CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit

Jianda Yuan[a,1], Sacha Gnjatic[b,1], Hao Li[a], Sarah Powel[c], Humilidad F. Gallardo[a], Erika Ritter[b], Geoffrey Y. Ku[a], Achim A. Jungbluth[b] , Neil H. Segal[a], Teresa S. Rasalan[a], Gregor Manukian[a], Yinyan Xu[a], Ruth-Ann Roman[c], Stephanie L. Terzulli[a], Melanie Heywood[c], Evelina Pogoriler[c], Gerd Ritter[b], Lloyd J. Old[b], James P. Allison[a,c,d,2], and Jedd D. Wolchok[a,c,2]

[a]Ludwig Center for Cancer Immunotherapy, Immunology Program, [b]Ludwig Institute for Cancer Research, New York Branch, and [d]Howard Hughes Medical Institute, and [c]Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065

Contributed by James P. Allison, October 17, 2008 (sent for review September 6, 2008)

Blockade of inhibitory signals mediated by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) has been shown to enhance T cell responses and induce durable clinical responses in patients with metastatic melanoma. The functional impact of anti-CTLA-4 therapy on human immune responses is still unclear. To explore this, we analyzed immune-related adverse events and immune responses in metastatic melanoma patients treated with ipilimumab, a fully human anti-CTLA-4 monoclonal antibody. Fifteen patients were selected on the basis of availability of suitable specimens for immunologic monitoring, and eight of these showed evidence of clinical benefit. Five of the eight patients with evidence of clinical benefit had NY-ESO-1 antibody, whereas none of seven clinical non-responders was seropositive for NY-ESO-1. All five NY-ESO-1 seropositive patients had clearly detectable $CD4^+$ and $CD8^+$ T cells against NY-ESO-1 following treatment with ipilimumab. One NY-ESO-1 seronegative clinical responder also had a NY-ESO-1 $CD4^+$ and $CD8^+$ T cell response, possibly related to prior vaccination with NY-ESO-1. Among five clinical non-responders analyzed, only one had a NY-ESO-1 $CD4^+$ T cell response and this patient did not have detectable anti-NY-ESO-1 antibody. Overall, NY-ESO-1-specific T cell responses increased in frequency and functionality during anti-CTLA-4 treatment, revealing a polyfunctional response pattern of IFN-γ, MIP-1β and TNF-α. We therefore suggest that CTLA-4 blockade enhanced NY-ESO-1 antigen-specific B cell and T cell immune responses in patients with durable objective clinical responses and stable disease. These data provide an immunologic rationale for the efficacy of anti-CTLA-4 therapy and call for immunotherapeutic designs that combine NY-ESO-1 vaccination with CTLA-4 blockade.

Cytotoxic T lymphocyte-associated antigen 4 | immunotherapy | tumor therapy

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a coinhibitory molecule expressed by activated T cells and a subset of regulatory T cells (1–4). CTLA-4 is of primary importance in maintaining immune homeostasis by downregulating T cell signaling to inhibit the CD28-B7 costimulatory pathway, limiting T cell responses and contributing to tolerance to self antigens (5, 6). Blockade of CTLA-4 signaling has been shown to augment T cell responses and induce tumor rejection in a number of animal models (7–9). Two monoclonal antibodies to human CTLA-4 have been found to elicit objective and durable tumor responses in clinical trials (10–14). However, the functional impact of anti-CTLA-4 therapy on human immune responses to antigens present on tumor cells is not yet fully understood.

NY-ESO-1 is a cancer/testis antigen that is expressed in a variety of human malignancies but not in normal tissues except for the testis and placenta (15). NY-ESO-1 is highly immunogenic and elicits spontaneous antibody and T cell responses in cancer patients, with a high frequency in patients with advanced NY-ESO-1-expressing tumors (16). We hypothesized that CTLA-4 blockade by ipilimumab may promote T cells responses to this prototypical antigen. In addition, we further postulated that spontaneous preexisting immune responses to NY-ESO-1 could be augmented by CTLA-4 blockade.

Extensive characterization of T cell effector functions can now be analyzed with advances in the number of parameters that can be simultaneously detected by flow cytometry. The delineation of T cells into distinct functional populations defines the quality of the response which is crucial to disease outcome (17). Polyfunctional T cells, which are able to produce multiple cytokines and chemokines in response to antigen stimulation, have been demonstrated in a number of preclinical models for infectious diseases as well as in patients infected with HIV-1 or immunized using vaccinia constructs (18). Polyfunctional T cell markers include surface CD107a mobilization for degranulation, IFN-γ, IL-2, macrophage inflammatory protein (MIP) 1β, and tumor necrosis factor (TNF) -α production. We proposed CTLA-4 blockade might enhance the quality of NY-ESO-1 antigen-specific T cell anti- tumor activity by inducing polyfunctional T cell responses.

Here we describe the clinical characteristics of 15 metastatic melanoma patients who received ipilimumab as part of several phase II clinical trials. We examined the presence of NY-ESO-1 antibody response before and after ipilimumab therapy, assayed NY-ESO-1 antigen-specific T cell responses in a subset of patients using polychromatic flow cytometry, and characterized the functional profiles of the T cells during CTLA-4 blockade.

Results

Patient Selection and Treatment. We selected 15 patients, both clinical responders and progressors from 25 refractory metastatic melanoma patients enrolled on two-phase II clinical trials of ipilimumab sponsored by Bristol-Myers Squibb (BMS). The trials were BMS CA 184–022, a blinded, randomized, dose-ranging trial, in which patients received ipilimumab at 0.3, 3, or 10 mg/kg every three weeks for four treatments, and CA

Author contributions: J.Y., S.G., L.J.O., J.P.A. and J.D.W. designed research. J.Y., S.G., H.L., S.P., H.F.G., E.R., G.Y.K., A.A.J., N.S., T.S.R., G.M., Y.X., R.-A.R., M.H., and E.P. performed research; S.L.T., G.R., L.J.O. contributed reagents; J.Y. and S.G. analyzed data; and J.Y., S.G., and J.D.W. wrote paper.

Conflict of interest statement: J.P.A. and J.D.W. are consultants to Bristol-Myers Squibb and Medarex. J.P.A. is an inventor of intellectual property related to anti-CTLA-4 that is held by the University of California and has been licensed to Medarex and Bristol-Myers Squibb.

[1]These authors contributed equally to this study.

[2]To whom correspondence may be addressed. E-mail: wolchokj@mskcc.org or allisonj@mskcc.org.

This article contains supporting information online at www.pnas.org/cgi/content/full/0810114105/DCSupplemental.

Table 1. NY-ESO-1 antigen specific response in patients treated with anti-CTLA-4 antibody

ID	Clinical response (months)	NY-ESO-1 Ab response	NY-ESO-1 CD4 response	NY-ESO-1 CD8 response	Polyfunctional CD4 and/or CD8 response
IMF-2*	PR (25+)	-	-	-	-
IMF-3*	PR (21+)	+++	+[a]	+	++[b]
IMF-8*	SD (22+)	++	+++[b]	++[b]	+++[b]
IMF-11*	CR (19+)	-	++[b]	++[b]	++[b]
IMF-13	SD (21+)	-	-	-	-
IMF-16	PR (20+)	+++	++	++	++
IMF-17	CR (19+)	+++	++	++	+
IMF-18	SD (23+)	+++	++[b]	+	+[a]
IMF-4	PD and death	-	-	-	-
IMF-6	PD and death	-	+	-	-
IMF-7	PD and death	-	ND	ND	ND
IMF-9	PD and death	-	-	-	-
IMF-10	PD and death	-	ND	ND	ND
IMF-15*	PD and death	-	-	-	-
IMF-19*	PD and death	-	-	-	-

ND = Not determined. NY-ESO-1 Ab titer: "-"=negative; "+"=100–1,000; "++"=1000–10,000; "+++"= >10,000. NY-ESO-1 T-cell response: "-" = <0.1%; "+"= 0.1–0.5%; "++"=0.5–5%; "+++"= >5%. Changes of T-cell response before and after-therapy: "a"=3~10 fold over baseline; "b"= higher than 10 fold over baseline. No availability of pre therapy, wk7/12 and wk20 PBMCs for patients IMF-2, 16, 17. "*"= tumor obtained for immunhistochemical analysis, Tumor tissues from patient IMF-3 and 8 were positive for NY-ESO-1, tumor tissues from patients IMF-2,11,15,18 were negative for NY-ESO-1 by immunhistochemical analysis.

184–008, an open-label trial where patients received treatment at 10 mg/kg on the same schedule. Patients without dose-limiting toxicity and with evidence of clinical benefit—defined as complete (CR) or partial responses (PR) or stable disease (SD)—at week 24 could continue to receive ipilimumab at the same respective doses every 12 weeks until disease progression, toxicity, withdrawal of consent, or trial closure occurred. Patients treated with ipilimumab at 0.3 or 3 mg/kg who experienced progressive disease (PD) before 12 weeks were offered re-induction with the drug at 10 mg/kg as part of BMS CA 184–025. The 15 patients were selected on the basis of availability of a suitable panel of specimens for immunologic monitoring. At least one pretherapy and one posttherapy peripheral blood mononuclear cell (PBMC) sample were required for immune assays.

Patient Demographics. Patients ranged in age from 33 to 86 years (supporting information (SI) Table S1). All patients had undergone more than one systemic therapy for metastatic melanoma before ipilimumab treatment, with six patients (40%) receiving three or more therapies. Ten patients (67%) had received temozolomide therapy; while two patients (13%) had received experimental vaccination (patient IMF-11: NY-ESO-1 protein vaccination; patient IMF-17: tyrosinase and gp100 peptide pulsed dendritic cell vaccination). Two patients had received IFNα-2b as adjuvant therapy, which is lower than that reported by other groups (19).

Clinical Response and Immune-Related Adverse Events (irAEs). Eleven of 15 patients received four doses of ipilimumab in the Induction Phase. Eight of 15 patients received more than four doses of ipilimumab in the Maintenance/Reinduction Phase. The number of ipilimumab doses per patient ranged from 2–10 for the entire cohort; clinical responders received 8–10 doses. Eight patients showed evidence of clinical benefit at week 24. Two patients (patient IMF-11 and IMF-17) had CRs, which were observed at week 36 and week 33, respectively. Three patients (patient IMF-2, 3 and 16) had PRs at week 32, 36, and 36, respectively and three patients (patient IMF-8, 13 and 18) had SD for >12 months (Table 1). Two of eight patients with objective response (patient IMF-2 and IMF-17) had a transient increase in tumor size or increase in the number of metastases between week 1 and week 12.

Thirteen patients (87%) had at least one or more grade I to grade III irAEs (Table S1). Most side effects were reversible with medical management (corticosteroids). All eight patients with clinical benefit had at least one irAE as indicated, and five of seven patients with PD also experienced irAEs. Four of 15 patients had dose delays or discontinued ipilimumab during the induction phase due to disease progression or irAEs.

Evaluation of NY-ESO-1 Antibody Response. Antibody response against NY-ESO-1 was analyzed by ELISA. Known NY-ESO-1 antibody positive and negative sera as well as irrelevant protein were used as controls for specificity and sensitivity. All five NY-ESO-1 seropositive patients showed evidence of clinical benefit, whereas none of seven clinical non-responders was seropositive for NY-ESO-1. Two patients who had preexisting antibody responses to NY-ESO-1 (IMF-3 and IMF-16) showed an increase in their anti-NY-ESO-1 titer. The NY-ESO-1 antibody titer decreased but still remained positive (>1:100) at week 35–42 for patients IMF-16 and IMF-18. Patients IMF-8 and IMF-17 seroconverted to NY-ESO-1 after two and four doses of anti-CTLA-4 antibody therapy, respectively. Patient IMF-11 who received prior vaccination with NY-ESO-1 protein had no detectable NY-ESO-1 antibody before or after CTLA-4 blockade (Fig. 1).

Evaluation of NY-ESO-1 Specific T Cell Response. We quantified the induction of antigen specific T cells after a ten day *in vitro* stimulation of pretherapy and posttherapy samples with a pool of overlapping peptides spanning the entire sequence of the NY-ESO-1 protein. NY-ESO-1-specific T cell activity was determined by intracellular cytokine and chemokine (IFN-γ, TNF-α, MIP-1β, IL-2) staining, and specificity was confirmed by quantifying responses in the absence of NY-ESO-1 peptides on antigen-presenting cells. NY-ESO-1 specific T cells were monitored from an early time point (before week 20) in five patients with evidence of clinical benefit (Patients IMF-3, -8, -11, -13, -18); PBMCs from the other three patients with evidence of clinical benefit (Patient IMF-2, 16, and 17) were collected in sufficient quantities for functional analyses only at week 40 or 48. Fig. 2 shows representative dot plots illustrating $CD4^+$ and $CD8^+$ T cell responses by a patient with clinical benefit (patient IMF-8). NY-ESO-1 seropositive patient IMF-8 showed a dramatic increase in IFN-γ, TNF-α, and MIP-1β production by

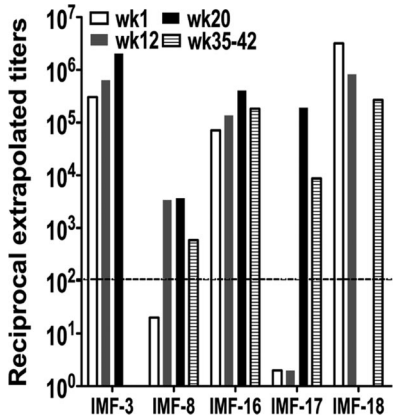

Fig. 1. Antibody responses to NY-ESO-1 before and after anti-CTLA-4 antibody therapy. Reciprocal antibody titers against *Escherichia coli*-derived NY-ESO-1 protein were measured by ELISA and are shown for each available time point as indicated. Antibody titers below 100 were defined as negative. Five NY-ESO-1 seropositive patients are shown, while the other 10 patients are NY-ESO-1 seronegative.

$CD4^+$ T cells specific for NY-ESO-1 from week 1 to 12, along with a more modest increase in CD8+ T cells producing IFN-γ and MIP-1β (Fig. 2).

All NY-ESO-1 seropositive patients had clearly detectable $CD4^+$ and CD8+ T cells recognizing NY-ESO-1 following ipilimumab treatment (Table 1). Four (IMF-2, -3, -8, and -17) of five patients (IMF-2, -3, -8, -17 and -18) tested had consistent NY-ESO-1 antigen specific T cell responses detectable as late as fifty weeks after initial treatment (data not shown). Among five of seven clinical non-responders analyzed (IMF-4, -6, -9, -15, and -19, Table 1), only patient IMF-6 had a $CD4^+$ T cell response to NY-ESO-1 and no observable CD8+ T cell response. This response was weak and was not accompanied by the presence of anti-NY-ESO-1 antibody.

IL-2, MIP-1β, TNF-α and IFN-γ were measured simultaneously to assess the quality of T cell responses. The NY-ESO-1 antigen-specific $CD4^+$ T cell response induced by CTLA-4 blockade revealed a polyfunctional response pattern of MIP-1β, TNF-α, and IFN-γ, but no IL-2 when restimulated with NY-ESO-1 overlapping peptides. NY-ESO-1 antigen-specific $CD8^+$ T cell responses were not as robust as $CD4^+$ T cell responses. Two patients with clinical benefit (IMF-8 and IMF-11) had either significant MIP-1β^+IFN-γ^+ or TNF-α^+IFN-γ^+ two-

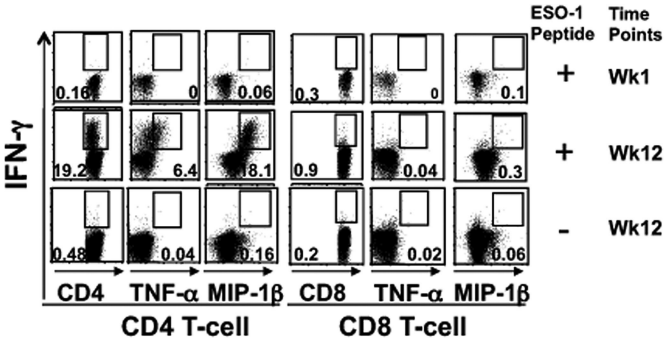

Fig. 2. NY-ESO-1 specific $CD4^+$ and CD8+ T cell responses were induced after CTLA-4 blockade. Representative intracellular cytokine and chemokine staining of both $CD4^+$ and CD8+ T cells responding to NY-ESO-1 pooled peptides from NY-ESO-1 seropositive patient IMF-8 at weeks 1 and 12. Single cytokine gates were set based on negative control (unstimulated) samples and were placed consistently across samples (*Bottom*).

cytokine production by CD8+ T cells (Fig. 3 *A* and *B*; Table 1). Polyfunctional $CD4^+$ T cell responses peaked at week 7 or 12, in most patients, followed by a small decrease (Fig. 3 *C* and *D*). Polyfunctional three-cytokine $CD4^+$ T cell response was not observed in any of the five clinical non-responders (Fig. 3*D*). The relative amount of each cytokine and chemokine per cell was measured for each functional population based upon the mean fluorescence intensity (MFI) of each functional marker, which is related to the quantitative expression of that function on a per-cell basis (18). An increase in IFN-γ MFI was associated with increasing polyfunctionality of T cells (Fig. 4). Both $CD4^+$ and $CD8^+$ NY-ESO-1 antigen specific T cells from patients with clinical benefit produced more than one cytokine, had higher IFN-γ MFIs, and produced more cytokine per cell after treatment with anti-CTLA-4 antibody.

Evaluation of NY-ESO-1 Protein Expression. Tumor tissue from 6 patients (IMF-2, -3, -8, -11, -15, and -19) was available for immunohistochemical analysis. All tissues were sampled before commencement of CTLA-4 treatment. Four of six available specimens were from patients experiencing clinical benefit and two were from non-responders. Tissues from two NY-ESO-1 seropositive patients (patient IMF-3 and IMF-8) were positive for NY-ESO-1 by immunohistochemistry, while tissues from patient 2, 11, 15, and 19 were negative for NY-ESO-1. Immunopositivity was present in >75% and <25%, respectively, of the tumor area. A representative NY-ESO-1 positive tumor (patient IMF-3) is shown in Fig. S1.

Discussion

We have conducted extensive immunologic monitoring on a panel of patients selected from a large cohort of metastatic melanoma patients treated with ipilimumab. Late onset of CRs or PRs was noted, occurring after more than 12 weeks of treatment in the majority of responding patients. Some patients demonstrated overt progression before eventually responding or showing disease stabilization. Such delayed onset of response and transient tumor progression should be considered when reviewing the progress of patients during ipilimumab treatment. This progression followed by regression represents an atypical pattern of response for cytotoxic therapies, yet may be the norm and has been reported for immunotherapies. A novel set of response criteria for immunotherapies has been proposed for more accurate evaluation of patients treated with novel biologics (20).

While prior and ongoing clinical trials have shown that anti-CTLA-4 antibody therapy can have potent anti-tumor effects in a subset of metastatic melanoma patients, there have been fewer studies of its functional impact on human antigen-specific immune responses (21, 22). For example, there have only been anecdotal observations of induced immunity to NY-ESO-1 in patients with ovarian and prostate cancer treated with ipilimumab (12, 23, 24). A detailed study of antibody as well as T cell responses is necessary for understanding the immunological impact of anti-CTLA-4 antibody therapy.

We chose to monitor immune responses to NY-ESO-1 in this panel of patients because of its relatively high frequency of expression in melanomas (30–40%) and because of the extensive knowledge of both antibody and T cell epitopes (16). Five of 15 patients had either preexisting (three of five) anti-NY-ESO-1 antibodies or developed it after anti-CTLA-4 treatment (two of five). It is intriguing that these five seropositive patients for NY-ESO-1 were all among the eight patients that experienced clinical benefit from treatment with ipilimumab. None of the seven patients who experienced PD was seropositive for NY-ESO-1 either before or after anti-CTLA-4 therapy.

As many patients on this trial had received their original diagnosis or prior therapy at other institutions, the availability of specimens for NY-ESO-1 protein analysis by immunohistochem-

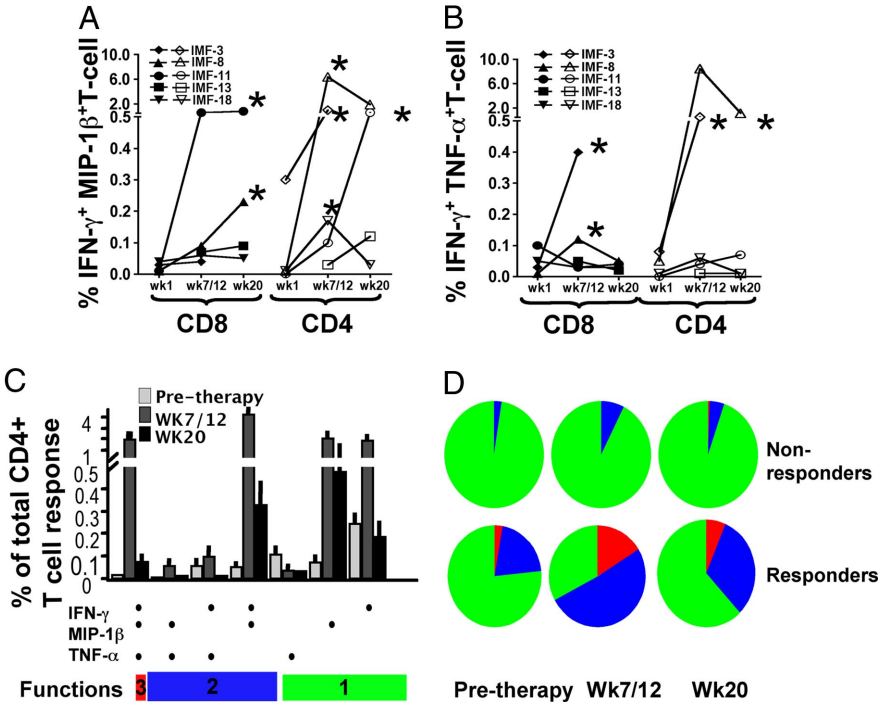

Fig. 3. CTLA-4 blockade induced T cell responses are polyfunctional. NY-ESO-1 specific T cells secrete MIP-1β and IFN-γ (*A*), or TNF-α and IFN-γ (*B*). Samples scored as positive are indicated with * over week1 baseline. (*C*) Functional composition of the CD4$^+$ T cell response. All possible combinations of three function responses are shown on the *x* axis. Responses are grouped and color-coded. (*D*) Each slice on the pie charts represents the fraction of the total response that is CD4$^+$ T cell positive for a given number of functions; (*Top*) calculated from five clinical non-responders, (*Bottom*) from eight patients with evidence of clinical benefit.

istry was limited. However, two of six available tissues were positive for NY-ESO-1 protein expression. Both positive samples were from patients who experienced clinical benefit. However, in previous studies, the presence of anti-NY-ESO-1 CD4$^+$ and CD8+ T cells did not always correlate with NY-ESO-1 expression in tumor tissues, and NY-ESO-1 expression varied according to tumor site and during the course of disease (25, 26). Tissue in the present study was obtained from samples collected before anti-CTLA-4 treatment was initiated. In the future, a study is warranted to examine the presence of tumor-associated antigens such as NY-ESO-1 during the course of treatment.

All five NY-ESO-1 seropositive patients receiving ipilimumab had CD4$^+$ and CD8$^+$ T cells against NY-ESO-1 following treatment. It was surprising to note that the three patients (IMF-3, -16, and -18) who were seropositive at baseline did not have significant NY-ESO-1 T cell responses before ipilimumab therapy. This stands in apparent contrast to prior studies in which the majority of NY-ESO-1 seropositive patients also had specific T cell responses and may be due to immunosuppression from prior cytotoxic chemotherapy in our cohort. One NY-ESO-1 seronegative clinical responder also had a NY-ESO-1 CD4$^+$ T cell response, possibly related to prior vaccination with NY-ESO-1. In these six patients who mounted NY-ESO-1 specific T cell responses and showed clinical benefit, T cells (either CD4$^+$ or CD8$^+$ or both) showed characteristics of antigen-specific polyfunctionality, with increased production of IFN-γ, MIP-1β, and/or TNF-α to NY-ESO-1 stimulation following anti-CTLA-4 antibody treatment. Among five of seven clinical non-responders analyzed, only one had a detectable but weak NY-ESO-1 CD4$^+$ T cell response that did not show polyfunctionality. Moreover, this patient did not develop an antibody response to NY-ESO-1.

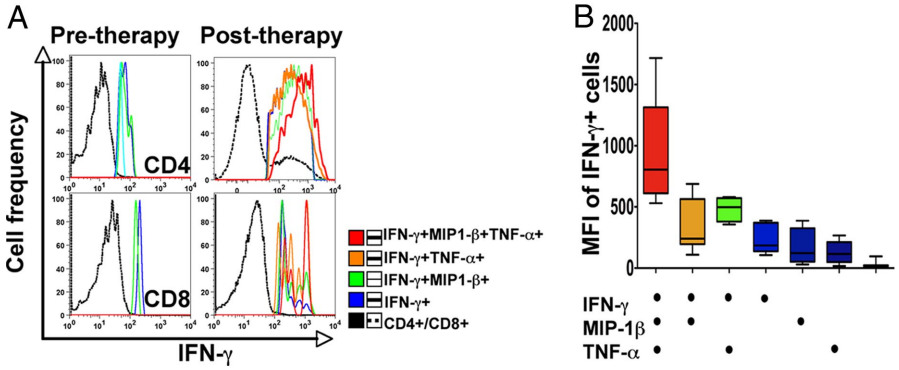

Fig. 4. Polyfunctional NY-ESO-1 antigen-specific T cells secreted higher levels of IFN-γ after anti-CTLA-4 antibody treatment. (*A*) IFN-γ fluorescence of CD4$^+$ (*Top*) and CD8$^+$ (*Bottom*) T cells with different functional properties before and after anti-CTLA-4 antibody treatment. Representative example from patient IMF-8. (*B*) Compiled IFN-γ MFI of CD4$^+$ T cells of different cytokine combinations.

Polyfunctional T cell responses have been shown to correlate with improved control of viral replication in infectious disease studies, suggesting that an effective vaccine should attempt to elicit this type of response (18, 27). NY-ESO-1 specific T cell responses induced by CTLA-4 blockade were polyfunctional and were not observed in clinical non-responders, suggesting that NY-ESO-1 antigen-specific polyfunctional T cells induced by CTLA-4 blockade might be potent effectors. Interestingly, these cells did not secrete high amounts of the cytokine IL-2, as had been shown for vaccinia-induced polyfunctional T cells (18), which may be attributed to different T cell signaling pathways triggered by CTLA-4 blockade.

We hypothesize that NY-ESO-1-specific T cell precursors may have been present at baseline in patients with clinical benefit and that their frequency was increased as a result of enhanced proliferation and/or decreased apoptosis during CTLA-4 blockade. It is not clear at this time whether the observed increase in T cell functionality was a consequence of the increase in the number of effectors to NY-ESO-1 leading to facilitated detection or whether it was a direct result of modulation of CTLA-4 activity or signaling on T cell precursors giving them functional and growth advantages.

It is not our intention to suggest that such immune responses to NY-ESO-1 were the only mediators of tumor growth control. It is more likely that detection of NY-ESO-1 immunity may be a surrogate for a more general immune activation to multiple targets, which may have allowed for a favorable clinical outcome. Several recent reports have shown that vaccination with NY-ESO-1 as a recombinant protein, formulated either with saponin-based adjuvant ISCOMATRIX or with cholesterol-bearing hydrophobized pullulan, induced strong NY-ESO-1 antibody as well as $CD4^+$ and $CD8^+$ responses in the majority of patients (28–31). It is reasonable to consider combining such vaccines with ipilimumab in an attempt to induce an integrated immune response to NY-ESO-1. Even if the specific response to NY-ESO-1 is not directly responsible for the anti-tumor effect, mechanisms such as intermolecular epitope spreading could result from the initial recognition by NY-ESO-1 specific T cells.

Materials and Methods

Patient Eligibility and Screening. Eligible patients had a diagnosis of unresectable stage III/IV melanoma and had experienced PD or intolerance to at least one prior systemic therapy. All pathology was confirmed at Memorial Sloan-Kettering Cancer Center. Patients were more than18 years of age and had normal hematologic and organ function and an Eastern Cooperative Oncology Group status of 0 or 1. Exclusion criteria included any other prior invasive malignancy, autoimmune disease or active infection, or pregnant or lactating women. All patients signed an informed consent approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. Additional blood draws were obtained for investigational purposes after patients gave further informed consent.

At baseline, patients underwent a complete history and physical examination, laboratory evaluation, and radiographic imaging appropriate for tumor evaluation. The same imaging modality was used for initial screening and posttherapy evaluation. Initial clinical responses were evaluated at week 12 and onward and adjudicated using modified World Health Organization criteria. Toxicity was assessed using Common Terminology Criteria for Adverse Events, version 3.0.

NY-ESO-1 Serum Antibody. NY-ESO-1-specific antibodies were measured in serum by ELISA before therapy (week 1) and after therapy at week 12, 20 or later time points as previously described (32).

Detection of NY-ESO-1 Antigen Specific T-Cells. For presensitization *in vitro*, thawed PBMCs were resuspended in 10% pooled human serum RPMI medium 1640 and plated at 2.5×10^6 cells per well. Autologous PBMCs (5×10^6/ml) were irradiated (8 Gy), pulsed with 20-mer NY-ESO-1 overlapping peptides (10 μg/ml) for one hour, and cultured with responder cells at antigen-presenting cell: responder ratio of 1:1 with cytokine IL-2 (10 IU/ml) and IL-15 (10 ng/ml) (33). Effector cells were harvested and restimulated with peptide (1 μg/ml) at day 10 and were enumerated for intracellular IL-2, MIP-1β, TNF-α, IFN-γ, and CD107, asecretion at the indicated time points by flow cytometry. $CD4^+$ and $CD8^+$ T cell responses were analyzed by using NY-ESO-1 antigen in the form of overlapping peptides covering the entire sequence of NY-ESO-1 pulsed on antigen-presenting cells (or unpulsed for specificity controls). Isotype controls for antibodies used in flow cytometry included the appropriate fluorochrome conjugated or unconjugated mouse IgG1 or IgG2a (DAKO). Cells were analyzed by flow cytometry using a CYAN flow cytometer with Summit software (DakoCytomation California Inc.). Analyses were performed using FlowJo software (version 8.7; TreeStar, Inc.).

Imunohistochemical Detection of NY-ESO-1 Protein. Formalin-fixed paraffin-embedded tissues from six patients were available (Table 1). Immunohistochemical detection of NY-ESO-1 was performed as previously described using monoclonal antibody E978 (34). Immunohistochemical staining was graded according to immunopositive tumor areas as follows: focal: $<$5%, +: 5%–25%; ++: $>$25%-50%; +++: $>$50%-75%; ++++: $>$75%.

Data Analysis and Statistical Methods. The data analysis program Simplified Presentation of Incredibly Complex Evaluations (SPICE software, version 4.1.6) was used to analyze and generate graphical representations of T cell responses detected by polychromatic flow cytometry. All values used for analyzing proportionate representation of responses are background subtracted. Specificity of NY-ESO-1 T cell responses was considered significant if $>$3-fold over control (unpulsed target cells). Patients were considered to have an increase in T cell response if the frequency of T cells detected in at least one posttherapy sample exceeded that found in the baseline sample by 3-fold, and the response was at least 0.1%.

ACKNOWLEDGMENTS. We thank Dr. M. Roederer for providing the SPICE software. This work was supported by NIH P01CA33049, Swim Across America, the Experimental Therapeutics Center of MSKCC and Ludwig Trust. JDW was supported by a Damon Runyon-Lilly Clinical Investigator Award and the Melanoma Research Alliance.

1. Walunas TL, Bakker CY, Bluestone JA (1996) CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med* 183:2541–2550.
2. Krummel MF, Allison JP (1995) CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 182:459–465.
3. Read S, Malmstrom V, Powrie F (2000) Cytotoxic T Lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192:295–302.
4. Salomon B, *et al.* (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431–440.
5. Karandikar NJ, Vanderlugt CL, Walunas TL, Miller SD, Bluestone JA (1996) CTLA-4: A negative regulator of autoimmune disease. *J Exp Med* 184:783–788.
6. Brunner MC, *et al.* (1999) CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol* 162:5813–5820.
7. Leach DR, Krummel MF, Allison JP (1996) Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271:1734–1736.
8. van Elsas A, Hurwitz AA, Allison JP (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and Granulocyte/Macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190:355–366.
9. van Elsas A, *et al.* (2001) Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. *J Exp Med* 194:481–489.
10. Hodi FS, *et al.* (2003) Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci USA* 100:4712–4717.
11. Phan GQ, *et al.* (2003) Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 100:8372–8377.
12. Hodi FS, *et al.* (2008) Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. *Proc Natl Acad Sci USA* 105:3005–3010.
13. Korman AJ, Peggs KS, Allison JP (2006) Checkpoint blockade in cancer immunotherapy. *Adv Immunol* 90:297–339.
14. Ribas A, *et al.* (2007) Tremelimumab (CP-675,206), a cytotoxic T lymphocyte associated antigen 4 blocking monoclonal antibody in clinical development for patients with cancer. *Oncologist* 12:873–883.
15. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 5:615–625.
16. Gnjatic S, *et al.* (2006) NY-ESO-1: Review of an immunogenic tumor antigen. *Adv Cancer Res* 95:1–30.

17. Seder RA, Darrah PA, Roederer M (2008) T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 8:247–258.
18. Precopio ML, *et al.* (2007) Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 204:1405–1416.
19. Downey SG, *et al.* (2007) Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin Cancer Res* 13:6681–6688.
20. Hodi FS, *et al.* (2008) Novel efficacy criteria for antitumor activity to immunotherapy using the example of ipilimumab, an anti-CTLA-4 monoclonal antibody. 2008 ASCO Annual Meeting, May 30th, Chicago USA, 3008 (abstr).
21. Maker AV, Attia P, Rosenberg SA (2005) Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. *J Immunol* 175:7746–7754.
22. Comin-Anduix B, *et al.* (2008) Detailed analysis of immunologic effects of the cytotoxic T lymphocyte-associated antigen 4-blocking monoclonal antibody tremelimumab in peripheral blood of patients with melanoma. *J Transl Med* 6:22.
23. Fong L, *et al.* (2008) CTLA-4 blockade for hormone refractory prostate cancer: Dose-dependent induction of CD8+ T cell activation and clinical responses. AACR, April 12th, San Diego, USA.
24. Liakou CI, *et al.* (2008) CTLA-4 blockade increases IFNgamma-producing $CD4^+$ $ICOS^{hi}$ cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci USA* 105:14987–14992.
25. Al-Batran SE, *et al.* (2005) Intratumoral T-cell infiltrates and MHC class I expression in patients with stage IV melanoma. *Cancer Res* 65:3937–3941.
26. Barrow C, *et al.* (2006) Tumor antigen expression in melanoma varies according to antigen and stage. *Clin Cancer Res* 12:764–771.
27. Duvall MG, *et al.* (2008) Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur J Immunol* 38:350–363.
28. Davis ID, *et al.* (2004) Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4+ and CD8+ T cell responses in humans. *Proc Natl Acad Sci USA* 101:10697–10702.
29. Uenaka A, *et al.* (2007) T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein. *Cancer Immun* 7:9–20.
30. Kawabata R, *et al.* (2007) Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients. *Int J Cancer* 120:2178–2184.
31. Tsuji K, *et al.* (2008) Induction of immune response against NY-ESO-1 by CHP-NY-ESO-1 vaccination and immune regulation in a melanoma patient. *Cancer Immunol Immunother* 57:1429–1437.
32. Adams S, *et al.* (2008) Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J Immunol* 181:776–784.
33. Valmori D, *et al.* (2007) Epitope clustering in regions undergoing efficient proteasomal processing defines immunodominant CTL regions of a tumor antigen. *Clin Immunol* 122:163–172.
34. Jungbluth AA, *et al.* (2001) Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. *Int J Cancer* 92:856–860.