

## Functional T Cells Targeting NY-ESO-1 or Melan-A Are Predictive for Survival of Patients With Distant Melanoma Metastasis

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Submitted October 25, 2011; accepted February 15, 2012; published online ahead of print at [www.jco.org](http://www.jco.org) on April 23, 2012.

Supported by Grants No. SFB 685 (B.W., C.G.), GK 794 (H.Z.), and PA 361/14-1 (G.P.) from the Deutsche Forschungsgemeinschaft; by Grant No. EU-FP7-259679 from the European Commission (E.D.); and by Grant No. IG 11746 from the Associazione Italiana per la Ricerca sul Cancro and by the Harry J. Lloyd Charitable Trust and Regione Toscana Regional Health Research Program 2009 (M.M.).

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Terms in [blue](#) are defined in the glossary, found at the end of this article and online at [www.jco.org](http://www.jco.org).

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/12/3015-1835/\$20.00

DOI: 10.1200/JCO.2011.40.2271

### ABSTRACT

#### Purpose

To analyze the prognostic relevance of circulating T cells responding to NY-ESO-1, Melan-A, MAGE-3, and survivin in patients with melanoma with distant metastasis.

#### Patients and Methods

We examined 84 patients with follow-up after analysis (cohort A), 18 long-term survivors with an extraordinarily favorable course of disease before analysis (> 24 months survival after first occurrence of distant metastases; cohort B), and 14 healthy controls. Circulating antigen-reactive T cells were characterized by intracellular cytokine staining after *in vitro* stimulation.

#### Results

In cohort A patients, the presence of T cells responding to peptides from NY-ESO-1, Melan-A, or MAGE-3 and the M category according to the American Joint Committee on Cancer classification were significantly associated with survival. T cells responding to NY-ESO-1 and Melan-A (hazard ratios, 0.29 and 0.18, respectively) remained independent prognostic factors in Cox regression analysis and were superior to the M category in predicting outcome. Median survival of patients possessing T cells responding to NY-ESO-1, Melan-A, or both was 21 months, compared with 6 months for all others. NY-ESO-1-responsive T cells were detected in 70% of cohort A patients surviving > 18 months and in 50% of cohort B patients. Melan-A responses were found in 42% and 47% of patients in cohorts A and B, respectively. In contrast, the proportion was only 22% for NY-ESO-1 and 23% for Melan-A in those who died within 6 months.

#### Conclusion

The presence of circulating T cells responding to Melan-A or NY-ESO-1 had strong independent prognostic impact on survival in advanced melanoma. Our findings support the therapeutic relevance of Melan-A and NY-ESO-1 as targets for immunotherapy.

*J Clin Oncol* 30:1835-1841. © 2012 by American Society of Clinical Oncology

### INTRODUCTION

The prognosis of patients with melanoma with unresectable distant metastasis is poor, with a median survival of 9 months and 5-year survival of less than 20%.<sup>1</sup> Lactate dehydrogenase (LDH) is the only well-accepted serum biomarker in malignant melanoma. It has been part of the [American Joint Committee on Cancer \(AJCC\) staging system](#) since 2001.<sup>2-4</sup> Other serum markers of melanoma cell origin (eg, S100b, MIA) have been investigated, but none are as yet widely accepted.<sup>5,6</sup> Current peripheral blood biomarkers do not allow the identification of either patients with the worst prognosis or patients who may survive longer term.

In contrast to factors related to or derived from the melanoma cell itself, immune-related biomark-

ers have rarely been described. Long-term survival even in advanced disease was observed in a subset of patients after treatment with interleukin-2 (IL-2)<sup>7</sup> or ipilimumab.<sup>8</sup> Those agents are believed to act indirectly through the immune system. Immune system-based prognostic markers might therefore be useful in tumor-free patients to indicate the likelihood of long-term survival and may serve as predictive markers for response to immunotherapies.

Thus far, interferon alfa (IFN- $\alpha$ )–induced autoantibodies and pretherapeutic serum cytokine profiles have been described to predict outcome after treatment with IFN- $\alpha$ .<sup>9,10</sup> C-reactive protein, IL-6, and IL-10 concentrations in serum have likewise been reported to serve as prognostic markers, but their specificity seems limited.<sup>11-14</sup> The humoral

immune response against TA90 has been found to be prognostically relevant in patients receiving an allogeneic melanoma vaccine.<sup>15,16</sup> Whether the frequency of circulating regulatory T cells, natural killer cells, myeloid-derived suppressor cells, or others does have prognostic impact has only been analyzed in small cohorts of patients with melanoma, with equivocal results.<sup>17-20</sup>

Specific immunotherapy of solid tumors mainly aims to induce or increase the number of T cells directed against epitopes of tumor antigens; it has been under intense investigation. Although a high frequency of circulating antigen-specific T cells after vaccination has been reported in many trials, correlations with clinical outcome have been notoriously inconclusive.<sup>21-23</sup> On the other hand, a direct impact of these types of cells has been demonstrated by impressive clinical responses in up to 70% of patients with melanoma on adoptive transfer of tumor-infiltrating lymphocytes or T-cell receptor-transduced lymphocytes.<sup>24,25</sup> T cells specific for melanoma-associated antigens have also been detected in the peripheral blood of patients with melanoma without vaccination or other immunotherapies.<sup>26,27</sup> So far, only a single, tetramer-based study has analyzed the correlation of spontaneously occurring circulating specific T cells in patients with melanoma with survival, but it did not apply functional assays and failed to report a prognostic impact.<sup>28</sup>

The aim of the present study was to investigate the prognostic relevance of functional circulating T cells responding to the tumor-associated antigens **NY-ESO-1**, **Melan-A**, **MAGE-3**, and **survivin** on overall survival of patients with melanoma with distant metastasis.

## PATIENTS AND METHODS

### Patients

Cryopreserved peripheral blood mononuclear cells (PBMCs) were accessed from the Departments of Dermatology, University Medical Centers of Tübingen and Essen (Tübingen and Essen, Germany), and the Department of Medical Oncology and Immunotherapy, University Hospital of Siena, (Siena, Italy). Peripheral blood lymphocytes were obtained from the Nijmegen Centre for Molecular Life Sciences (Nijmegen, the Netherlands). Additional fresh blood samples were received from the Blood Bank and Department of Dermatology, University Medical Center of Tübingen. PBMCs were immediately isolated from fresh blood by Ficoll-Hypaque density gradient centrifugation and cryopreserved until use.

Available biobanked samples from patients with melanoma who fulfilled the following criteria were obtained from the clinical centers: cohort A, patients with unresectable distant metastases at the time of blood draw and available follow-up data after blood draw; cohort B, no radiologic or clinical evidence of disease at blood draw,  $\geq 24$  months since first diagnosis of distant metastasis, history of visceral metastasis other than lung or elevated LDH (long-term survivors); cohort C, no history of cancer. All patients provided written informed consent for biobanking. This study was approved by the ethics committee in Tübingen (approvals 147/2011BO2 and 432/2011BO2).

### Detection of Antigen-Responsive T Cells

T-cell responses against NY-ESO-1, Melan-A, MAGE-3, and survivin were measured as described previously.<sup>29</sup> Briefly, cells were stimulated with protein-spanning overlapping peptides (1  $\mu\text{g}/\text{mL}$ ; PepMix; JPT Peptide Technologies, Berlin, Germany). After culture for 12 days, T cells were restimulated at a ratio of 1:2 with autologous, carboxyfluorescein succinimidyl ester (CFSE) –stained PBMCs (5  $\mu\text{mol}/\text{L}$  CFSE; Invitrogen, Karlsruhe, Germany) either unpulsed (negative control) or presenting one of the antigens in the presence of Golgi-Plug (1  $\mu\text{L}/\text{mL}$ ; BD Biosciences, Toronto, Ontario, Canada). After 12 hours of cocubation, Fc receptors were blocked with Gamunex (human immunoglobulin; Bayer, Le-

**Table 1.** Patient Characteristics and Results of Survival Analysis Based on Kaplan-Meier Method

Factor	Patients			1-Year Survival Rate*		P†
	No.	%	% Dead	%	95% CI	
Age, years						.755
< 55	41	48.8	73.2	35.8	20.9 to 50.7	
$\geq 55$	43	51.2	81.4	50.1	35.0 to 65.2	
Sex						.715
Male	56	66.7	75.0	40.1	27.0 to 53.2	
Female	28	33.3	82.1	48.9	30.1 to 67.7	
M category						.024
M1a or b	22	26.5	68.2	58.7	37.9 to 79.5	
M1c	61	73.5	82.0	36.3	24.0 to 48.6	
NY-ESO-1-responsive T cells						< .001
Present	36	42.9	61.1	63.3	47.4 to 79.2	
Absent	48	57.1	89.6	27.5	14.6 to 40.4	
Melan-A-responsive T cells						.011
Present	22	31.9	63.6	59.1	38.5 to 79.7	
Absent	47	68.1	83.0	37.2	23.1 to 51.3	
Missing	15		80.0	37.5	12.0 to 63.0	
MAGE-3-responsive T cells						.042
Present	38	60.3	71.1	49.3	33.2 to 65.4	
Absent	25	39.7	92.0	28.0	10.4 to 45.6	
Missing	21		71.4	49.5	27.2 to 71.8	
Survivin-responsive T cells						.374
Present	8	15.1	62.5	58.3	21.8 to 94.8	
Absent	45	84.9	80.0	34.4	20.3 to 48.5	
Missing	31		77.4	51.6	34.0 to 69.2	
Prior systemic treatments						
Anti-CTLA4						.218
Yes	9	10.7	44.4	64.8	32.5 to 97.1	
No	75	89.3	81.3	40.2	28.8 to 51.6	
Other immunotherapy						.260
Yes	39	46.4	74.4	49.7	33.6 to 65.8	
No	45	53.6	80.0	37.6	23.5 to 51.7	
Monochemotherapy						.505
Yes	22	26.2	72.7	36.2	14.4 to 58.0	
No	62	73.8	79.0	45.1	32.8 to 57.4	
Polychemotherapy						.802
Yes	7	8.3	71.4	35.7	74.5 to 74.5	
No	77	91.7	77.9	43.5	32.3 to 54.7	
Biochemotherapy						.447
Yes	7	8.3	57.1	53.6	14.2 to 93.0	
No	77	91.7	79.2	42.0	30.8 to 53.2	
Subsequent systemic treatments						
Anti-CTLA4						.421
Yes	18	21.4	61.1	54.5	31.2 to 77.8	
No	66	78.6	81.8	39.9	27.9 to 51.9	
Other immunotherapy						.656
Yes	17	20.2	88.2	47.1	23.4 to 70.8	
No	67	79.8	74.6	42.0	30.0 to 54.0	
Monochemotherapy						.171
Yes	36	42.9	86.1	37.6	21.5 to 53.7	
No	48	57.1	70.8	47.0	32.7 to 61.3	
Polychemotherapy						.164
Yes	19	22.6	89.5	35.1	13.0 to 57.2	
No	65	77.4	73.8	45.4	33.2 to 57.6	
Biochemotherapy						.713
Yes	12	14.3	91.7	66.7	40.0 to 93.4	
No	72	85.7	75.0	39.0	27.4 to 50.6	

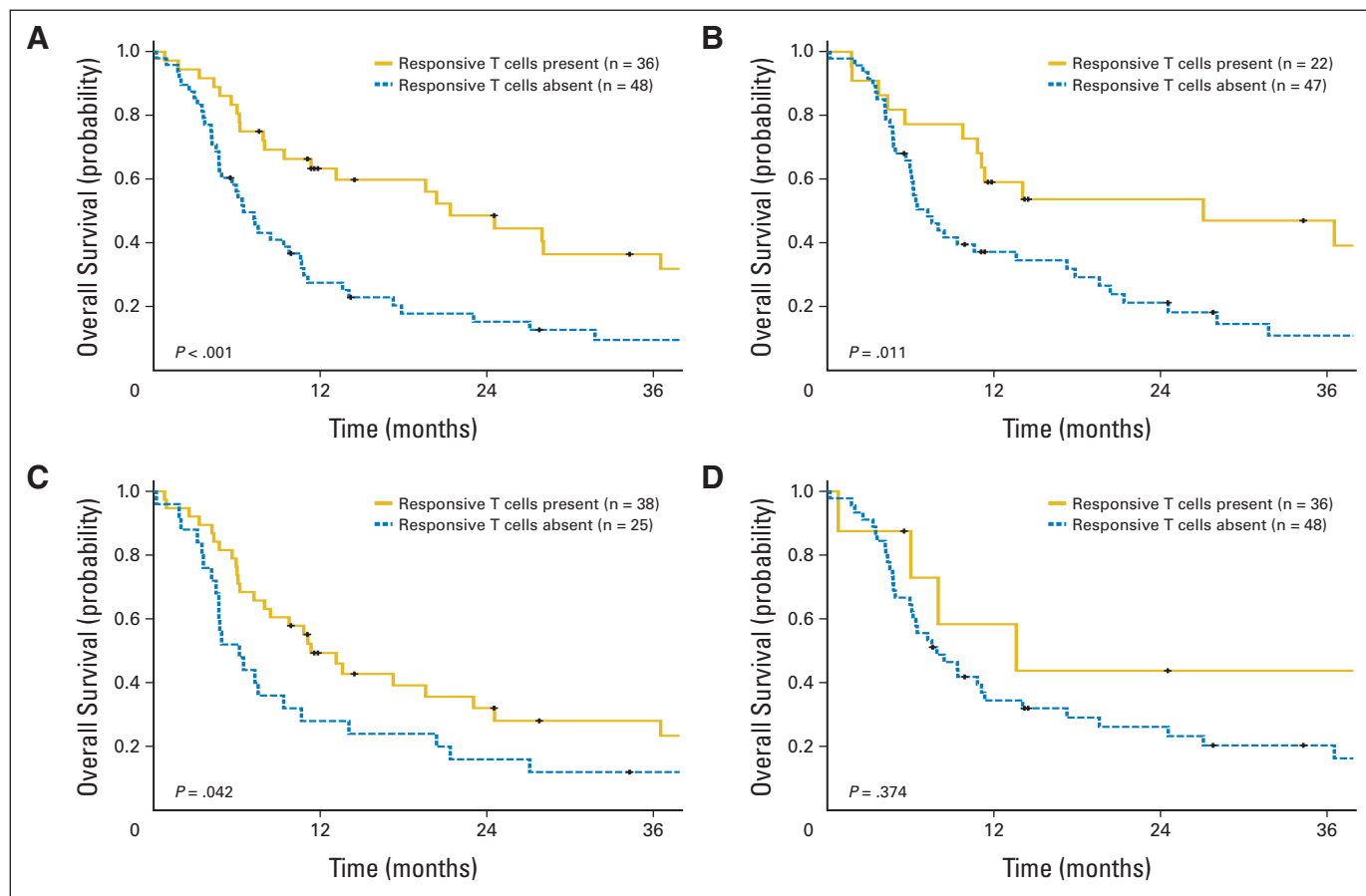
\*Two- and 3-year survival rates provided in Appendix Table A1, online only.  
†P values are results of log-rank tests, excluding patient cases with missing values.

verkusen, Germany), and dead cells were labeled with ethidium monoazide (Invitrogen, Karlsruhe, Germany). Cells were fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences) and stained with the following antibodies: CD3/Qdot655 and anti-CD4/Pacific Orange (Invitrogen); CD8/APC-H7, IL-4/APC, and IFN- $\gamma$ /PE-Cy7 (BD Biosciences); TNF/PE (Miltenyi Biotec, Bergisch Gladbach, Germany); IL-10/Pacific Blue and IL-17/PerCP-Cy5.5 (eBioscience, San Diego, CA); and IL-2/Alexa700 (BioLegend, San Diego, CA). Samples were measured immediately using LSR II and FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). After removal of the duplicates using the forward-scatter area versus forward-scatter height plot, autologous stimulator cells were excluded by gating on the CFSE-negative cells. Next, CD4+ and CD8+ cells were gated within viable CD3+ lymphocytes and analyzed separately for the production of cytokines. For each cytokine, we evaluated the percentage of cytokine-producing cells among all gated T cells in sample one (restimulated with antigen-pulsed PBMCs) and sample two (restimulated with unpulsed PBMCs). For each cytokine, antigen-responsive T cells were defined as present if the stimulation index was  $\geq 2$  (sample one divided by sample two) and a clearly separate cytokine-producing population distinguishable from the nonproducing cells was present in sample one, as described previously.<sup>30</sup> The patient was defined as having antigen-responsive T cells for the analyzed cytokine if these criteria were met either for CD8+ or CD4+ T cells. The interpretation of fluorescence-activated cell sorting data and the response evaluation were performed according to established criteria for intracellular cytokine staining, as described previously.<sup>29</sup> All experiments were performed and analyzed centrally by one investigator (H.Z.). To ensure the quality of samples and the functional capacity of T cells, the cytokine response after stimulation by influenza matrix protein 1 and nucleocapsid protein (1

$\mu\text{g/mL}$ ; PepMix; JPT Peptide Technologies) was assessed as for the tumor-associated antigens. Only patients with detectable influenza-responsive T cells were further analyzed. Assay reproducibility was assessed in 61 additional independent experiments performed exclusively in patients participating in this study. The initial result (detection of antigen-responsive T cells, yes or no) was confirmed in 51 of these assays (84%).

**Statistics**

The presence of antigen-responsive T cells was analyzed separately for NY-ESO-1, MAGE-3, Melan-A, and survivin. Additional prognostic factors considered were age (dichotomized using the median of the distribution), sex, AJCC M category (M1a or b v M1c), and systemic treatments applied before and after blood draw. Therapies were aligned to the following categories: treatment with anti-CTLA4 antibody, immunotherapy other than anti-CTLA4 antibody, monotherapy, polychemotherapy, and biochemotherapy. Follow-up time was defined from the date when blood was drawn for T-cell analysis to the date of last follow-up or death. Disease-specific survival probabilities were calculated, and only deaths resulting from melanoma were considered, whereas deaths resulting from other causes were regarded as censored events. Estimates of cumulative survival probabilities according to the Kaplan-Meier method were described together with 95% CIs and compared using log-rank tests. Median survival times (MSTs) are presented. Multivariable Cox proportional hazards analyses were used to determine the independent effect of prognostic factors. All variables were considered in multivariable analysis. In the first model (model A), patients with missing values of significant factors were excluded. In the second model (model B), missing values were dummy coded to allow the inclusion of all patients. Both models were established using backward and forward stepwise procedures.



**Fig 1.** Survival of patients with unresectable stage IV disease according to the presence or absence of T cells responding to (A) NY-ESO-1, (B) Melan-A, (C) MAGE-3, and (D) survivin peptides.

Remaining nonsignificant factors were assessed for potential confounding effects. Changes in the estimates of factors in a model by more than 5% were regarded as indicative for confounding. Results of the Cox model were described by means of hazard ratios (HRs) together with 95% CIs, and *P* values were based on the Wald test. Throughout the analysis, *P* values <.05 were considered statistically significant. All analyses were carried out using SPSS version 19 (SPSS, Chicago, IL).

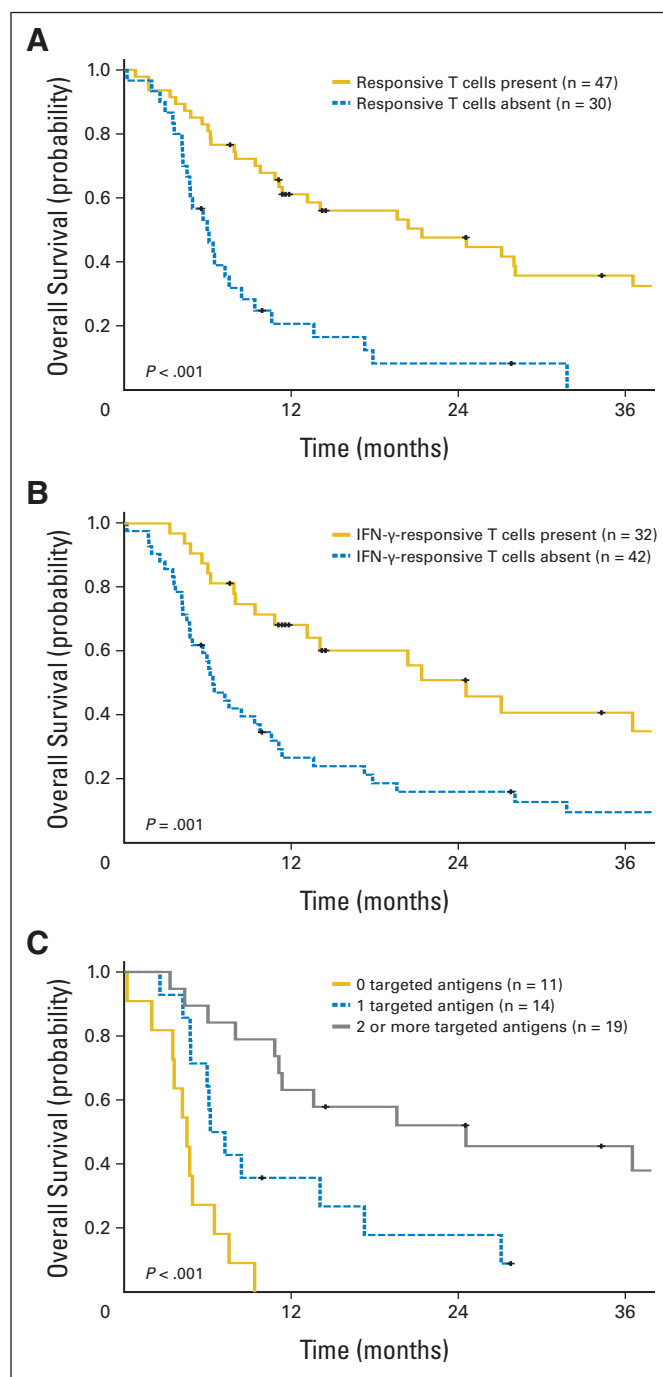
## RESULTS

### Patients

PBMCs initially collected for biobanking purposes were requested by the participating clinical centers according to the inclusion criteria. One of 117 samples was excluded from further analysis because influenza-responsive functional T cells could not be detected. The other 116 patients were assigned to three cohorts: 84 patients with unresectable distant metastasis and clinical follow-up after T-cell analysis (cohort A), 18 tumor-free long-term survivors (cohort B), and 14 healthy controls (cohort C). In cohort A (67% male), the MST was 9.7 months. Median follow-up was 24 months for 19 patients who were alive at the last follow-up and 6.5 months for 65 patients who died; 73.5% of patients were assigned to the M category M1c. Median age was 55 years, with an interquartile range (IQR) of 45 to 67 years. The 18 tumor-free long-term survivors of cohort B (also 67% male) had a median age of 56.5 years (IQR, 48 to 68 years). Healthy controls had a median age of 45 years (IQR, 41 to 59 years), and 50% were men (cohort C). No patient had received NY-ESO-1 or Melan-A vaccines before blood draw for T-cell analysis.

### T-Cell Analysis Correlates With Overall Survival

In univariate analysis of 84 cohort A patients, the presence of T cells responding to either NY-ESO-1, Melan-A, or MAGE-3 was associated with improved overall survival (Table 1; Fig 1; Appendix Table A1, online only). The most significant difference in survival for patients with responsive T cells versus those without was seen for NY-ESO-1 ( $P < .001$ ), with Melan-A less significant at  $P < .011$  and MAGE-3 at  $P < .042$ , whereas the presence or absence of T cells responsive to survivin peptides was not associated with survival in this analysis. The MST of patients possessing T cells responding to NY-ESO-1 and/or Melan-A was 21 months, compared with 6 months for all others (Fig 2A). The survival benefit was also evident when the analysis was limited to the detection of IFN- $\gamma$ -producing T cells (Fig 2B) and when CD4+ and CD8+ T cells were analyzed separately (Appendix Fig A1, online only). The individual frequencies of IFN- $\gamma$ -producing cells after stimulation with NY-ESO-1 and Melan-A for cohort A patients are listed in Appendix Table A2 (online only). As expected, the M category (Table 1) was also prognostic: the MST was 21 months for patients with soft tissue or lung metastases and normal LDH (M1a/b), whereas it was 8 months for those with other visceral metastases or elevated LDH (M1c;  $P = .024$ ). Multivariable Cox proportional hazards analysis of 53 patients with complete data on all significant factors (model A) showed that the presence of T cells targeting NY-ESO-1 (HR, 0.29;  $P = .001$ ) or Melan-A (HR, 0.18;  $P < .001$ ) were independently associated with a survival benefit, whereas for the M category and T cells responding to MAGE-3, no additional significant prognostic impact was found (Table 2). Model B included patients with missing data. In agreement with model A, this analysis of 83 patients confirmed the prognostic impact of NY-ESO-1- and Melan-A-responsive T cells, whereas significant independent roles of the M



**Fig 2.** (A) Patients with circulating T cells responding to Melan-A and/or NY-ESO-1 by producing at least one of six cytokines have a median survival time of 21 months, compared with 6 months for those who are negative for both. (B) Impact of Melan-A- and/or NY-ESO-1-responding interferon- $\gamma$ -positive T cells on survival. (C) Survival according to the number of targeted antigens per patient. Only patients with analysis of responses to all four antigens are considered.

category and MAGE-3 were likewise not observed (Table 3). A complete analysis of T-cell responses against all four antigens was performed in 44 patients. There was a strong correlation between the number of targeted antigens and survival. Patients with no response to any antigen, a response to a single antigen, or responses to at least two of the four antigens had an MST of 4, 6, or 24 months, respectively (Fig 2C).

**Table 2.** Final Model A of Multivariable Cox Proportional Hazards Analysis\*

Factor	Patients			HR	95% CI	P
	No.	%	% Dead			
M category						.943
M1a or b	18	34.0	77.8	0.97	0.48 to 2.00	
M1c	35	66.0	77.1	1		
NY-ESO-1-responsive T cells						<b>.001</b>
Present	23	43.4	60.9	<b>0.29</b>	<b>0.14 to 0.61</b>	
Absent	30	56.6	90.0	<b>1</b>		
Melan-A-responsive T cells						<b>&lt; .001</b>
Present	16	31.9	56.3	<b>0.18</b>	<b>0.08 to 0.42</b>	
Absent	37	68.1	86.5	<b>1</b>		
MAGE-3-responsive T cells						.341
Present	33	62.3	69.7	0.73	0.39 to 1.39	
Absent	20	37.7	90.0	1		

NOTE. Bold font indicates statistical significance.  
Abbreviation: HR, hazard ratio.  
\*Model A included 53 patient cases (those with missing values were excluded) and was adjusted for confounding effects of M category, presence of MAGE-3-responsive T cells and anti-CTLA4 treatment after T-cell analysis.

No association between survival and the frequency of influenza-specific IFN- $\gamma$ -producing CD4/CD8+ T cells was observed (data not shown).

**Antigen-Responsive T Cells in Long-Term Survivors**

T cells responding to NY-ESO-1 and Melan-A peptides were detectable in 50% and 47%, respectively, of long-term survivors in cohort B. Such T cells were also found in a high proportion of cohort A patients who survived > 18 months after analysis. In contrast, only 22% or 23% of patients who died within 6 months after T-cell analysis possessed detectable peripheral blood NY-ESO-1- or Melan-A-specific T cells, respectively. This was similar to the background level in

**Table 3.** Final Model B of Multivariable Cox Proportional Hazards Analysis\*

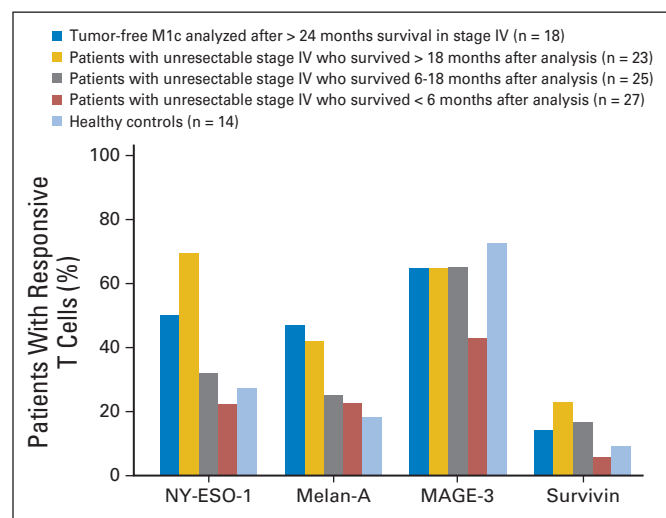
Factor	Patients			HR	95% CI	P
	No.	%	% Dead			
M category						.064
M1a or b	22	26.5	68.2	0.56	0.31 to 1.04	
M1c	61	73.5	82.0	1		
NY-ESO-1-responsive T cells						<b>.002</b>
Present	35	42.2	62.9	<b>0.42</b>	<b>0.24 to 0.73</b>	
Absent	48	57.8	89.6	<b>1</b>		
Melan-A-responsive T cells						<b>.002</b>
Present	22	32.4	63.6	<b>0.36</b>	<b>0.19 to 0.69</b>	
Absent	46	67.6	84.8	<b>1</b>		
MAGE-3-responsive T cells						.103
Present	37	59.7	73.0	0.62	0.35 to 1.10	
Absent	25	40.3	92.0	1		

NOTE. Bold font indicates statistical significance.  
Abbreviation: HR, hazard ratio.  
\*Model B included 83 patient cases (one had missing information for M category and was excluded; those with missing values were assigned their own category per characteristic; missing value categories for Melan-A-specific T cells [n = 15] and MAGE-3 specific T cells [n = 21] were included) and was adjusted for confounding effects of M category and presence of MAGE-3-responsive T cells.

healthy controls. MAGE-3-specific T cells were detectable in a high proportion of healthy controls (cohort C) but were equally frequent in patients of cohort A with the worst prognosis. Survivin-responsive T cells were rare and were found in only 14% of long-term survivors (Fig 3). Five recurrences but no deaths were observed among 18 cohort B patients after a median follow-up of 18 months. Interestingly, NY-ESO-1-responsive T cells were detected in only one (20%) of five patients who experienced relapse, in contrast to eight (62%) of 13 long-term survivors without recurrences thus far. Similarly, only one (25%) of four long-term survivors who experienced relapse versus seven (54%) of 13 long-term survivors who did not assessed for Melan-A had responsive T cells. Appendix Tables A3 and A4 (online only) list frequencies of antigen-responsive CD4+ and CD8+ T cells according to cytokine response to antigen stimulation.

**DISCUSSION**

Functional circulating tumor antigen-reactive T cells were found to have an important impact on survival of patients with advanced melanoma in this study. Melan-A- and NY-ESO-1-reactive T cells contributed independently to prognosis and predicted survival better than the AJCC M category in patients with distant metastasis. Moreover, patients possessing T cells reactive to more than one antigen had better survival than those with T cells reactive to fewer. This finding further supports but does not prove the hypothesis that antigen-specific T cells play a causative role in the control of tumor cells. To our knowledge, this is the first report to associate spontaneously occurring functional T-cell responses to survival in patients with melanoma. The proportion of patients with an NY-ESO-1- or Melan-A-stimulated T-cell response was likewise high in the cohort of tumor-free long-term survivors, indicating a prognostic value independent of tumor burden. MAGE-3-reactive T cells were frequently found in healthy controls, and the proportion was lower in all analyzed melanoma cohorts, suggesting a disease-related decrease of responsiveness to this antigen. The M category was strongly associated with prognosis according to univariate analysis but did not remain independently significant when



**Fig 3.** Proportion of patients with T cells responding to NY-ESO-1, Melan-A, MAGE-3, and survivin in long-term survivors, patients with unresectable stage IV disease with follow-up after T-cell assay, and healthy controls.

tested in conjunction with NY-ESO-1- and Melan-A-responsive T cells in any model of the multivariate analysis. Nevertheless, a strong trend was found according to model B ( $P = .064$ ), whereas model A negated any independent impact of the M category on survival. These differences might have been caused by the small sample size and need to be clarified in larger patient cohorts.

Biobanking efforts of different centers enabled us to include samples from patients for whom clinical follow-up was already available. Antigen-reactive T cells, either occurring spontaneously or after vaccination, have been investigated before, but their correlation with survival remains inconclusive, and most studies have not assessed any aspects of the actual functional integrity of the T cells.<sup>21,22,26,28,31</sup> Previous analyses have focused on different tumor-associated antigens and methods to analyze T cell status. Van Oijen et al<sup>28</sup> used tetramer staining to analyze spontaneously occurring T cells in 62 patients with distant metastasis. Specific T cells were detected in 68% of patients, but no correlation of any of these cells with survival was observed. It has been reported that the majority of tumor antigen-specific T cells in the blood of patients with melanoma are not able to lyse tumor cells or to produce any cytokines.<sup>26</sup> In contrast, here we analyzed functional response by measuring cytokine production on antigenic stimulation. To minimize the risk of underestimating the frequency of T cells in principle capable of responding to melanoma-associated antigens, we quantified production of six cytokines. Because of different and partially opposing functions of these cytokines in adaptive immunity, we also compared survival of cohort A patients based only on detection of IFN- $\gamma$ -releasing T cells, which confirmed the strong impact of these functional T cells on survival. The frequency of tumor antigen-reactive memory T cells in vivo is too low to be detected directly ex vivo by intracellular cytokine staining. Therefore, we initially applied a limited 12-day in vitro stimulation to ensure expansion of memory and not naive T cells to a measurable extent. For stimulation, nested overlapping 15-mer peptides, spanning entire protein antigens, were used as stimuli, allowing us to include patients of any HLA type. Furthermore, considering the increasing appreciation of the importance of T helper responses for tumor rejection over the last few years,<sup>32,33</sup> it is noteworthy that both CD8 as well as CD4 responses are stimulated and analyzed simultaneously with this approach.<sup>34</sup>

The prognostic impact of spontaneously occurring memory T cells responsive to NY-ESO-1 and Melan-A was not restricted to patients with limited stage IV disease; 73.5% of cohort A patients presented with visceral metastases other than lung and/or elevated LDH. The analysis of their T-cell responses enabled us to identify patients with a chance of long-term survival, even among those in the M1c category.

Our work provides a rationale for refining vaccination and T-cell transfer strategies by targeting Melan-A and NY-ESO-1. Poor clinical responses in previous trials might be explained by tumor-induced escape mechanisms like downregulation of HLA class I or recruitment of regulatory T cells to metastatic sites. For upcoming specific immunotherapies, combinations with newly available agents targeting immune escape mechanisms should therefore be considered.

This analysis might also serve as a rationale for investigating the pretherapeutic detection of circulating T cells reactive to melanoma-associated antigens as a predictive marker for outcome after ipilimumab.<sup>8</sup> Although the mode of action of this agent is incompletely understood, it breaks tolerance by blocking CTLA-4 and amplifies preexistent memory immune responses, which might be detectable by our assay. Recent data implicate an association between outcome after ipilimumab treatment and the existence of post-treatment T-cell responses targeting NY-ESO-1.<sup>35,36</sup>

In conclusion, circulating functional T cells targeting melanoma-associated antigens in patients with melanoma with distant metastasis have strong prognostic impact. T cells responding to Melan-A peptides (HR, 0.18;  $P < .001$ ) or NY-ESO-1 peptides (HR, 0.29;  $P = .001$ ) were independently associated with a survival benefit and superior to the M category in predicting outcome according to [Cox regression analysis](#). In addition, our findings provide a rationale for pursuing vaccination and T-cell transfer strategies targeting Melan-A and NY-ESO-1.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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## GLOSSARY TERMS

**AJCC/UICC-TNM staging:** The TNM Classification of Malignant Tumours (TNM) is a cancer staging system that describes the extent of cancer in a patient's body. T describes the size of the tumor and whether it has invaded nearby tissue; N describes regional lymph nodes that are involved; M describes distant metastasis (spread of cancer from one body part to another). TNM is developed and maintained by the International Union Against Cancer (UICC) to achieve consensus on one globally recognized standard for classifying the extent of spread of cancer. The TNM classification is also used by the American Joint Committee on Cancer (AJCC). In 1987, the UICC and AJCC staging systems were unified into a single staging system. Prognosis of a patient is defined by the TNM classification.

**Cox regression analysis:** The Cox proportional hazards regression model is a statistical model for regression analysis of censored survival data. It examines the relationship of censored survival distribution to one or more covariates. It produces a baseline survival curve, covariate coefficient estimates with their standard errors, risk ratios, 95% CIs, and significance levels.

**MAGE-3:** Protein encoded by the MAGE A locus. *MAGE* genes are normally silent in normal tissues but expressed in several cancers.

**Melan-A:** A melanoma-related antigen. MART-1 is absent in all normal cells except for melanocytes and the retina. In addition, apart from melanomas, MART-1 has not been observed in any other cancers. Consequently, the MART-1 antigen is considered to have a melanocyte lineage.

**NY-ESO-1:** Also known as CTAG1B or cancer/testis antigen 1B, the gene codes for antigens recognized on neoplastically transformed T cells.

**Survivin:** IAPs suppress host cell death in response to viral infection. By binding to caspases, they directly inhibit apoptosis. Survivin and XIAP are members of this family, differing perhaps in binding to selective caspases. BIRC5: Tumor-associated protein belonging to the group of shared overexpressed antigens.