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

Achim A. Jungbluthb, Neil H. Segala, Teresa S. Rasalana, Gregor Manukiana, Yinyan Xua, Ruth-Ann Romanc, ESO-1 seronegative clinical responder also had a NY-ESO-1 CD4+ T cell response 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 co-inhibitory 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 cell 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


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.

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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 IFNo-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 weeks 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-2, 16, and IMF-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
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) had tested 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), 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-3 and IMF-11) had either significant MIP-1β ‘IFN-γ’ or TNF-α ‘IFN-γ’ two-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. 3D). 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 to 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 biologies (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 antibody 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-

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**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.

**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 on negative control (unstimulated) samples and were placed consistently across samples (Bottom).

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**Table 1.** Summary of NY-ESO-1 antigen-specific T cell responses.
...try 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\(^+\)/H11001 and CD8\(^+\)/H11001 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. 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 week 1 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.

**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 were not observed in clinical non-responders, suggesting that NY-ESO-1 antigen-specific polyfunctional T cells 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 be 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, it is 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.

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 than 18 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. Patients were informed of the study and enrolled after 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.

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 x 10^6 cells per well. Autologous PBMCs (5 x 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). Effector cells were harvested and restimulated with peptide (1 µg/ml) at day 10 and were enumerated for intracellular IL-2, MIP-1α, TNF-α, IFN-γ, and CD107, ascretion 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.).

Immunohistochemical 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 E97B (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%.

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Supporting Information

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Fig. S1. Immunohistochemical staining for NY-ESO-1. Representative immunohistochemical staining for NY-ESO-1 with monoclonal antibody D978 in patient IMF-3. Homogeneously immunopositive tumor cells with E978-negative interspersed stroma and lymphocytes.
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<td>Colitis: 1, Diarrhea: 1, Dermatitis: 1, Pruritus: 1, Vitiligo: 1, Hepatitis: 1</td>
<td></td>
</tr>
</tbody>
</table>

CVT: Cisplatin, vinblastine and temozolomide; IFN: Interferon [alpha]-2b; CVB: Carboplatin, vinblastine and bleomycin. ULN: Upper limit of normal.

*Patients with progressive disease at 0.3 or 3 mg/kg received re-induction with ipilimumab at 10 mg/kg.