Supporting Information

Yuan et al. 10.1073/pnas.1110814108

SI Materials and Methods

Patient Demographics, Eligibility, and Selection. A total of 144 patients with metastatic melanoma at MSKCC or Yale University were analyzed, and complete demographics are available for the 99 patients treated at MSKCC, who ranged in age from 32 to 86 y, with a median age of 61 y. There was a male predominance of 66 men, compared with 33 women. The majority of patients had American Joint Committee on Cancer stage IV disease at the time of treatment (95%), with the remainder having unresectable stage III disease (5%). Most patients had a favorable Karnofsky performance status, with 92 patients at 80% or greater. Ninety-one patients had undergone treatment before the initiation of ipilimumab therapy, with 20 having four or more previous lines of therapy. All patients provided informed consent. Patients received ipilimumab at 0.3 mg/kg ($n = 1$), 3 mg/kg ($n = 4$), or 10 mg/kg ($n = 139$) every 3 wk for four treatments. Those without dose-limiting toxicity and with evidence of clinical benefit, defined as an objective response or SD at week 24, could continue to receive ipilimumab at 10 mg/kg every 12 wk until progressive disease, death, toxicity, or withdrawal of consent occurred. All five patients who started ipilimumab treatment at 0.3 mg/kg ($n = 1$) or 3 mg/kg ($n = 4$) eventually received ipilimumab at 10 mg/kg, following dose switching during the initial treatment phase or maintenance phase. Responses were adjudicated by recently proposed immune-related response criteria (1). All patients, with and without clinical benefit at week 24, were selected on the basis of having at least one pretherapy blood sample available for determination of NY-ESO-1 serology by standard ELISA.

Antibody Responses Against NY-ESO-1 Protein as Detected by ELISA. Sera were tested in fourfold serial dilutions, starting from 1/100, for IgG reactivity against full-length NY-ESO-1 and against a series of other control proteins including DHFR to assess specificity. Specificity of NY-ESO-1 humoral responses was established based on the absence of detectable reaction to control antigens. An extrapolation of antibody titers was performed based on a pool of healthy donor sera with no reactivity to NY-ESO-1. Results were expressed as reciprocal titers observed in a representative assay from at least three repeat experiments (which showed high reproducibility). Reciprocal titers were considered significant if greater than 100.

T-Cell Stimulation in Vitro. Thawed PBMCs were incubated at a 1:1 ratio with irradiated autologous PBMCs pulsed with 10 μg/mL of 20-mer NY-ESO-1 overlapping peptides spanning the entire protein for the in vitro stimulation (JPT Peptide Technologies). The cells were harvested at day 10, rechallenged with NY-ESO-1 overlapping peptides, and analyzed on day 11 for ICS. ICS was performed on cells obtained after 10 d T-cell stimulation, as described earlier (2). The following fluorochrome-labeled antibodies were used: APC-Cy7-CD8, FITC-IFN-γ, Pacific blue-CD3, APC-CD3, PE-Macrophage inflammatory protein (MIP)-1β, PE-Cy5-CD107a (BD Pharmingen), PE-Cy7-TNF-α (eBioscience), and ECD-CD4 (Beckman Coulter). Cells were analyzed by flow cytometry using a CYAN flow cytometer (DakoCytomation) and FlowJo software (version 9.1; TreeStar).

Data Analysis and Statistical Methods. All values used for analyzing T cells are background-subtracted. The specificity of NY-ESO-1 T-cell responses was considered significant if at least 0.1% of cells produced the indicated cytokine. Comparison of serum antibody frequency and $CD4^+$ and $CD8^+$ T-cell response between patients was performed by two-tailed Fisher exact test. Overall survival was analyzed by the Kaplan–Meier method stratified by baseline NY-ESO-1 antibody positivity. A time-dependent Cox model was fit to evaluate any association between NY-ESO-1 antibody, CD4, and/or CD8 positivity and survival. This model accounts for CD4/CD8 positivity at different time points.

1. Wolchok JD, et al. (2009) Guidelines for the evaluation of immune therapy activity in solid tumors: Immune-related response criteria. *Clin Cancer Res* 15:7412–7420.

2. Lin Y, et al. (2009) Optimization and validation of a robust human T-cell culture method for monitoring phenotypic and polyfunctional antigen-specific CD4 and CD8 T-cell responses. *Cytotherapy* 11:912–922.

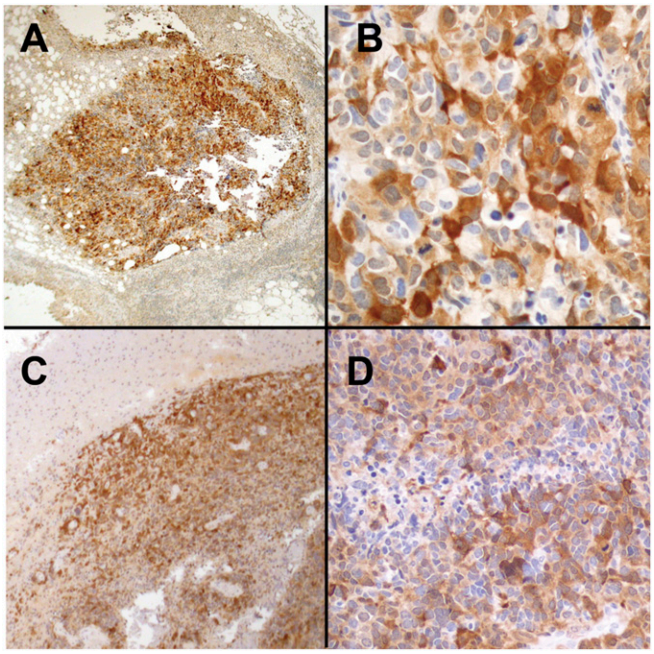


Fig. S1. Immunohistochemical staining for NY-ESO-1. Representative immunohistochemical staining for NY-ESO-1 with monoclonal antibody D978 in patient IMF-38. Representative images demonstrate positive staining at magnifications of ×4 (*A*) and ×20 (*B*) of the dermal metastasis. NY-ESO-1 positivity is demonstrated at magnifications of 4× (*C*) and 10× (*D*) of the brain metastasis.

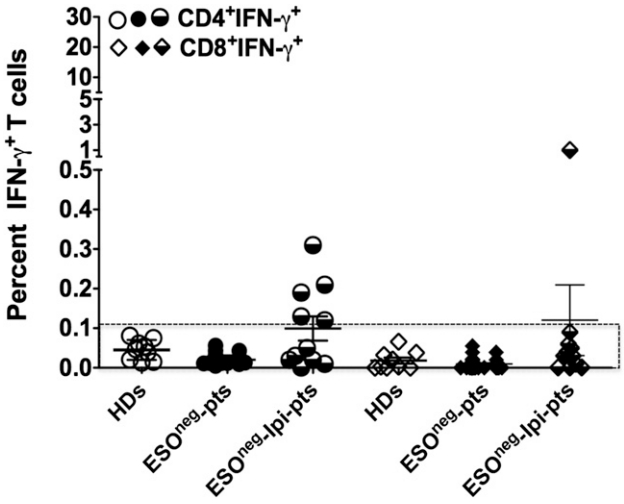


Fig. S2. NY-ESO-1–specific CD4$^+$ and CD8$^+$ T-cell response from healthy donors and patients with NY-ESO-1–seronegative melanoma with or without ipilimumab treatment. ICS staining was performed on three healthy donors (HDs); 10 patients with NY-ESO-1–seronegative metastatic melanoma without ipilimumab treatment (ESO^{neg}-pts) and 11 NY-ESO-1–seronegative patients treated with ipilimumab (ESO^{neg}-Ipi-pts) after a 10-d culture with NY-ESO-1 overlapping peptide. The frequency of NY-ESO-1–specific CD4$^+$IFN-γ^+ and CD8$^+$IFN-γ^+ for NY-ESO-1–seronegative patients not treated with ipilimumab are 0.02 ± 0.01% and 0.01 ± 0.013%, respectively. The frequency of CD4$^+$IFN-γ^+ and CD8$^+$IFN-γ^+ for healthy donors are 0.045 ± 0.025% and 0.018 ± 0.02%, respectively. These data support the use of 0.1% as the threshold value for a positive response to therapy for CD4$^+$IFN-γ^+ and CD8$^+$IFN-γ^+ T cells. Five of 11 NY-ESO-1–seronegative patients treated with ipilimumab had detectable CD4$^+$ T-cell response to NY-ESO-1; one patient (IMF-11) developed NY-ESO-1–specific CD8 T-cell response.

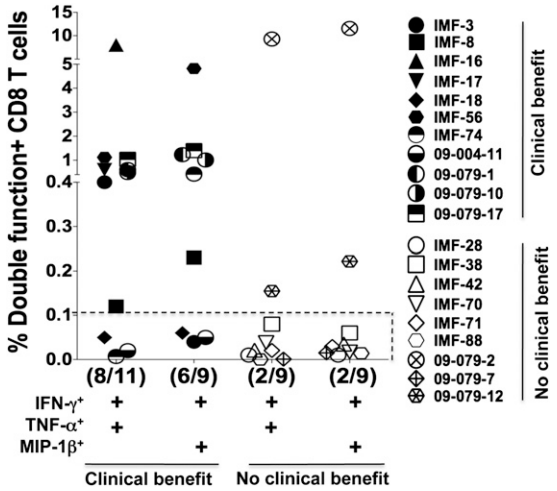
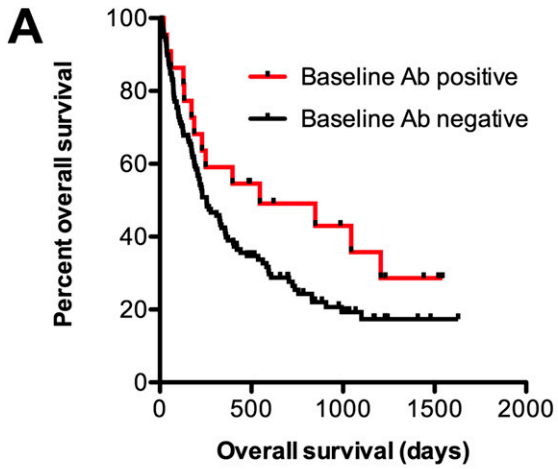


Fig. S3. CTLA-4 blockade-induced T-cell responses are polyfunctional. Maximal NY-ESO-1–specific $CD8^+$ T cells produce IFN-γ^+TNF-α^+ or IFN-γ^+MIP1β^+, as indicated at the bottom, at any timepoint during ipilimumab treatment. There is a trend of $CD8^+$IFN-γ^+TNF-α^+ T cells that patients who experienced clinical benefit (closed symbols; $n = 11$) had more double function-positive NY-ESO-1–specific $CD8^+$ T cells than patients who did not experience clinical benefit (open symbols; $n = 9$; $P = 0.07$).



B

Variable	HR	95% CI Lower	95% CI Upper	p-value
Ab + vs -	0.617	0.374	1.017	0.0584

C

Variable	HR	95% CI Lower	95% CI Upper	p-value
CD4 +	0.415	0.097	1.787	0.2380
CD8 +	0.181	0.047	0.706	0.0139

Fig. S4. Clinical correlations. (*A*) Association of NY-ESO-1 Ab status at baseline with overall survival. Curves of patients with baseline seropositivity to NY-ESO-1 ($n = 22$, red line) or baseline seronegativity to NY-ESO-1 ($n = 118$, black lines) were compared by using the log-rank (Mantel–Cox) test (median survival of baseline Ab positive patients, 546 d vs. 255 d in baseline Ab-negative patients; $P = 0.0973$). (*B*) Impact of seropositivity to NY-ESO-1 was also analyzed by a time-dependent Cox regressional model (CI, confidence interval). (*C*) Impact of presence of $CD4^+$ or $CD8^+$ T-cells to NY-ESO-1 was analyzed by a time-dependent Cox regressional model.

Table S1. NY-ESO-1 gene/antigen expression in 13 tumors from patients with melanoma treated with ipilimumab

Patient code	Tumor	NY-ESO-1 gene by RT-PCR	NY-ESO-1 expression by IHC
NY-ESO-1 seropositive			
IMF-8	Mel-329	+++	+++
IMF-16	Mel-332	++	+++
IMF-38	Mel-301	+	+++
09–079-1	Mel-445	+++	++
09–079-7	Mel-381	++	++
09–079-10	Mel-351	+++	ND
NY-ESO-1 seronegative			
IMF-44	Mel-308	–	–
IMF-52	Mel-352	–	–
IMF-55	Mel-362	+	–
IMF-68	Mel-392	–	–
09–004-5	Mel-363	–	–
09–079-6	Mel-394	–	–
09–079-11	Mel-398	+++	ND

Six of 13 patients had NY-ESO-1 antibody response. RT-PCR according to testis positive control. IHC: –, <5%; +, 5~25%; ++, 25~50%; +++, 50~75%; ++++, >75%. ND, not done.

Table S2. Correlation of NY-ESO-1 Ab status at any time point during ipilimumab treatment (includes seroconversions) with clinical status at week 24 after ipilimumab treatment

Response at wk 24	Total (%)	NY-ESO-1 seronegative (%)	NY-ESO-1 seropositive (%)
Clinical benefit	51 (35.4)	34 (30.1)	17 (54.8)
Complete response	4 (2.8)	2	2
Partial response	15 (10.4)	9	6
Stable disease	32 (22.2)	23	9
No clinical benefit	93 (64.6)	79 (69.9)	14 (45.2)
Total	144	113	31

Patients seropositive for NY-ESO-1 are more likely to experience disease control than seronegative patients [$P = 0.0186$, RR = 1.8 (1.2–2.8), two-tailed Fisher test].

Table S3. NY-ESO-1–specific response in NY-ESO-1–seronegative patients treated with ipilimumab

	Clinical benefit			Ab response		CD4 T-cell response			CD8 T-cell response		
Patient code	Wk 12	Wk 24	Survival, mo	Pre	Post	Pre	Post	Polyf	Pre	Post	Polyf
Clinical benefit											
IMF-11	CR	CR	49+	–	–	++	++	++	++	++	++
IMF-13	SD	SD	29+	–	–	–	–	–	–	–	–
09–079-3	PD	SD	17+	–	–	+	+	–	–	–	–
09–079-4	SD	SD	17+	–	–	–	+	–	–	–	–
09–079-8	SD	SD	17+	–	–	–	–	–	–	–	–
Total				0/5	0/5	2/5	3/5	1/5	1/5	1/5	1/5
No clinical benefit											
IMF-4	PD	DOD	7	–	–	–	–	–	–	–	–
IMF-6	PD	DOD	5	–	–	+	+	–	–	–	–
IMF-9	PD	DOD	6	–	–	–	–	–	–	–	–
IMF-15	PD	DOD	4	–	–	–	–	–	–	–	–
IMF-19	PD	DOD	8	–	–	–	–	–	–	–	–
09–079-6	PD	PD	6	–	–	+	+	–	–	–	–
Total				0/6	0/6	2/6	2/6	0/6	0/6	0/6	0/6

NY-ESO-1 Ab titer: –, negative; +, 100~1,000; ++, 1,000~10,000; +++, >10,000. NY-ESO-1 T-cell response: –, <0.1%; +, 0.1~0.5%; ++, 0.5~5%; +++, >5%. Polyfunctional T-cell response is defined as T cells producing double functions for IFN-γ, TNF-α, MIP-1β, CD107a; and the value ≥0.1%. Patient IMF-11 received vaccination with NY-ESO-1 protein and imiquimod in an independent study conducted 18 mo before receiving anti–CTLA-4 treatment. DOD, disease of death; PD, progressive disease; polyf, polyfunctionality of T cells.

Table S4. Patterns of humoral and cellular immune responses to NY-ESO-1

Category	NY-ESO-1 Ab response	NY-ESO-1 CD4 T-cell response	NY-ESO-1 CD8 T-cell response	Clinical benefit (%)	Individual patients	
					Clinical benefit	No clinical benefit
I	+	+	+	9/11 (81.8)	IMF-3, 8, 16, 17, 18, 56, 74; 09-79-01, 17	IMF-38, 09-079-02
II	+	–	+	1/2 (50)	09-079-10	09-079-12
III	+	+	–	1/5 (20)	09-004-11	IMF-28, 42, 70, 88
IV	+	–	–	0/2	NA	IMF-71, 09-079-7

NA, not applicable.