Clinical Cancer Research

Modulation of Lymphocyte Regulation for Cancer Therapy: A Phase II Trial of Tremelimumab in Advanced Gastric and Esophageal Adenocarcinoma

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Abstract

Purpose: Cytotoxic T lymphocyte antigen 4 (CTLA4), a key negative regulator of T-cell activation, is targeted by the antibody tremelimumab to release potentially useful antitumor activity.

Experimental Design: This phase II trial investigated tremelimumab as a second-line treatment for patients with metastatic gastric and esophageal adenocarcinomas. Tremelimumab was given every 3 months until symptomatic disease progression. Safety, clinical efficacy, and immunologic activity were evaluated.

Results: Eighteen patients received tremelimumab. Most drug-related toxicity was mild; however, there was a single death due to bowel perforation that complicated colitis. Four patients had stable disease with clinical benefit; one patient achieved a partial response after eight cycles (25.4 months) and remains well on study at 32.7 months. Markers of regulatory phenotype, forkhead box protein 3 and CTLA4, doubled transiently in $CD4^+CD25^{high}$ lymphocytes in the first month after tremelimumab before returning to baseline. In contrast, CTLA4 increased in $CD4^+CD25^{low/negative}$ lymphocytes throughout the cycle of treatment. *De novo* proliferative responses to tumor-associated antigens 5T4 (8 of 18 patients) and carcinoembryonic antigen (5 of 13) were detected. Patients with a posttreatment carcinoembryonic antigen proliferative response had median survival of 17.1 months compared with 4.7 months for nonresponders ($P = 0.004$). Baseline interleukin-2 release after T-cell activation was higher in patients with clinical benefit and toxicity.

Conclusion: Despite the disappointing response rate of tremelimumab, one patient had a remarkably durable benefit for this poor-prognosis disease. *In vitro* evidence of enhanced proliferative responses to relevant tumor-associated antigens suggests that combining CTLA4 blockade with antigen-targeted therapy may warrant further investigation. *Clin Cancer Res; 16(5); 1662–72. ©2010 AACR.*

Gastric and esophageal adenocarcinomas are common tumors with poor prognoses. In 2006, age-standardized incidence in the United Kingdom was similar: 9.5 per 100,000 population for esophageal carcinoma and 8.9 per 100,000 population for gastric adenocarcinoma.[4] A minority of patients present with disease amenable to surgical cure, and even for these patients, the 5-year survival with combination chemotherapy and surgery is around 36% (1). For the majority who present with advanced disease, the median survival with combination cytotoxic chemotherapy is only 10 months (2). There is no standard second-line therapy. Although a number of active agents

have been investigated in this setting, there is no trial data confirming benefit over supportive care alone (3). Response rates for active salvage therapies are around 20%, but toxicity can be high (4). Durable responses are very rare. There is a clear need for alternative therapeutic agents.

There is, however, some evidence that esophagogastric cancer can respond to immunotherapy, and nonspecific immune activation is widely used in Japan and Korea as an adjuvant to surgery. Recent phase III trial data show a 12% 15-year survival benefit of adjuvant chemoimmunotherapy over chemotherapy alone (5–7). The necessity for evasion of immunosurveillance by a developing cancer is well established. This involves both "immunologic sculpting" (8) of the tumor, such as loss of human leukocyte antigen (HLA) expression or downregulation of tumor-associated antigens (TAA), and reciprocal changes within the patient's immune system, leading to a dominance of regulatory over activating mechanisms. Many cancer patients, for example, show an increased proportion of natural regulatory T lymphocytes (Treg) in both the tumor microenvironment and

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Translational Relevance

Gastric and esophageal adenocarcinomas are common cancers with poor prognoses. There is no standard second-line therapy, and durable responses are very rare. Clearly, there is a need for alternative therapeutic agents with some evidence that esophagogastric cancer can respond to immunotherapy. In this phase II trial, anti-CTLA4 antibody was used as a second-line therapy against advanced gastric and esophageal cancer, to release potentially useful antitumor activity. Four of 18 patients had stable disease with clinical benefit, and one of them developed a partial response after eight cycles. In this study, changes in lymphocyte regulatory phenotype and enhanced *in vitro* proliferative responses to two relevant tumor-associated antigens were detected after treatment. This enhanced tumor-specific proliferative response suggests that combining CTLA4 blockade with antigen-targeted therapy may warrant further investigation in this challenging setting.

the peripheral blood, which seems to correlate with survival (9, 10). Natural Tregs are characterized by their expression of the transcription factor forkhead box protein 3 (FoxP3) and constitutive expression of cell surface markers CD25 and cytotoxic T lymphocyte antigen 4 (CTLA4; refs. 11, 12). In health, they have a role in controlling autoimmunity (13); in cancer, they may suppress an antitumor response. Targeting immunoregulation could allow the immune system to respond toward even established cancer.

CTLA4 is a key negative regulator of T-cell activation. It is constitutively expressed on the cell surface of Treg and inducibly expressed on activated T lymphocytes and monocytes. It shares structural features with the costimulatory molecule CD28 and reciprocally targets the same receptor complex (CD80/86), for which it has a higher affinity (14). Upregulation of CTLA4 leads to reduced interleukin-2 (IL-2) production and IL-2 receptor expression, and arrest of T cells at the G_1 phase of the cell cycle (15). Preclinical models have explored CTLA4 as a target in the treatment of cancer (with blocking monoclonal antibodies; ref. 16) and autoimmunity (with CTLA4-Ig; ref. 17). Two fully humanized monoclonal antibodies, ipilimumab and tremelimumab, have shown clinical activity in man (18, 19).

Tremelimumab (CP-675,206, Pfizer) is a fully humanized anti-CTLA4 monoclonal antibody. It was developed as an IgG2 isotype to minimize complement activation and reduce the risk of cytokine storm; this has resulted in a long terminal phase half-life of 19.6 days, and a dosing schedule of once every 3 months. Early-phase studies in melanoma showed an improved toxicity profile of 15 mg/kg every 3 months compared with 10 mg/kg every 3 weeks, with equivalent objective response rates of around 10% (18).

This phase II, single-center, open-label, nonrandomized study investigated the use of tremelimumab as a second-line therapy for patients with metastatic gastric and esophageal adenocarcinomas. The primary end point was antitumor efficacy as described by the objective response rate. The secondary objectives were to further assess efficacy and safety in the clinic and to explore changes in lymphocyte phenotype and function with anti-CTLA4 treatment in the laboratory. Absolute counts of T-cell, B-cell, and natural killer cell populations were investigated in whole blood, and the regulatory phenotype was investigated in cryopreserved peripheral blood mononuclear cells (PBMC). To explore potential antitumor effects of any phenotypic changes, *in vitro* proliferative response to two relevant TAAs, 5T4 and carcinoembryonic antigen (CEA), were measured. 5T4 is an oncofetal antigen found in around half of gastric adenocarcinomas, for which expression has been correlated with poor prognosis (20, 21). CEA has established diagnostic and prognostic significance in colorectal cancer (22) and is also widely expressed in esophageal and gastric adenocarcinomas, being found in up to 86% of biopsies (23) and in the serum of nearly half of patients with advanced disease.

Patients and Methods

Study design and treatment. Tremelimumab was given by slow i.v. infusion at a dose of 15 mg/kg every 90 d (a cycle). The protocol was approved by the Medicines and Healthcare Products Regulatory Agency and Regional Research Ethics Committee and complies with the Helsinki Declaration. All patients signed an informed consent and were screened and treated at the Christie Hospital, Manchester, United Kingdom.

Patients. Eligible patients were adults (≥18 y) with biopsy-proven locally advanced or metastatic gastric or esophageal adenocarcinomas, who had previously received at least one cisplatin-based chemotherapy. They had an Eastern Cooperative Oncology Group performance status of 0 or 1; adequate bone marrow, hepatic, and renal function (defined as neutrophil count $≥1.5 × 10^9$ cells/L, platelets $≥100 × 10^9$ cells/L, hemoglobin ≥9.0 g/dL, total bilirubin ≤2 × Upper Limit of Normal, and creatinine ≤170 μmol/L); and had recovered from all prior treatment-related toxicities. Patients were excluded for any history of chronic inflammatory or autoimmune disease, or colitis of any origin, and also for prior or concurrent malignancy within the last 5 years, except basal cell carcinoma or *in situ* carcinoma of the uterine cervix. Those who had required systemic steroids in the last 4 wk, who were deemed likely to require them, or who had known brain metastases were also excluded.

Patients underwent prestudy computed tomography (CT) scan of the chest, abdomen, and pelvis within 28 d of enrollment. All patients had at least one measurable lesion by Response Evaluation Criteria in Solid Tumors (RECIST; ref. 24).

Clinical monitoring. Therapeutic response was assessed by CT scan using RECIST at the end of each cycle of treatment, by monthly serum tumor markers and by clinical review. The serum tumor markers investigated were CEA (reference range <3 μg/L) and CA19-9 (reference range

<31 units/mL). Safety was assessed using National Cancer Institute Common Toxicity Criteria (version 2; ref. 25). The protocol anticipated a potentially slow mechanism of drug action and allowed patients with asymptomatic disease progression after the first cycle of treatment to receive a single further dose.

Samples. Peripheral venous blood (45 mL) was collected pretreatment and at days 15, 30, 60, and 90 of each cycle. PBMCs were separated from whole blood using Lymphoprep (Axis-Shield) and cryopreserved in liquid nitrogen in heat-inactivated fetal calf serum with 10% dimethyl sulfoxide (VWR) for use in later assays.

Lymphocyte phenotype. Additional whole blood samples (3 mL) were collected into BD vacutainers (BD Bioscience) containing 5.4 mg potassium EDTA. Absolute counts of T-cell, B-cell, and natural killer cell populations were obtained contemporaneously by staining for the surface markers CD3 (anti-CD3 FITC; clone UCHT1), CD4 (anti-CD4 phycoerythrin (PE); clone 13B8.2), CD8 (anti-CD8 PE; clone B9.11), CD16 (anti-CD16 PE; clone 3G8), CD19 (anti-CD19 PE; clone J4.119), CD45 (anti-CD45 FITC; clone J33), CD56 (anti-CD56 PE; clone N901

N901-NKH-1; all Beckman Coulter), and CD25 (anti-CD25 PECy5; clone M-A251, BD Pharmingen). Briefly, 10 μL of relevant antibodies were added to a BD Trucount tube (BD Biosciences) plus 50 μL of whole blood. After 20-min incubation, red blood cells were lysed by the addition of 1 mL IOTest 3 lysing solution (Beckman Coulter). Following a further 10-min incubation, the cells were analyzed on the flow cytometer (FC500; Beckman Coulter) without washing. The lymphocyte population was selected for analysis using either forward scatter versus side scatter or CD45 versus side-scatter gating.

Cryopreserved PBMCs were used to investigate changes in regulatory lymphocyte phenotype. Intracellular FoxP3 staining was done using the human regulatory T-cell staining kit (eBioscience) and a FACSCalibur flow cytometer (Becton Dickinson). Briefly, cryopreserved PBMCs were carefully thawed, washed, resuspended at 3×10^6/mL, and stained with a cocktail of anti-CD4 FITC and anti-CD25 APC before permeabilizing and staining with anti-FoxP3 PE (clone PCH101). Results were analyzed using WinMDI 2.8. Lymphocytes were gated against forward and side scatter, and then against CD4 and CD25, to identify $CD4^+CD25^+$

Table 1. Patient characteristics and clinical response

Patient	Age (y)	Sex	Primary	Palliative chemotherapy	Sites of disease at screening	Trial: cycles	Best CT response	Serum tumor antigen change	OS (mo)
1	41	M	E	ECF	L, N, liver, lung	1	PD	Increase	2.73
2	49	M	E	ECF	L	2	SD	Stable	5.07
3	63	M	GEJ	CF	L, N	1	PD	Decrease	10.17
4	37	F	G	MCX	Duodenal mass, ascites, peritoneal, pelvic	2	SD	Stable	12.20
5	55	M	E	CF	L, N, liver, lung, bone	1	PD	Increase	1.70
6	39	M	G	ECF, ECX	L, N	1	PD	Increase	3.63
7	63	M	GEJ	CF	L, N, lung	2	PD	Increase	7.27
8	43	F	G	ECX, D/I	L, omental, serosal, ascites	1	PD		12.03
9	57	M	G	C/TS1	L, N, pleural effusion, ascites	1	PD		4.27
10	75	F	G	MCX	L, N, liver	1	PD	Increase	4.60
11	49	M	E	ECF	L, adrenal, chest wall, muscle	1	PD	Increase	2.20
12	67	M	E	ECX	L, N, liver, lung, adrenal, pleural, bone	11	PR	Decrease	32.70+
13	75	M	GEJ	MCX	L, N, lung, pleural effusion	1	*	Increase	3.73
14	61	F	G	ECF, MCF	N, splenic	1	PD	Decrease	17.93
15	68	M	GEJ	ECX	L, N, liver, lung	2	PD	Increase	17.10
16	41	M	GEJ	CF	L, N	1	PD	Increase	4.83
17	62	M	E	MCX	L (pericardium and diaphragm)	2	SD	Increase	12.83
18	53	M	GEJ	ECF	L, liver, chest wall	1	PD	Increase	4.70

Abbreviations: D/I, Docetaxel/Irinotecan; OS, overall survival; ES, esophageal; G, gastric; GEJ, gastroesophageal junction; E, epirubicin; C, cisplatin; F, 5-fluorouracil; M, mitomycin; X, capecitabine; TS1, an oral 5-fluorouracil derivative; L, local; N, lymph node; PD, progressive disease; SD, stable disease; PR, partial response; +, patient still alive.

T lymphocytes. The method was adapted to analyze intracellular CTLA4 staining using anti-CTLA4 PE antibody (clone BNI3, BD Biosciences) and PE mouse IgG2a isotype (Serotec).

Antigens and cellular responses. To explore changes in *in vitro* responses to relevant TAAs, overlapping 5T4 32-mer peptides (18 patients, 24 cycles) and purified CEA (13 patients, 15 cycles) were used in a thymidine incorporation proliferation assay (26). All time points for each patient were investigated in the same assay: cryopreserved PBMCs were thawed and 1.5×10^5 viable cells per well were cultured in Iscove's modified Dulbecco's medium with 10% AB serum, with six pools of four overlapping 5T4 peptides (defined as A-X) spanning the whole sequence of human 5T4 (10 μg/mL of each peptide); media controls with and without the appropriate concentration of dimethyl sulfoxide were used to prepare peptide stocks (27, 28). In parallel assays, PBMCs were cultured with purified CEA (Calbiochem) at 100, 200, and, if sufficient cells are present, 300 ng/mL. PHA-M (Sigma-Aldrich) at 2 and 5 μg/mL was used as a polyclonal stimulant. Each target was investigated using three replicates. Plates were incubated for 5 d at 37°C in 5% CO_2 before pulsing with 1 μCi/mL of [^{3}H]TdR for an additional 18 h before harvesting and counting with a Packard cell harvester and Topcount (Perkin-Elmer). Stimulation indices (SI) were calculated by dividing the counts per minute of the test wells by the mean count per minute of the appropriate control wells; mean results are presented. A pretreatment response was defined by a mean SI of ≥2. A posttreatment response was defined as a mean SI of ≥2, with a doubling of pretreatment response (27). Patients with new posttreatment responses were considered responders.

Cytokines. To investigate changes in T-lymphocyte responsiveness, cryopreserved PBMCs were thawed and cultured at 1×10^6 per well for 48 h in RPMI (Sigma-Aldrich) with 10% AB serum in the presence of 1 μg/mL anti-CD3 (OKT3, Janssen-Cilag) and 1 μg/mL anti-CD28 (clone 37407, R&D Systems). The supernatant was collected and frozen at –20°C. At a later time point, human IL-10 and IL-2 (n = 15) were quantified using ELISA kits (Diaclone) according to the manufacturer's instructions.

Statistical analysis. The primary end point was the best overall response rate, defined as the percentage of patients with complete response or partial response measured by RECIST. Survival analysis was summarized using the Kaplan-Meier method and log-rank test.

Pooled analysis of laboratory data across patients was done using StatsDirect. Nonparametric data were described using the median and interquartile range (IQ); changes with time were compared using Wilcoxon signed ranks. Patients were stratified by laboratory results as described, and the log-rank test was used to explore the relationship between laboratory results and clinical data.

Results

Patient characteristics. Between September 2006 and April 2007, 18 patients were recruited and treated. Individual

Table 2. Common and serious treatment-related toxicities for patients with metastatic gastric and esophageal cancer receiving tremelimumab

Toxicity	Grade	Cycle 1 (n = 18)	Cycle 2 (n = 6)	Cycle 3+ (n = 1)
Itch	1	8	2	5
Rash	1	4	3	2
Fatigue	1	5		1
Arthralgia	1	3		
Nausea	1	3		
Dizziness	1	1	1	6
Hypertension	1	2		
	2			1
Atrial fibrillation	2			1
	3	1		
AST increase	3	1		
Diarrhea	1	4	1	3
	5	1		
Eosinophilia	1	6	2	

NOTE: Most toxicities were grade 1. There was one toxic death from bowel perforation that complicated diarrhea. *n* indicates the number of patients. Only one patient received more than two cycles of tremelimumab.

Abbreviation: AST, aspartate transaminase.

characteristics are shown in Table 1. In summary, the majority of the patients were male (n = 14). Their median age was 56 years, with a range from 37 to 75 years. All patients had metastatic esophageal (n = 6), gastric (n = 6), or gastroesophageal junctional (n = 6) adenocarcinomas. Median time from diagnosis of advanced disease was 10.5 months [95% confidence interval (95% CI), 8.4-12.7 months]. All patients had previously received treatment with appropriate cisplatin-based chemotherapy in a metastatic disease setting, and three had also received second-line chemotherapy. The objective response rate of these patients to first-line chemotherapy in the metastatic setting was only 28% (two complete responses, three partial responses), with a 72% disease control rate (responses and stable disease). Most patients had an Eastern Cooperative Oncology Group performance status of 1 (n = 14) and had disease-related symptoms. Pretreatment CT scans showed a significant burden of disease: a majority of the patients had visible local involvement (n = 16), measurable lymph node metastases (n = 12), and/or visceral metastases (n = 11) at screening. Simple demographics of the further 30 screened patients who did not enter the trial were similar; entry was most commonly declined on the grounds of poor performance status (n = 13) or patient preference (n = 4).

Treatment and follow-up. Twelve patients received only a single cycle of tremelimumab. Five patients received two cycles of treatment, and a single patient remains on treatment after 11 cycles (32.7 months). No patients were lost

to follow-up and all were assessed for toxicity. A single patient was not objectively assessed for response but had symptomatic progression by 3 months.

Toxicity. All serious or common tremelimumab-related toxicity is summarized in Table 2. There was no treatment-related bone marrow toxicity but anemia commonly occurred with disease progression. Three patients experienced no drug-related toxicity. The most common toxicities were immunomediated but mild (18, 19): itch (50% of patients); rash and eosinophilia (both 33%); and diarrhea and fatigue (both 28%). The rash was an itchy maculopapular eruption, generally distributed on the torso and upper arms. It was managed with emollients and mild steroid cream, but recurred in subsequent cycles of treatment. Most diarrhea was mild and self-limiting but a single

patient (no. 11) developed colitis. This patient had onset of mild diarrhea 3 weeks after tremelimumab. Symptoms worsened but initially responded to systemic steroids, before recurring in week 7, complicated by fatal bowel perforation. Imaging and autopsy confirmed features of a pan-colitis from cecum to rectum with mucosal ulceration, friable bowel wall, and at least three small perforations. The tumor was confined to the known sites of disease and was not seen in the large bowel. This toxicity has been described as a rare class effect with anti-CTLA4 blockade (29). The other two tremelimumab toxicities greater than grade 3 were transient. Patient 4 had asymptomatic transaminitis (G3); serum bilirubin peaked on day 56 at 42 μmol/L, alkaline phosphatase at 792 IU/L, and aspartate transaminase at 499 IU/L, before resolving spontaneously within 10 days.

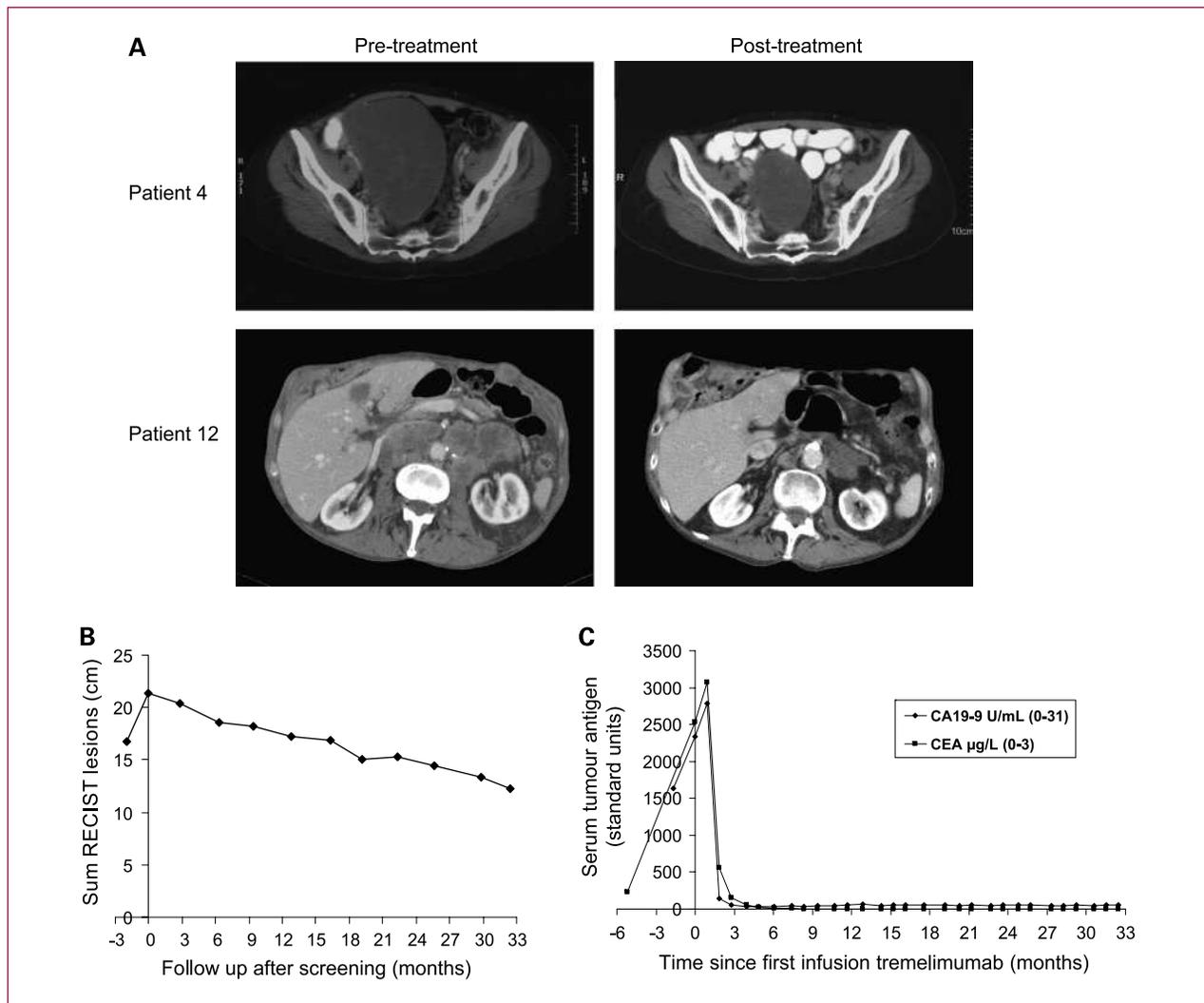Fig. 1. Clinical response to tremelimumab. A, top scans show a 12% reduction in size of large pelvic metastasis (13.1 × 8.8 cm) with first cycle, which was associated with cessation of vaginal bleeding in patient 4. The bottom scans show baseline and recent (post cycle 10, 32.4 months) assessment of intra-abdominal disease for patient 12. B, cumulative reduction in measurable disease following 11 cycles of tremelimumab (patient 12). C, serum tumor antigen response in patient 12 who continued to benefit from tremelimumab after 32.7 mo.
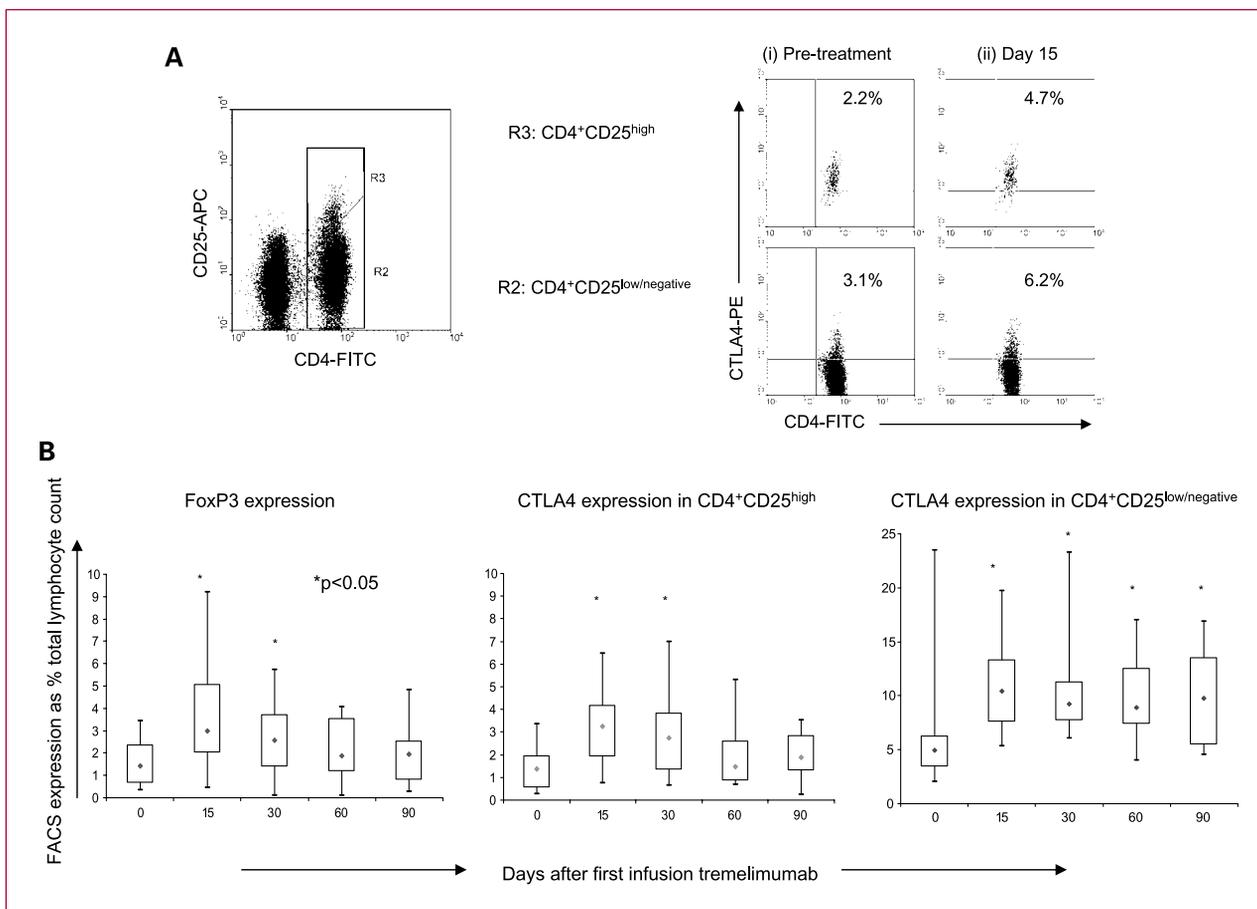
Fig. 2. Flow cytometry analysis of PBMCs. A, patient 12 as an example: lymphocytes are gated against forward and side scatter, then against CD4+CD25high (R3) and CD4+CD25low/negative (R2). The upper right quadrant shows CD4+CTLA4+ lymphocytes in the gated population as a percentage of total lymphocytes. Pretreatment levels of CTLA4+ lymphocytes (i) increase in both populations at day 15 (ii). B, changes in lymphocyte phenotype after treatment with tremelimumab for all patients. Pooled data are expressed as box-and-whisker plots of median expression, with quartile range and minimum and maximum results marked. Statistical significance is by Wilcoxon signed ranks.

Patient 4 had no evidence of viral hepatitis or liver metastases, and signs of liver inflammation did not recur on retreatment. Tremelimumab caused infusion-related hypertension in three patients: patient 12 required antihypertensive medication, but the other episodes resolved spontaneously. Two patients had new onset of atrial fibrillation with no clear precipitant, although as both episodes occurred toward the end of the treatment cycle, causality remains unclear. Despite the single fatality, tremelimumab was well tolerated.

Responses. There were no objective responses measurable by RECIST at the end of the first cycle of tremelimumab. However, four patients had stable disease with clinical benefit, including weight stabilization, pain resolution, cessation of vaginal bleeding from a pelvic metastasis, and return of libido. Figure 1A shows CT scan changes following treatment for patients 4 and 12. One of these patients (no. 12) achieved durable benefit with incremental reduction in tumor burden following each cycle of treatment and a partial response after 25.4 months (8 cycles) of treatment, as shown in Fig. 1B.

Median time to progression was 2.83 months (95% CI, 2.65-3.02), and median overall survival was 4.83 months (95% CI, 4.06-5.59), reflecting the rapid course of disease progression in the majority of patients. A minority of patients survived longer than expected, and 12-month survival was 33% (95% CI, 14-54%). The one patient with a durable response remains alive, well, and on treatment after 32.7 months.

Serum tumor antigen levels were used as a supplementary method for assessing response. Elevated levels were found in 13 patients pretreatment: CA19-9 in 11 patients (range 35-15,091 units/mL) and CEA in 6 patients (range 12-2,537 μg/L). During the first cycle of tremelimumab, serum TAA levels increased in 10 of these patients and decreased in 3. For the minority of patients with increased serum levels of both TAAs, changes occurred in parallel. One patient (no. 16) developed increased serum CA19-9 and CEA *de novo* by day 60 of the first cycle. Two patients (nos. 2 and 4) had mildly increased levels of CA19-9 alone at day 60 but later samples were normal. Median overall

survival for patients with stable or decreasing serum tumor antigens was 12.20 months (95% CI, 7.84-16.59) compared with 4.6 months (95% CI, 3.45-5.75) for those whose levels increased ($P = 0.03$).

Patient 12, who achieved durable benefit, had the most marked decrease in serum tumor antigen levels, as shown in Fig. 1C. CEA and CA19-9 both peaked at day 30 of the first cycle of tremelimumab, at 3,071 µg/L and 2,785 units/mL, respectively, before decreasing to 56 µg/L and 34 units/mL by the end of the first cycle. By the end of the second cycle, serum levels of both markers were just above the normal threshold, and this effect has been sustained for 32.7 months.

Lymphocyte phenotype. Absolute counts were available from paired fresh samples for the last 12 patients (nos. 7-18). Median absolute lymphocyte count was 1.10×10^9/L (range 0.66-3.00) at baseline. Total lymphocyte count and CD8+ lymphocyte count (median 0.35×10^9/L) did not vary with treatment, but median CD4+ lymphocyte count increased transiently from 0.39×10^9/L to 0.72×10^9/L at day 15 ($P = 0.009$). Within this population, there was an increase in CD4+CD25high cells from 0.02×10^9/L to 0.035×10^9/L at day 15 ($P = 0.003$), which was maintained until day 30 before decreasing.

Baseline samples were obtained for later analysis of regulatory phenotype for all patients. For comparative analysis of results from the first cycle, data were pooled for day 15 ($n = 17$), day 30 ($n = 17$), day 60 ($n = 13$), and day 90 ($n = 9$). The changes in patient lymphocyte phenotype with tremelimumab treatment are summarized in Fig. 2. There was a strong correlation between the proportion of CD4+CD25high lymphocytes expressing FoxP3 or CTLA4 (not shown, $\tau = 0.45$, $P < 0.0001$). By both markers, 1.4% lymphocytes were Treg pretreatment, increasing transiently to 3% and 3.2%, respectively, on day 15 ($P < 0.005$) before returning to baseline by day 60.

CTLA4 is also found on activated T cells. A more sustained increase with tremelimumab was seen in these CD4+CD25low/negativeCTLA4+ lymphocytes, from 5.0% lymphocytes pretreatment to 8.9% to 10.4% from day 15 to 90 ($P < 0.005$). This population may represent potential effector lymphocytes.

Lymphocyte function: proliferation responses to tumor antigens. Lymphocyte proliferative responses to 5T4 were investigated in all study patients using pooled peptides. From the first cycle of treatment, at least three time points were available for all patients, and a complete data set (five time points) was available for 10. From the second cycle of treatment, 1 ($n = 2$), 3 ($n = 1$), or 4 ($n = 2$) time points were available.

Six of 18 patients had preexisting baseline responses, and 8 of 18 had at least one *de novo* posttreatment response. Pretreatment responses occurred with equal frequency against all the peptide pools, except IJKL and VWX. Posttreatment responses occurred against all peptide pools but were most frequent against QRST (5 of 18), and IJKL and VWX (3 of 18 each). The timing of posttreatment responses was complex: median time to first posttreatment response was 35 days but the range was large (19-120 days), and three patients did not develop a *de novo* posttreatment response until after day 90. Responses were most frequent around 30 days after tremelimumab, with three responses at this time after the first cycle and two responses after the second cycle.

Lymphocyte proliferative responses to CEA were investigated in 13 patients for whom material was available using the whole protein. From the first cycle of tremelimumab, at least three time points were available for 12 patients, and a complete data set (5 time points) was available for 6 patients. The second cycle of tremelimumab was also investigated in three patients, using 2 ($n = 1$) or 4 ($n = 2$) time points.

Two of 13 patients had pretreatment responses, and 5 of 13 had posttreatment responses. Posttreatment responses were most frequent mid cycle: three responses occurred 30 days after the first cycle, two at 60 days after the first cycle, and one at 30 days after the second cycle of tremelimumab. In the subset of patients with increased serum CEA at baseline ($n = 4$), none had a pretreatment proliferative response to CEA, but two developed a proliferative response posttreatment at day 30 (nos. 7 and 12). In both cases, this apparently precedes a decrease in serum CEA, albeit of very different magnitudes (6% for patient 7; 85% for patient 12). Figure 3A shows responses to 5T4 and 200 ng CEA during the first two cycles of treatment for patient 12 who had durable benefit from treatment: significant responses to CEA occur at day 30 of both cycles of tremelimumab, and to 5T4 at days 30 and 60 of the second cycle. Only three patients had posttreatment responses to both tumor antigens, and whereas only one of these experienced durable benefit from treatment, the other two (nos. 7 and 15) had stable visceral disease on CT scan and survived for 7.27 and 17.20 months, respectively.

The patients were stratified post hoc by first cycle posttreatment proliferative responses to CEA to explore any relationship between the *in vitro* data and outcome. Median survival for the responders was 17.1 months (95% CI, 7.9-26.2 months) compared with 4.7 months (95% CI, 4.4-5.0 months) for the nonresponders (Fig. 3B, $P = 0.004$). Parallel stratification by first cycle posttreatment proliferative responses to any pool of 5T4 showed no relationship with overall survival.

Lymphocyte function: cytokines. To explore the effect of tremelimumab on T-lymphocyte responsiveness, PBMCs were stimulated with anti-CD28 and anti-CD3 before harvesting the supernatant for cytokine ELISA ($n = 15$). Analysis of the results shows no significant pattern of change with treatment, although median pretreatment IL-2 increased from 153 pg/mL (IQ range, 64-419) to 328 pg/mL at day 30 ($P = 0.15$). Median IL-10 release was 1,254 pg/mL (IQ range, 581-2,479) and did not change with treatment. Interestingly, median pretreatment IL-2 release was higher for those who later had stable disease at the first CT scan (399 pg/mL; IQ range, 304-611) compared with those with disease progression and no significant toxicity (73 pg/mL; IQ range 17-136, $P = 0.054$ by Mann

Fig. 3. Lymphocyte proliferative responses to tumor antigens. A, example proliferative response to 5T4 peptides (i) and CEA protein (ii) for patient 12, with parallel responses at day 30 of cycle 2. B, Kaplan-Meier overall survival by *de novo* posttreatment proliferative response to CEA ($n = 13$).
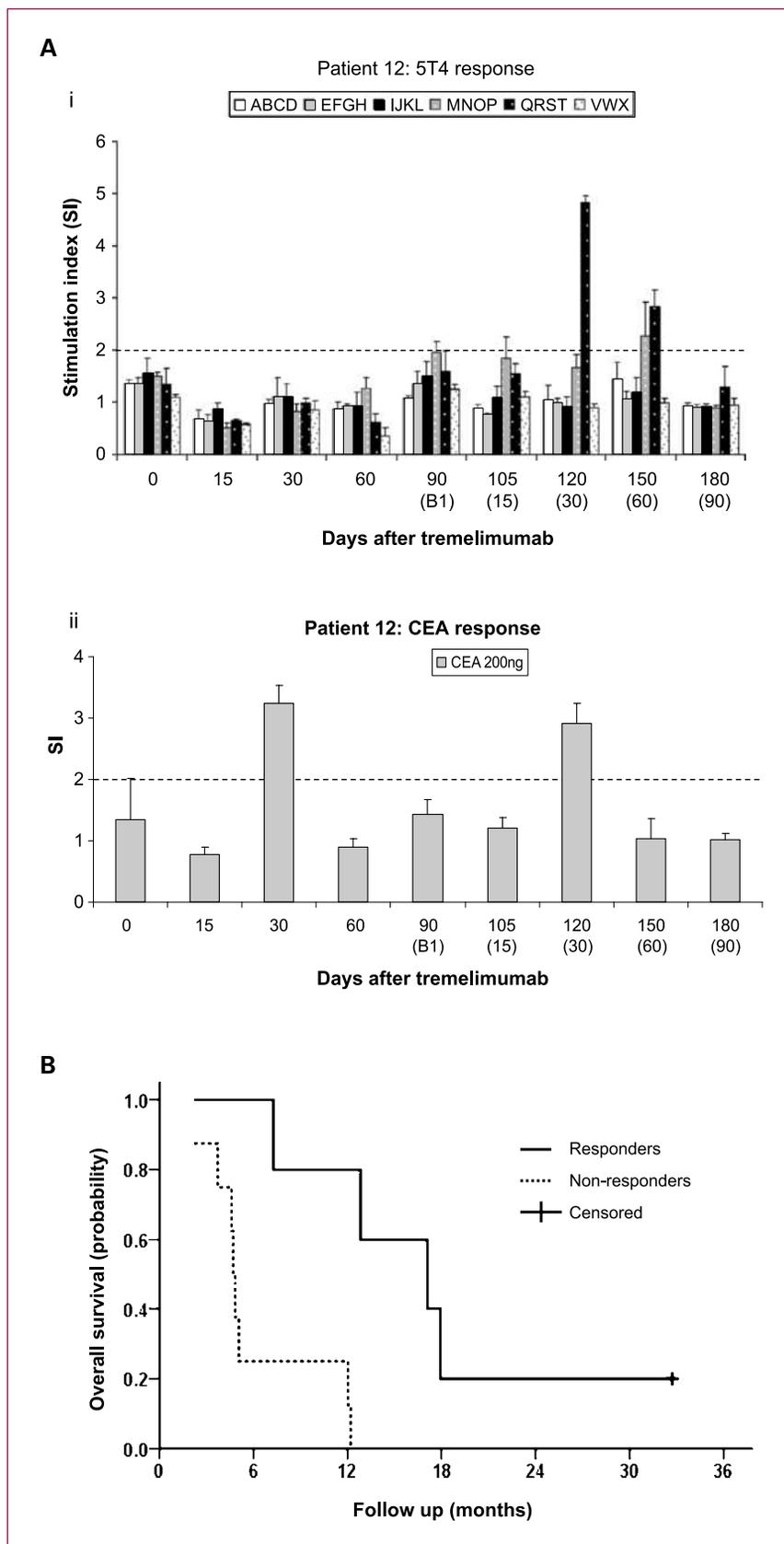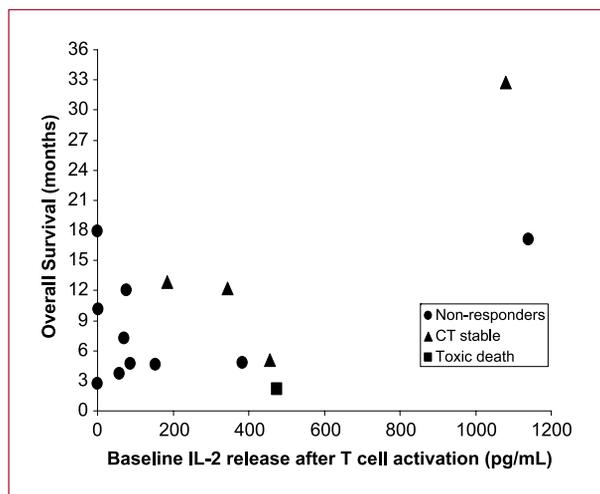
Fig. 4. Baseline IL-2 release after T-cell activation against overall survival.

Whitney U test). As shown in Fig. 4, the four patients with the highest IL-2 levels included two with stable disease at first scan (nos. 2 and 12) and the patient who developed fatal toxicity (no. 11). Moreover, when median pretreatment IL-2 release after T-cell activation was used to divide patients with high and low IL-2 responses, there was an increased time to progression [5.1 months (95% CI, 1.1-9.0) versus 2.8 (95% CI, 2.6-2.9), Mantel-Cox $P = 0.014$] and a trend toward increased overall survival (median 12.2 months versus 4.7, $P = 0.27$) with high baseline IL-2.

Discussion

The objective response rate for tremelimumab as a second-line treatment for metastatic esophageal and gastric adenocarcinomas in this phase II study is only 5%; however, there was clinical benefit with evidence of disease control in a small cohort of patients, as assessed by stable CT scan (4 of 18) and/or decline or stabilization of serum TAAs (5 of 18).

In this study, the one partial response was achieved incrementally over 24.8 months, with very different kinetics from cytotoxic chemotherapy responses, and has proven durable. The patient (no. 12) has recent CT scan evidence of ongoing benefit and remains well on treatment 32.7 months after trial enrollment. Such long-lived benefit is remarkable in this poor-prognosis group. Literature review did not identify any reports of spontaneous remission in metastatic esophageal adenocarcinomas. No clinical features at screening identify this patient as unusual, and some, such as the presence of multiple visceral metastases, have been reported to predict poor prognosis (30). Review of pathology did not identify any unusual features. His best response to first-line chemotherapy was only stable disease, and at screening he was underweight with a body mass index of 16.2, and had symptomatic disease progression with recurrence at his surgical anastomosis, nodal,

liver, lung, adrenal, and bone metastases, and increasing serum CEA (2537 µg/L) and CA19-9 (2333 units/mL).

Despite the low objective response rate in this trial, the median overall survival at 4.8 months is similar to that reported in a review of 12 phase II trials of second-line chemotherapy for gastric cancer, in which the average reported median survival was 5.6 months (range 2.5-11 months; ref. 3). At 12.2 months, survival for patients who had stable disease following tremelimumab compares favorably with that of chemotherapy responders whose mean survival was 9.1 months (range 5.5-12 months).

Tremelimumab was well tolerated in the majority of these patients, despite increasingly symptomatic disease. The single treatment-related death was due to bowel perforation that complicated autoimmune colitis, a recognized complication of CTLA-4 blockade with a frequency of 4 of 700 patients (0.6%) at doses of ipilimumab of 3 mg/kg or more (31). This study is clearly too small to characterize whether the frequency of severe colitis and perforation varies with tremelimumab and gastroesophageal primary disease; however, there was no increased frequency of diarrhea (all grades) or evidence of upper gastrointestinal inflammation at gastroscopy, which several patients required for management of dysphagia.

The two anti-CTLA4 antibodies in clinical development, tremelimumab and ipilimumab, have both shown interesting antitumor activity in melanoma. Objective response rates for both drugs have been modest at around 10% to 15% when measured using classic CT response criteria (18, 31). However, some responses have been reported either late or after apparent disease progression (32). This unusual time course of responses has led to concern that reliance on established CT response guidelines such as RECIST or WHO (33), developed to validate rapid responses to traditional cytotoxic chemotherapy, will underestimate the activity of such agents. Wolchok et al. have even suggested an alternative "immune-related response criteria" (34). Where responses have occurred in melanoma with anti-CTLA4 blockade, many are durable (35). For example, four of eight responders in the original tremelimumab phase I/II studies have achieved durable benefit for more than 2 years (18).

Targeting of tremelimumab to potential immune responders requires a better understanding of the effects and kinetics of CTLA4 blockade in treated patients. CTLA4 blockade may act by suppressing Treg control of lymphocyte activation or more directly on activated lymphocytes, which transiently express the target. Rosenberg's group showed an increase in relative FoxP3 expression by PCR after ipilimumab, and no loss of the suppressive activity of $CD4^+CD25^+$ lymphocytes *in vitro* (36). CTLA4 blockade induced expansion of $FoxP3^+$ Treg in addition to activated effector $CD4^+$ T cells in prostate cancer patients (37). In agreement with these studies, we found that Tregs, as defined by FoxP3 and CTLA4 expression, were expanded after treatment with tremelimumab. Natural Tregs remain hard to characterize. CD25, the IL-2 receptor α chain, is expressed on the surface of both activated and regulatory $CD4^+$ T cells; thus, in this study, Tregs were further

characterized using FoxP3 and intracellular CTLA4, which they constitutively express (38). Intracellular CTLA4 expression was chosen because the frequency of expression at the cell membrane at any given time is very low due to rapid trafficking within the cell (39). Both markers suggest that the proportion of Tregs increases in a reproducible temporal pattern after tremelimumab. These results support the idea that CTLA4 blockade is not acting primarily through Tregs (37). In contrast, the percentage of $CD4^+CD25^{low/negative}CTLA4^+$ lymphocytes shows a sustained increase after tremelimumab. As CTLA4 expression is a late event in lymphocyte activation, these cells may represent a population with potentially sustained activation. This inference is supported by development of *de novo in vitro* lymphocyte proliferative responses to two TAAs, 5T4 and CEA, after tremelimumab. Within the cohort investigated, the development of a response to CEA after tremelimumab correlated with survival. Interestingly, the patient with a durable clinical response developed recurring posttreatment lymphocyte proliferative responses to both TAAs after tremelimumab. These results need to be interpreted with caution and warrant further investigation.

Analysis of prognostic factors shows a relationship between immune-related adverse events and clinical responses to CTLA4 blockade (35). Both toxicity and responses seem to occur in a minority of potential immune responders. In this small study, pretreatment IL-2 release after T-cell activation seems to identify both those patients with potentially beneficial and serious toxic responses to tremelimumab, and warrants future prospective investigation.

The possible induction of antitumor-specific lymphocytes by CTLA4 blockade requires further investigation in man, but may suggest a rationale for combining anti-CTLA4 blockade with one of the antigen-targeted therapies currently in clinical development, such as CEA or 5T4 vaccination (27, 40), 5T4 superantigen (41), or CEA-targeted cytotoxic T cells. CTLA4 blockade has previously been administered to melanoma and ovarian cancer patients who had received vaccination with antitumor immunity being shown (42). In view of the unusual durability of the best response to tremelimumab, and the *in vitro* evidence of enhanced proliferative responses to relevant TAAs, further investigation of drug activity may be warranted in this challenging tumor type despite the disappointing objective response rate in this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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