

Potentiating Endogenous Antitumor Immunity to Prostate Cancer through Combination Immunotherapy with CTLA4 Blockade and GM-CSF

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Abstract

CTL-associated antigen 4 (CTLA4) is a costimulatory molecule expressed on activated T cells that delivers an inhibitory signal to these T cells. CTLA4 blockade with antibody treatment has been shown to augment antitumor immunity in animal models and is being developed as a treatment for cancer patients. As has been seen in preclinical models, combining CTLA4 blockade and granulocyte macrophage colony-stimulating factor (GM-CSF)-based immunotherapies can enhance the antitumor efficacy of this approach. We therefore examined whether CTLA4 blockade could be combined with GM-CSF administration. We treated 24 patients with metastatic, castration-resistant prostate cancer in a phase I trial where sequential cohorts were treated with increasing doses of ipilimumab, a fully human anti-CTLA4 antibody. Study subjects also received s.c. injections of GM-CSF at a fixed dose. Of the six patients treated at the highest dose level, three had confirmed PSA declines of >50%, including one patient that had a partial response in visceral metastases. Expansion of activated, circulating CD25⁺ CD69⁺ CD8⁺ T cells occurred more frequently at higher doses of treatment and was greater in magnitude than was seen in patients who received the same doses of either ipilimumab or GM-CSF alone. By screening sera with protein arrays, we showed that our treatment can induce antibody responses to NY-ESO-1. These results show that this combination immunotherapy can induce the expansion not only of activated effector CD8 T cells *in vivo* but also of T cells that are specific for known tumor-associated antigens from the endogenous immune repertoire. [Cancer Res 2009;69(2):609–15]

Introduction

Cancer immunotherapy often relies on the induction of effector T cells to mediate tumor regression. CTL-associated antigen 4 (CTLA4) signaling provides negative feedback to activated T cells, thereby dampening an immune response (1, 2). Blocking CTLA4 with anti-CTLA4 antibodies enhances effector T-cell responses and can induce T-cell-mediated rejection of certain tumors in mouse models (3). With less immunogenic tumors such as the mouse B16

melanoma, however, CTLA4 blockade is only effective if combined with the injection of GVAX (4–6). Human anti-CTLA4 antibodies have entered clinical trials and have shown antitumor responses in patients, predominantly in melanoma and kidney cancer patients (7–10). These treatments have been associated with immune induced toxicities, termed immune-related adverse events, manifesting as inflammation within skin, colon, eye, and pituitary gland (11, 12).

We have previously conducted a phase I trial with anti-CTLA4 antibody as monotherapy in prostate cancer patients using ipilimumab (Medarex, Inc./Bristol-Myers Squibb), a fully human immunoglobulin (IgG1; ref. 13). Fourteen patients with progressive metastatic castration-resistant prostate cancer (CRPC) received a single 3 mg/kg i.v. dose of ipilimumab. There was no evidence of polyclonal T-cell activation, and clinical autoimmunity was limited to one patient with a grade 3 rash/pruritus that resolved with steroids. Two of the 14 patients showed PSA declines of $\geq 50\%$. Pharmacokinetic analysis from this study showed a terminal half-life of 12.5 days. These data indicated that anti-CTLA4 antibody administration is safe in prostate cancer patients and may have some anticancer effects. These effects are presumably mediated by preexisting tumor-specific T cells that were primed by endogenous tumor-derived antigens and are receptive to CTLA4 blockade.

Whereas these results were intriguing, combining ipilimumab with other immunotherapies could be an important avenue to enhancing immune and clinical responses to CTLA4 blockade, as has been shown in mouse models (4–6). Interestingly, we have previously shown that systemic granulocyte macrophage colony-stimulating factor (GM-CSF) administration in patients with prostate cancer can modulate prostate-specific antigen (PSA) levels, leading to long-term disease control in $\sim 15\%$ of patients (14, 15). We therefore undertook a clinical trial to determine whether CTLA4 blockade could be successfully combined with systemic GM-CSF administration.

Materials and Methods

Study subjects. Study participants were at least 18 y old with histologically confirmed, metastatic prostate cancer, with disease evident on computed tomography (CT), magnetic resonance imaging, and/or bone scans. Study subjects were required to have CRPC with disease progression as defined by the PSA Working Group Consensus Criteria (16). Study subjects remained on androgen deprivation therapy and were required to have undergone antiandrogen withdrawal, when applicable. Subjects must not have received prior chemotherapy or immunotherapy. They could not receive radiation therapy within 4 wk of participation on the study.

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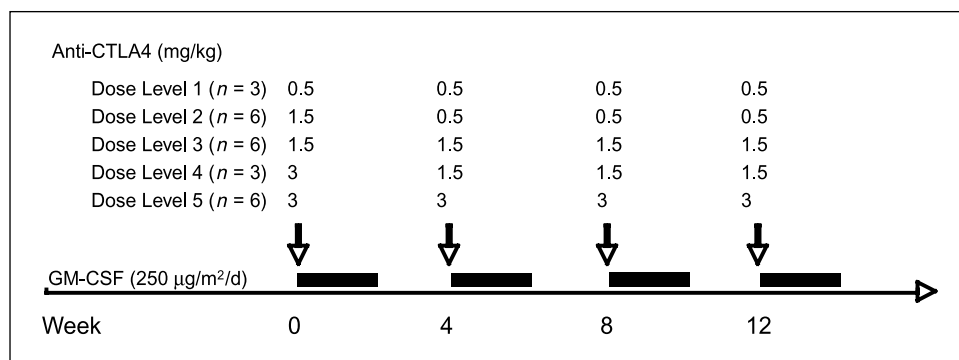


Figure 1. Administration of anti-CTLA4 antibody and GM-CSF. The dose of anti-CTLA4 antibody (mg/kg) and number of patients (*n*) on each dose level are presented.

Participants also could not have a history of autoimmune disease, nor could they take systemic corticosteroids during the study. All participants provided written informed consent.

Clinical trial. The phase I trial combined escalating doses of ipilimumab (BMS/Medarex) with a fixed dose of GM-CSF (sargramostim, Bayer HealthCare Pharmaceuticals) to assess for safety, feasibility, and immunogenicity of this treatment (Fig. 1). Initially, cohorts of three patients were sequentially enrolled into each of five dose cohorts at escalating dose levels of anti-CTLA4 antibody. If a single patient experienced significant treatment-related side effects potentially related to ipilimumab at a given dose, that cohort was expanded to six subjects. Patients received up to four doses of anti-CTLA4 antibody at the specified doses. These doses were given in 4-wk cycles, with GM-CSF administered daily on the first 14 d of these cycles. Cycles of GM-CSF treatment could continue until disease progression or toxicity. Treatment for any patient was stopped with any significant (grade 3 or 4) treatment-related side effects. If none of three or only one of six patients at a given dose level encountered dose-limiting side effects, the dose of anti-CTLA4 was escalated for the next cohort of patients. A total of 24 patients were accrued to this phase I study. Twenty-four study subjects were enrolled in this clinical trial (Table 1).

Clinical end points. The primary end point of safety was assessed while subjects were on study and graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events version 3.0.⁶ Additional exploratory end points include assessing T-cell activation, clinical responses by Response Evaluation Criteria in Solid Tumors,⁷ and the serum tumor marker PSA according to PSA Working Group Consensus Criteria (16).

Flow cytometry. Peripheral blood mononuclear cells (PBMC) were obtained from patients before treatment and monthly while on treatment. PBMC were either stained fresh and assessed by flow cytometry or cryopreserved for later study. The fluorochrome-labeled antihuman antibodies to CD3, CD8, CD25, and CD69 were purchased from BD Biosciences. Stained cells were washed and analyzed with a FACSCalibur or LSR II (BD Biosciences) flow cytometer. All data analysis was done with FlowJo software (Treestar).

Detection of antigen-specific immune responses by spotted arrays. To determine if IgG antibodies to known cancer-testis antigens are modulated by combination treatment with GM-CSF and CTLA4 blockade, phage high-throughput immunoblot analysis was carried out (17). Blots were spotted in duplicate with phage expressing 30 different cancer-testis antigens including Mage, LAGE, and Gage family members, as well as prostatic acid phosphatase and p53. Phages encoding human IgG were also spotted in duplicate as a positive control. Sera from baseline and month 6 from cohort 5 were diluted 1:200 in blocking buffer and were incubated with these blot membranes. The membranes were washed with buffer and detected with antihuman total IgG conjugated to alkaline phosphatase

(Sigma) and the colorimetric detection reagent 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Pierce).

IFN γ enzyme-linked immunosorbent spot assay. PBMC (300,000 per well) were cultured in 96-well MAIP plates (Millipore) precoated with anti-IFN γ antibody (BD Biosciences). Cells were incubated for 24 h at 37°C in a 5% CO₂ in a humidified incubator with NY-ESO-1₁₅₇₋₁₆₅, CMV pp65₄₉₅₋₅₀₄, and HIV gag₇₆₋₈₄ peptides (Synthetic Biomolecules) at a concentration of 10 µg/mL. Plates were then washed, incubated with detection anti-IFN γ antibody, and then developed with AEC substrate (BD Biosciences). Spots were then enumerated on an automated AID enzyme-linked immunosorbent spot (ELISPOT) plate reader.

Statistical analysis. The primary outcome for this phase I study was to determine the safety of anti-CTLA4 given with GM-CSF. The standard dose escalation procedure for phase I trials was carried out with 3 to 6 patients accrued per dose cohort. The maximum tolerated dose level was defined as the dose of anti-CTLA4 resulting in none of three patients or only one of six patients for an expanded dose cohort experiencing dose-limiting toxicity. A total of 24 patients were accrued to five different dose levels (Fig. 1). Due to the small sample size for each dose cohort, no formal statistical testing was done comparing outcomes between different cohorts. Outcomes for immunologic and clinical outcomes were summarized graphically with descriptive statistics.

Results

Clinical responses and side effects. Overall, 3 of 24 patients experienced a >50% decline in PSA, whereas no significant clinical responses were seen in the initial four dose levels. In the highest dose cohort of 3 mg/kg Ipilimumab, three of six patients

Table 1. Patient characteristics

	Median (range)
Age (y)	70 (60–82)
Gleason score	7 (6–10)
PSA (ng/mL)	35.3 (6.7–435.1)
ECOG performance status	0 (0–1)
Hemoglobin	13.4 (11.7–15)
WBC	6.6 (3.7–10.7)
LDH	159 (136–381)
Metastases	
Bone	22/24
Nodal	6/24
Visceral	3/24

Abbreviations: ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase.

⁶ http://ctep.cancer.gov/reporting/ctc_v30.html

⁷ <http://ctep.cancer.gov/guidelines/recist.html>

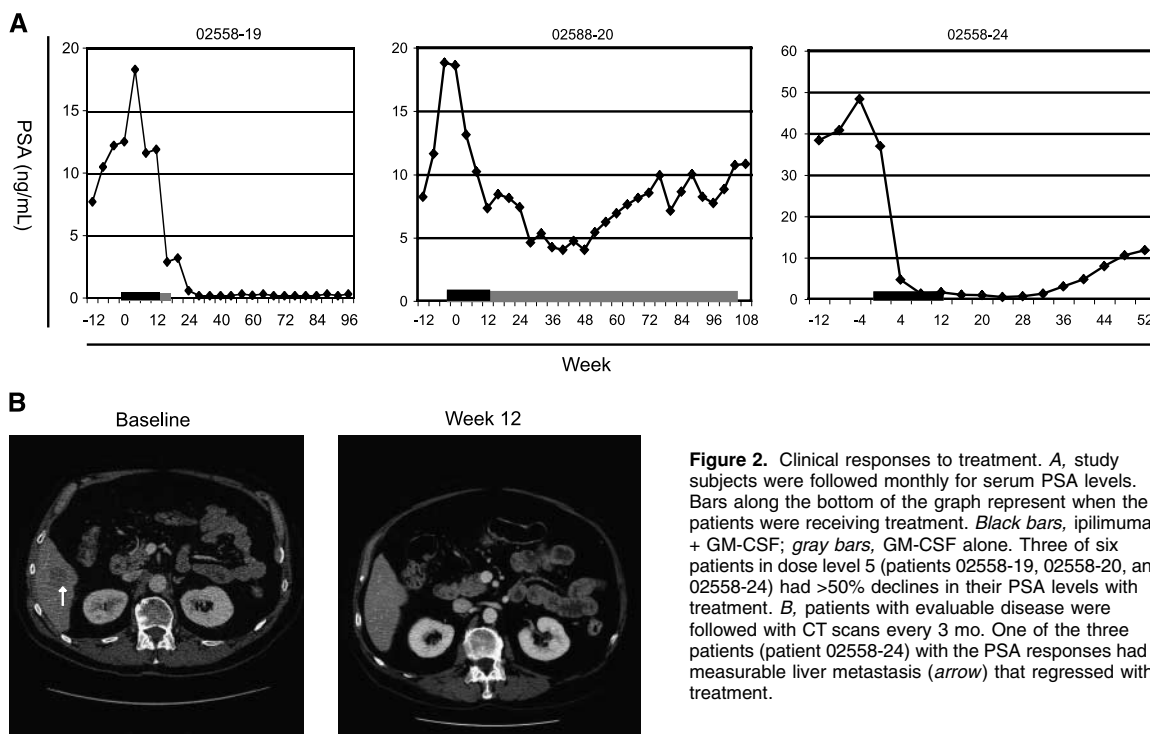


Figure 2. Clinical responses to treatment. *A*, study subjects were followed monthly for serum PSA levels. Bars along the bottom of the graph represent when the patients were receiving treatment. *Black bars*, ipilimumab + GM-CSF; *gray bars*, GM-CSF alone. Three of six patients in dose level 5 (patients 02558-19, 02558-20, and 02558-24) had >50% declines in their PSA levels with treatment. *B*, patients with evaluable disease were followed with CT scans every 3 mo. One of the three patients (patient 02558-24) with the PSA responses had a measurable liver metastasis (*arrow*) that regressed with treatment.

(02558-19, 02558-20, and 02558-24) in the highest dose cohort experienced a PSA decline, defined by PSA Working Group Consensus Criteria (16) as a >50% decline in PSA level (Fig. 2A). One of these patients (patient 02558-24) also developed a near-complete radiological response of liver metastasis with treatment by week 12, confirmed by a follow-up scan 12 weeks later, and was deemed a partial response by Response Evaluation Criteria in Solid Tumors (Fig. 2B). This radiographic response was confirmed with a follow-up scan 12 weeks later. As has been seen in other trials with anti-CTLA4 blockade, we observed immune-related adverse effects, particularly in the higher dose cohorts (Table 2). The three patients who achieved a PSA response also experienced an immune-related adverse event. One patient (subject 02558-19) was diagnosed with pan-hypopituitarism at week 16 of treatment and was taken off treatment. This patient also developed a durable PSA response that is ongoing, now almost 2 years since initiating study therapy. A second patient (subject 02558-20) experienced a mild rash and ultimately developed progressive disease by PSA levels at almost 2 years of treatment. The third patient (subject 02558-24) developed grade 3 diarrhea at week 12, which was successfully treated with systemic steroids. Subsequently, treatment with ipilimumab and GM-CSF was stopped. One subject in dose level 5 developed biopsy-proven temporal arteritis, which has not previously been described with CTLA-4 blockade. Whereas this subject did not achieve a clinical response as defined in our study, he did have a >30% reduction in his PSA with treatment. Finally, one subject in dose level 2 had a stroke at the initiation of therapy, which was likely unrelated to treatment, although any causality could not be excluded.

Expansion of activated CD8⁺ T cells with combination GM-CSF and ipilimumab therapy. The activation of circulating CD8⁺ T cells by staining patient-derived PBMC at baseline and at week 2 for activation markers CD25 and CD69 (Fig. 3A). We could detect an increase in the percentage of activated CD8⁺ T cells that coexpress both CD25 and CD69. This effect, however, was primarily seen in dose levels 4 and 5 (3 mg/kg initial dose), with seven of nine patients having at least a doubling in the percentage of CD25⁺ CD69⁺ CD8⁺ T cells following treatment compared to baseline. To determine the individual contribution of ipilimumab or GM-CSF on CD8⁺ T-cell activation, we also stained PBMC derived from participants with advanced prostate cancer from two separate clinical trials wherein ipilimumab or GM-CSF was administered as a monotherapy. Prostate cancer patients receiving ipilimumab alone received the antibody at the 3 mg/kg dose (13). Prostate cancer patients receiving GM-CSF alone were treated with the same schedule and dose received by patients on combination therapy. Ipilimumab treatment alone did not induce an increase in CD25⁺ CD69⁺ CD8⁺ T cells (Fig. 3B). Whereas there was an increase in CD25⁺ CD69⁺ CD8⁺ T cells with GM-CSF alone, the magnitude was less compared with combination treatment (Fig. 3C).

Given these results, we quantified the number of activated CD25⁺ CD69⁺ CD8⁺ T cells through the 16 weeks of treatment in the different dose cohorts. Subjects entering the study had variable CD8 T-cell counts at baseline, and there was no consistent change in the total CD8⁺ T-cell counts during treatment across the different dose levels (Fig. 4A). With dose levels 1 to 3, small, if any, increases were seen in the number of circulating activated CD25⁺ CD69⁺ CD8⁺ T cells per blood volume following treatment (Fig. 4B).

Table 2. Overview of clinical and immune responses

Patient	Dose level	No. of cycles	Adverse event	Clinical response	Immune response	
					Pre	Post
1	1	3			NY-ESO-1, p53	NY-ESO-1, p53
2	1	3				
3	1	7				
4	2	1	G3 CVA			
5	2	3				
6	2	5			NY-ESO-1	NY-ESO-1
7	2	5				
8	2	4				
9	2	2				
10	3	5				
11	3	2	G3 rash			
12	3	4				
13	3	3				
14	3	2				
15	3	1				
16	4	4				
17	4	6				NY-ESO-1
18	4	3			NY-ESO-1	NY-ESO-1
19	5	5	G3 pan-hypopituitarism	PSA		
20	5	24		PSA		NY-ESO-1
21	5	4				
22	5	8				
23	5	5	G3 temporal arteritis			
24	5	4	G3 diarrhea	PSA, CT		

Abbreviation: CVA, cerebral vascular event.

At dose levels 4 and 5, however, increases in the number of activated CD25⁺ CD69⁺ CD8⁺ T cells per volume of blood following treatment could be seen: Seven of the nine patients in dose levels 4 and 5 experienced at least a doubling in their activated CD25⁺ CD69⁺ CD8⁺ T-cell counts following treatment. These results are consistent with a dose-response relationship between ipilimumab dose and CD8⁺ T-cell activation.

Detection of immune responses to a cancer-testis antigen.

Antigen-specific T-cell immune responses against known prostate antigens including PAP, PSA, PSMA, EphA2, and survivin were assessed using ELISPOT and MHC-peptide tetramers but could not be detected (data not shown). We therefore turned to assessing for IgG antibody responses to known cancer-testis antigens. High-throughput array-based screening with phage-spotted immunoblots was carried out (17). Blots are spotted in duplicate with phage expressing 30 different cancer-testis antigens including Mage, LAGE, and Gage family members, as well as prostatic acid phosphatase and p53. With this approach, IgG antibodies to NY-ESO-1 were detected in the posttreatment serum, but not in the pretreatment serum, of one of the three clinical responders (02558-20), showing that antibodies to cancer-testis antigens could be induced by this treatment (Fig. 5A). One of the clinical non-responders also had a detectable induction of NY-ESO-1 antibodies (Table 2). Interestingly, three additional subjects had detectable preexisting NY-ESO-1 antibodies that persisted with treatment, and one of these patients also had pretreatment and posttreatment antibodies to p53.

Because immunoglobulin class switching to an IgG isotype requires T-cell help, NY-ESO-1-specific T cells were presumably induced in these patients. Because patient 02558-20 happened to be HLA-A*02⁺, we assessed this patient for CD8⁺ T-cell responses to the HLA-A*02 restricted epitope NY-ESO-1₁₅₇₋₁₆₅ by IFN γ ELISPOT. Whereas no significant NY-ESO-1₁₅₇₋₁₆₅ reactivity was detected at baseline, IFN γ -producing T cells in response to NY-ESO-1₁₅₇₋₁₆₅ could be detected following treatment, consistent with an induced immune response to this antigen (Fig. 5B).

Discussion

CTLA4 blockade may hold significant promise as an immunotherapy for cancer, but enhancing its clinical efficacy will be important if this approach is to gain broad applicability. The failure of a recent randomized phase III clinical trial to show benefit of another anti-CTLA4 antibody, tremelimumab, as a single agent in melanoma underscores the importance of developing combination therapies (18). Here, we show that combining CTLA4 blockade with systemic GM-CSF treatment is safe and can lead to clinically significant antitumor responses in patients with CRPC. Moreover, we show that this combination treatment can enhance the activation of circulating CD8⁺ T cells. In contrast, we could not detect the activation of circulating CD8⁺ T cells with ipilimumab alone. These results are consistent with prior reports of other trials that administered CTLA4 blockade alone or in combination with

peptide vaccines in which the expansion of activated CD25⁺ and/or CD69⁺ CD8⁺ T cells following treatment was not seen (8, 19, 20). Importantly, we also observed a dose-response relationship where the expansion of CD25⁺ CD69⁺ CD8⁺ T cells was seen primarily above the 3 mg/kg dose of ipilimumab. These results are consistent with the dose-response relationship that we have seen in the activation of effector CD4⁺ T cells (21) as well as with the observed clinical responses and immune-related adverse events.

GM-CSF can enhance T-cell activation with CTLA4 blockade through different potential mechanisms. We have previously shown that systemic GM-CSF expands the number of circulating antigen presenting cells, including dendritic cells in prostate cancer patients (22). GM-CSF may therefore enhance antigen presentation of endogenous tumor antigens. Alternatively, GM-CSF may increase the numbers of Fc receptor-bearing antigen presenting cells as has been postulated in a clinical trial that showed the capacity of GM-CSF to enhance the efficacy of another antibody, rituximab (23). In that study, however, the expansion of FcγRIIIa- and FcγRIIIa-expressing cells presumably magnifies the antibody-dependent cellular cytotoxicity mediated by the tumor-targeting antibody. We hypothesize that in our study, the expansion of Fc receptor-bearing cells could alternatively provide a cellular scaffold for ipilimumab to enhance cross-linking on the effector T cells or provide more costimulation by increasing the number of cells expressing CD80 and CD86.

As has been seen in prior clinical trials with CTLA4 blockade, subjects on this trial experienced immune-related adverse events in a dose-dependent fashion (8, 10, 12). The three subjects that

experienced clinical responses also had immune-mediated toxicities: grade 3 pan-hypopituitarism in one, grade 3 colitis in another, and grade 2 rash in the third. All of these immune-related toxicities were clinically manageable. Whereas pan-hypopituitarism resulting in impaired adrenal androgenesis could conceivably account for the clinical activity observed, nevertheless we saw clinical responses in the absence of autoimmune endocrinopathies. These results would indicate that tumor-specific immune responses were induced from the endogenous T-cell repertoire.

Importantly, immune responses to prostate-restricted proteins such as prostate acid phosphatase were not seen, suggesting that tumor antigens that have been historically considered because of tissue-specific expression may not represent the relevant antigens in this group of patients. In fact, several of our subjects had detectable levels of activated CD8⁺ T cells within their blood at baseline beyond what we have typically seen in healthy individuals. These subjects could therefore possess a pool of tumor-reactive or autoreactive T cells that are held in check by inhibitory signals such as CTLA4. With the CTLA4 blockade, these cells may expand and/or develop an effector function. The immediacy of the PSA declines in relation to the initiation of treatment in our trial further supports this notion. Preexisting IgG antibodies reactive to prostate-derived peptides have been detected in prostate cancer patients, consistent with prostate-reactive helper T cells already existing in these patients (24). Moreover, we also detected preexisting immune responses to NY-ESO-1 and p53 in some of our patients. Overexpression of NY-ESO-1 has been described in advanced prostate cancer (25–27). p53 mutations have also been

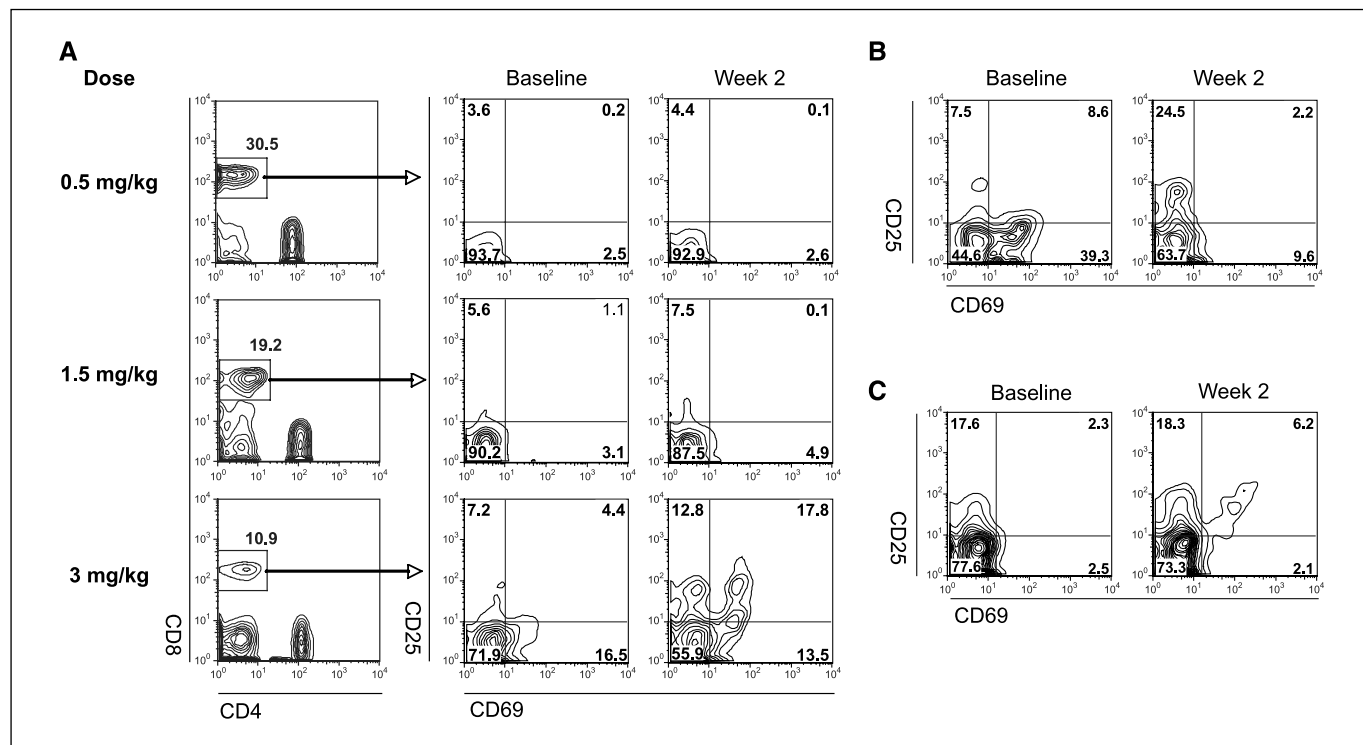


Figure 3. Treatment effects on CD8 T-cell activation. PBMC derived from study subjects were stained with antibodies to CD4, CD8, CD25, and CD69 and assessed by flow cytometry. CD8⁺ T cells were gated on and assessed for expression of CD25 and CD69. **A**, PBMC from baseline and at week 2 from subjects who received GM-CSF and ipilimumab at the indicated doses of ipilimumab were stained. **B**, PBMC from baseline and at week 2 from subjects participating in a separate clinical trial of advanced prostate cancer who received anti-CTLA4 antibody alone at 3 mg/kg were also assessed. **C**, PBMC from baseline and at week 2 from subjects participating in a separate clinical trial of advanced prostate cancer who received GM-CSF alone at the same dose and schedule of the combination trial were also assessed. Numbers represent the percentage of cells for each gate or quadrant. Gating for CD25 and CD69 expression was set with results from staining with isotype-matched control IgG. Results are representative of three patients treated at this dose level. Data are derived from one representative patient in each of the cohorts.

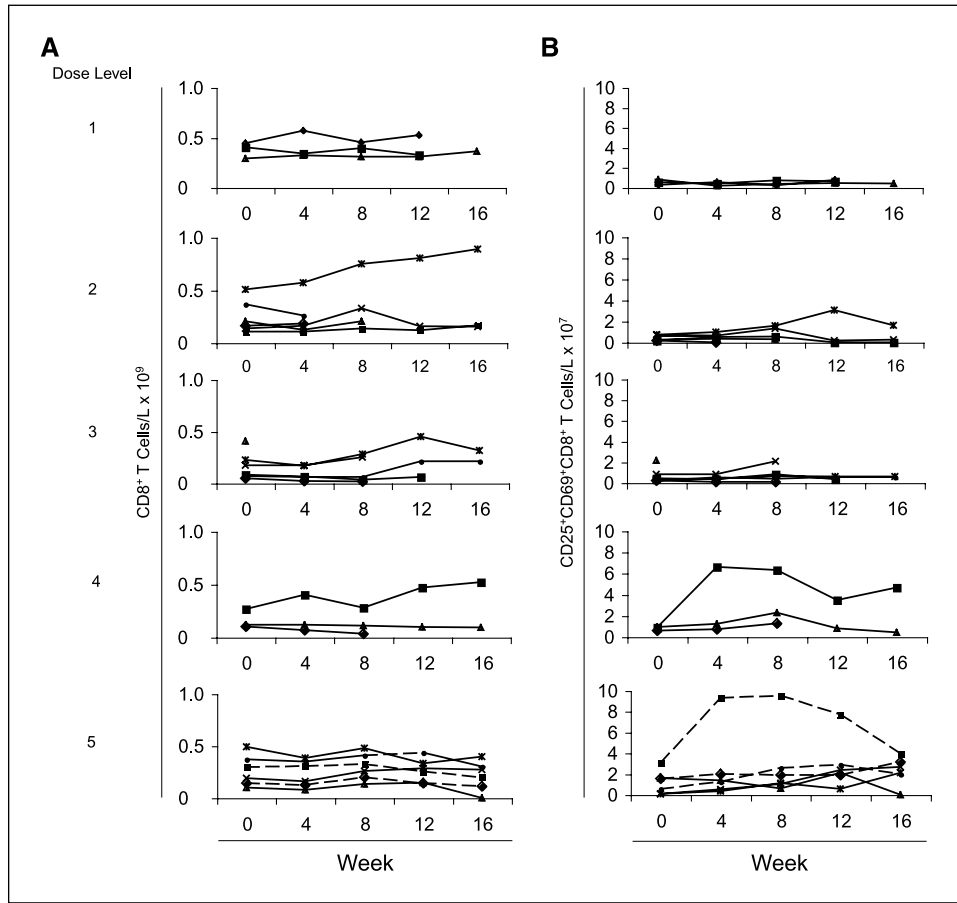


Figure 4. Activation of CD8⁺ T cells with escalating doses of anti-CTLA4 antibody. *A*, the total CD8 blood counts were calculated at baseline and at weeks 4, 8, 12, and 16 of treatment by multiplying the percent CD8⁺ T cells by the absolute lymphocyte counts measured simultaneously. Each row represents the counts for each evaluable subject within that specified dose level. *B*, PBMC at baseline and at weeks 4, 8, 12, and 16 of treatment from study subjects were stained with antibodies to CD3, CD8, CD25, and CD69. Stained cells were then assessed by flow cytometry and gated on CD8⁺ T cells. Gating for CD25 and CD69 expression was set with results from staining with isotype-matched control IgG. The counts of CD25⁺ CD69⁺ CD8⁺ T cells per volume of blood were calculated by multiplying the percent CD25⁺ CD69⁺ CD8⁺ T cells by the CD8⁺ T-cell counts. Each line again represents the counts for each evaluable subject within the specified dose level. *Dotted lines*, subjects who experienced clinical responses with treatment.

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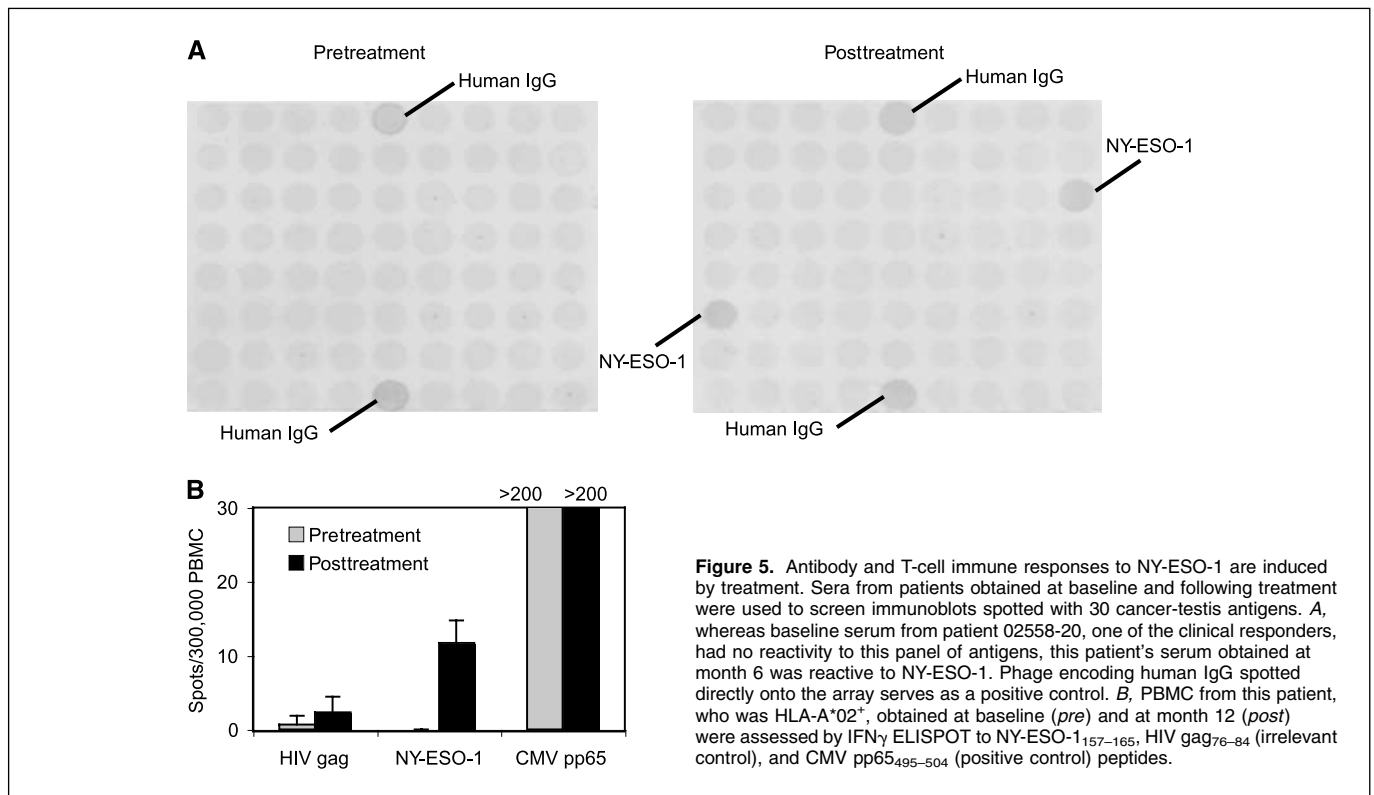


Figure 5. Antibody and T-cell immune responses to NY-ESO-1 are induced by treatment. Sera from patients obtained at baseline and following treatment were used to screen immunoblots spotted with 30 cancer-testis antigens. *A*, whereas baseline serum from patient 02558-20, one of the clinical responders, had no reactivity to this panel of antigens, this patient's serum obtained at month 6 was reactive to NY-ESO-1. Phage encoding human IgG spotted directly onto the array serves as a positive control. *B*, PBMC from this patient, who was HLA-A*02⁺, obtained at baseline (*pre*) and at month 12 (*post*) were assessed by IFN γ ELISPOT to NY-ESO-1₁₅₇₋₁₆₅, HIV gag₇₆₋₈₄ (irrelevant control), and CMV pp65₄₉₅₋₅₀₄ (positive control) peptides.

infrequently seen in prostate cancer (28). These molecular derangements could render these proteins more immunogenic in prostate cancer patients.

Induced antibody responses to NY-ESO-1 were observed in two patients, one of whom was a clinical responder. Induction of antigen-specific CD8⁺ T cells could also be detected in this clinical responder. The induction of NY-ESO-1 immunity in the absence of a clinical response would suggest that immunity to this antigen is not sufficient for an antitumor response. A larger trial would be necessary to assess the relevance of immune responses to this antigen in prostate cancer. Nevertheless, CTLA4 blockade provides a unique opportunity to identify immune responses that may be relevant for tumor rejection (29–31).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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