significantly increased in the PBMC of CRC patients with more advanced disease and concomitantly reduced T cell responses (stage III vs. HD: 10.8% ± 0.8% vs. 7.8% ± 0.68%; P = 0.016 and T3 vs. HD: 11.4% ± 0.6% vs. 7.8% ± 0.7%; P = 0.0026) (Figure 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 (Figure 4B), indicative of Treg involvement in suppressing 5T4-specific responses in certain cases, as suggested in previous ex vivo analyses (7, 12). Additionally, these cultured responses were confined to the CD45RO^+ T cells (Figure 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 CRC patients. For this purpose, a pilot proof-of-principle study was performed recruiting ten cancer patients with metastatic CRC. 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 six patients exhibited a transient reduction in the proportion of peripheral blood-derived Tregs by day 22 (Figure 4C), in marked contrast to none of the 4 controls not given cyclophosphamide (Figure 4E). Five out 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 (Figure 4D, Day 0 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 in the 4 patients not given cyclophosphamide (Figure 4F).

Thus, a partial reduction in Treg proportion appears 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. did not increase the 5T4-specific responses, there was marked radiological progression in the tumor metastases (Supplementary Figure 3). None of the other patients in the cyclophosphamide group had
clinical or radiological evidence of tumor progression after 12 weeks (data not shown).
Discussion

Effective adaptive immunity is dependent on activation of helper CD4+ T cells. In CRC 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 proven challenging, in part due to the difficulties in identifying and accurately measuring tumor antigen-specific CD4+ T cell responses. The results in this paper advance previous studies by both demonstrating a robust method for measuring tumor antigen (i.e. 5T4)-specific T cell responses, and showing that the magnitude of anti-tumor CD4+ T cell responses in the peripheral blood is inversely correlated to the stage of the CRC. 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 biomarkers to screen for and defined 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 et al. also recently demonstrated 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. Utilizing 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 tumor associated antigens in cancer-free individuals (23). Collectively, these findings raised important questions regarding how and why these T cells are maintained at such a frequency in the CD45RO+ memory pool. One possibility is transient up-regulation of 5T4 in subjects with periods of inflammation of the colon (Supplementary Figure 4). 5T4-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 CRC drives a population of Tregs that inhibit antitumor immune responses to tumor-associated antigens (TAAs) (5T4 and CEA), and although excision of the tumor lead to normalization of Treg numbers, suppression of T cell responses prior to resection was still associated with tumor recurrence at 12 months (6). Here, we have demonstrated that decreased CD4+ T cell responses to 5T4 significantly correlated with a steadily worse histopathological 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 pre-operative 5T4 T cell responses, were less likely to develop metastatic disease by 12-months post-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 appears 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 CRC patients pre-surgery compared to responses in healthy controls, responses to PPD remained unimpaired, confirming a tumor-antigen specific defect. In the cohort of CRC patients 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, since using low-dose cyclophosphamide to reduce peripheral Treg proportion resulted in elevated anti-5T4 T cell responses in 5 of 6 metastatic CRC patients 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 CD25hi CD4+ T cells (>90% Foxp3+), 5T4 responses can be enhanced. Since Tregs are stimulated by cancer vaccines incorporating TAAs in vivo (27), this might inhibit effective cancer
immunotherapy (28). Thus, targeting both effector and regulatory T cells 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 pancreatic cancer patients (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 underway 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 CRC. 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 non-invasive 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 TB testing (29). These results also provide evidence for the key role Tregs play in controlling antitumor immune responses in CRC, and a rationale for planning further therapeutic approaches for boosting IFN-γ+ T cell responses in patients.
Acknowledgments: We thank Dr. Emma Jones for assistance with immunohistochemistry and Dr. Audrey Yong for providing and interpreting patient CT scans.

Funding: This work was funded by Cancer Research Wales.

Author contributions: Research design: MS, AG and AG. Performed experimental work: MS, AB, KS, HB and RS. Provision of samples: TP, AC, TH, MD, SP and RH. Wrote paper: MS, AG and AG.

Competing interests: The authors have no conflicting financial interests.
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Figure Legends

Figure 1. Culturing PBMC in 5T4 peptide pools for 14-days enriches for measureable 5T4-specific T cell responses using IFN-\(\gamma\) 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 5-7 peptides as indicated, to allow for easier identification of peptide-specific responses. Freshly isolated PBMC from a representative CRC patient (C) or healthy donor (D) were cultured with peptide pools and IL-2 as detailed in methods. After 14 days of culture IFN-\(\gamma\) production to the 5T4 peptide pools was enumerated from T cells by ELISpot assay (see also supplementary figure 1). * indicates positive response, defined as a minimum of 20 spot-forming cells (sfc) per 10\(^5\) cultured cells, after subtraction of the background, and an increase of at least 50% above background.

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

Figure 3. The effect of colorectal tumor resection on anti-5T4 T cell responses. Five CRC patients (1 Stage II, 4 Stage III) with relatively poor pre-operative 5T4-specific IFN-\(\gamma^+\) T cell responses (i.e. total 5T4 response magnitude <250 and 5T4 magnitude / epitope <75) were analyzed for total 5T4 responsiveness (A) and average 5T4 responsiveness per epitope (B) post-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-\(\gamma^+\) responses. Significant differences indicate the results of a paired t-test; *\(p < 0.05\).

Figure 4. Increased Foxp3\(^+\) Treg proportions amongst CRC patients can be reduced using low-dose cyclophosphamide, resulting in enhanced anti-5T4 T cell responses. Freshly isolated PBMC from CRC patients 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. Results from CRC patients were correlated to histopathological tumor score (A). Individual 5T4 peptide specific IFN-γ T cell responses can be enhanced in vitro by depleting CD25+ T cells prior to culture in identical conditions. These responses are completely abrogated by removal of CD45RO+ memory T cells. An example of IFN-γ responses to 5T4 peptide 23 in one individual is shown (B). Six metastatic (Stage IV) CRC patients were given 50mg B.D. cyclophosphamide at indicated time-points (grey bars) and Treg proportion amongst peripheral blood was analysed throughout (C). 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 the six patients taking cyclophosphamide; *p < 0.05 (D). The same analysis was performed in another four metastatic CRC patients who did not receive any treatment over the same time period (E and F).
Figure 1

A

No. | Sequence
---|---
1 | MPGSCGSPGAPASDAGLRLAR
2 | AGDGRLRARLALVLLGWS
3 | LALVLLGWSVSSSPTSSASS
4 | SSSPTSSASSFSFSSAPLAS
5 | FSSSAPFLASAVSAQPPPLD
6 | AVSAQPLPDCPACPCECSE
7 | QCPALCEGSEAARTVKCVNR
8 | AARTVKCVNRLTETVPTDLP
9 | NLTEVPTDLPIYVRNRLFTG
10 | AYVRNLFITGQLAVLPAGA
11 | NQLAVPAGAFARRPPLAE
12 | FARRPPLAEALNLSRSLG
13 | AALNLSGSRLEVRAGAFEH
14 | DEVRAGAFEHPLSRQLDLS
29 | LQGLPHIRVF
30 | LDNPWVCDC
31 | HADMTWKLK
32 | EVGGKDLRLT
33 | LTMKMRNLVL
34 | NRDCPILPP
35 | DLDCPILPP
36 | SLQTSYFGL
37 | IVLALIGAFLLVLYN
38 | LLVLYNKG
39 | IKKWMHNRD
40 | ACRDMEFYH
41 | YRINCAPRNL
42 | KLSNLSLTVSTY
43 | LSNNLTVSTY
44 | YSVRLNTLH
45 | ESHLDEDNLK
46 | KVLHGNTLAE

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 |

C: CRC Patient

D: Age-Matched Healthy Donor

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Figure 2

A

B

C

D

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Figure 3

A  
Total 5T4 Response Magnitude

NS (P = 0.058)

Pre-Op  Post-Op

B  
5T4 Magnitude / Epitope

*  
Pre-Op  Post-Op
Figure 4

A

**% CD4+ Expressing Foxp3**

Healthy Age-Matched Donors

<table>
<thead>
<tr>
<th>Healthy Age-Matched Donors</th>
<th>CRC Patients (TNM Stage)</th>
<th>CRC Patients (T Stage)</th>
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</tbody>
</table>

B

**% Treg Depletion**

- Whole PBMC
- CD25-depleted PBMC
- CD45RO-depleted PBMC

C: Cyclophosphamide Group (6 patients)

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<td>30</td>
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</tbody>
</table>

D

**Total 5T4 Response Magnitude**

E: Control Group (4 patients)

F

**Total 5T4 Response Magnitude**
Cancer Immunology Research

Escalating regulation of 5T4-specific IFN-\(\gamma\)+ CD4+ T cells distinguishes colorectal cancer patients from healthy controls and provides a target for in vivo therapy

Martin Scurr, Anja Bloom, Tom Pembroke, et al.

_Cancer Immunol Res_ Published OnlineFirst September 17, 2013.

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Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-13-0035

Supplementary Material
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