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Myeloid-Derived Suppressor Cells Predict Survival of Patients with Advanced Melanoma: Comparison with Regulatory T Cells and NY-ESO-1- or Melan-A–Specific T Cells

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Abstract

Purpose: To analyze the prognostic relevance and relative impact of circulating myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) compared with functional tumor antigen–specific T cells in patients with melanoma with distant metastasis.

Experimental Design: The percentage of $CD14^+CD11b^+HLA-DR^{-/low}$ MDSCs, $CD4^+CD25^+FoxP3^+$ Tregs, and the presence of NY-ESO-1- or Melan-A–specific T cells was analyzed in 94 patients and validated in an additional cohort of 39 patients by flow cytometry. Univariate survival differences were calculated according to Kaplan–Meier and log-rank tests. Multivariate analyses were performed using Cox regression models.

Results: NY-ESO-1–specific T cells, the M-category, and the frequency of MDSCs were associated with survival. The absence of NY-ESO-1–specific T cells and the M-category M1c independently increased the risk of death. In a second Cox model not considering results on antigen-specific T cells, a frequency of >11% MDSCs showed independent impact. Its association with survival was confirmed in the additional patient cohort. Median survival of patients with a lower frequency of MDSCs was 13 months versus 8 months for others ($P < 0.001$, combined cohorts). We observed a strong correlation between high levels of MDSCs and the absence of melanoma antigen–specific T cells implying a causal and clinically relevant interaction. No prognostic impact was observed for Tregs.

Conclusions: Circulating $CD14^+CD11b^+HLA-DR^{-/low}$ MDSCs have a negative impact on survival and inversely correlate with the presence of functional antigen–specific T cells in patients with advanced melanoma. Our findings provide a rationale to investigate MDSC-depleting strategies in the therapeutic setting especially in combination with vaccination or T-cell transfer approaches. *Clin Cancer Res; 20(6); 1601–9. ©2013 AACR.*

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Introduction

The prognosis of patients with melanoma with unresectable distant metastasis is poor, with a median survival time of 9 months (1). Lactate dehydrogenase (LDH) is the only well-established blood biomarker, and has been part of the AJCC staging system since 2001 (2). We have previously demonstrated that circulating functional T cells targeting certain melanoma-associated antigens have strong prognostic impact in patients with melanoma (3). Whether other circulating immune cells such as regulatory T cells or myeloid-derived suppressor cells also have prognostic impact has so far only been analyzed in small cohorts of patients with melanoma (4–7). In addition to prognostic information, which may influence therapeutic decisions, immune system–based biomarkers might serve as predictive markers for response to immunotherapies

Translational Relevance

The level of myeloid-derived suppressor cells (MDSC) in the peripheral blood is strongly associated with survival of patients with melanoma with distant metastasis. After further validation, the MDSC level may represents a candidate to serve as a prognostic factor for stratification/classification purposes and for patient counseling. The correlation between high levels of MDSCs and the absence of melanoma antigen–specific T cells emphasizes their key role in the adaptive immune system. Our observations provide a rationale to investigate a potential predictive function of the MDSC levels about outcome of immunotherapies and to investigate MDSC-depleting strategies in the therapeutic setting, especially in combination with vaccination or T-cell transfer approaches.

such as systemic high-dose interleukin (IL)-2 (8) or ipilimumab (9) and could shed light on the underlying mechanisms of (treatment-induced) immunologic tumor rejection.

Myeloid-derived suppressor cells (MDSC) are heterogeneous populations of immature cells of myeloid origin, at different stages of differentiation (6, 7, 10, 11). Various populations of MDSCs with several distinct phenotypes have been described over the last years, with a shared common suppressive function on adaptive and innate immunity (12, 13). Circulating MDSCs have been shown to correlate with grading, stage, and tumor burden (14–17) or with clinical outcome in patients with different types of cancer (18–20). At present, there are conflicting data on the prognostic significance of circulating MDSCs in melanoma (6, 21, 22).

Regulatory T cells (Treg) are essential for maintaining self-tolerance and are assumed to hamper antitumor immune responses (23). Tregs are overrepresented in the blood of patients with metastatic melanoma (4, 24–27), and specifically recognize a broad range of melanoma-associated antigens (28). They are highly enriched in the tumor microenvironment (29, 30) and suppress ambient immune cells in an antigen nonspecific manner (bystander effect) after activation by specific antigen through their T-cell receptor (23). Tregs are identified as expressing CD4, high levels of the α-chain of the IL-2 receptor (CD25), and the forkhead box P3 (FoxP3) transcription factor. The impact of circulating Tregs on the course of disease in patients with melanoma has only been analyzed in small patient cohorts thus far and was recently reviewed by Jacobs and colleagues (31).

The aim of this study was to investigate the prognostic relevance of circulating immune cell subsets including Tregs, MDSCs, and their relative impact compared with functional NY-ESO-1- and Melan-A–specific T cells on survival of patients with melanoma with distant metastasis.

Patients and Methods

Patients

Cryopreserved peripheral blood mononuclear cells (PBMC) were accessed from the Department of Medical Oncology and Immunotherapy (Siena, Italy) and the Departments of Dermatology (Essen and Tübingen, Germany). PBMCs had been immediately isolated from fresh blood by Ficoll/hypaque density gradient centrifugation and cryopreserved until usage. Inclusion criteria were presence of unresectable distant melanoma metastasis at the time of blood draw and available follow-up data after blood draw. If the number of available PBMCs allowed the full spectrum of analyses or if results of the analysis of NY-ESO-1 or Melan-A-responsive T cells was already known ($n = 48$) from the already published related project (3) patients were allocated to cohort 1. In samples with a low cell number, the analysis was limited to the *ex vivo* analysis of Tregs and MDSCs (cohort 2). All patients gave their written informed consent for biobanking. This study was approved by the Ethics Committee, University of Tübingen (approvals 425/2012BO2 and 524/2012BO2).

Analysis of immune cell subsets

All analyses were performed centrally by 2 investigators (A. Martens and H. Zelba). PBMCs were thawed using a step 2 procedure by incubation for 1 to 2 minutes in a 37°C water bath, followed by addition of equal amounts of 4°C cold RPMI 1640 (Life Technologies GmbH), before and after incubation for 5 minutes at room temperature. After thawing, different populations of immune cells were evaluated immediately using multicolor flow cytometry. For analysis of MDSCs and Tregs, Fc receptors were initially blocked with Gamunex (human immunoglobulin; Bayer), and dead cells were labeled for exclusion with ethidium monoazide (EMA; Invitrogen).

MDSCs were characterized by the $CD14^+CD11b^+HLA-DR^{-/low}$ phenotype (6, 7, 18, 19, 32). PBMCs were stained with CD3/PerCP, CD4/PerCP, CD8/PerCP, HLA-DR/PerCP-Cy5.5, CD11b/APC-Cy7 (BD Biosciences), and CD14/Pe-Cy7 (BioLegend). The percentage of MDSCs was calculated as the relation between $CD14^+CD11b^+HLA-DR^{-/low}$ cells and all viable PBMCs.

Tregs, which were characterized by the $CD25^+FoxP3^+$ phenotype (33), were stained with CD25/PE, CD4/PerCP, and CD8/APC-H7 (BD Biosciences). After fixation and permeabilization with Human FoxP3 buffer (BD Biosciences), cells were surface stained with CD3/Pacific Orange (Invitrogen) and intracellularly stained with FoxP3/Alexa647 (BD Biosciences). The percentage of Tregs was calculated as the relation between $CD25^+FoxP3^+$ cells and all $CD4^+$ cells.

NY-ESO-1- and Melan-A–specific T-cell responses were detected as described previously (3). Briefly, cells were stimulated with protein-spanning overlapping peptides (1 µg/mL; PepMix; JPT Peptide Technologies). After culture for 12 days, T cells were restimulated at a ratio of 1:2 with autologous, fluorescent-labeled PBMCs (5 µmol/L CFSE;

Invitrogen; replaced after February 2012 by 10 µmol/L Cell Proliferation Dye eFluor 450; eBioscience) either unpulsed (negative control) or presenting one of the antigens in the presence of Golgi-Plug (1 µL/mL; BD Biosciences) for 12 hours. Alternatively, if cell numbers were limited, T-cell cultures were restimulated by addition of peptides alone. After blocking with Gamunex and labeling with EMA cells were fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences) and stained with the following antibodies: CD3/Qdot655, CD4/Pacific Orange (both Invitrogen), CD8/APC-H7, IL-4/APC, IFN-γ/PE-Cy7 (all BD Biosciences), TNF/PE (Miltenyi Biotec), IL-10/Pacific Blue, IL-17/PerCP-Cy5.5 (both eBioscience), and IL-2/Alexa700 (BioLegend). After February 2012, subsequent analyses were performed with an optimized panel that was identical except for the following positions: CD3/eFluor605 (eBioscience), CD4/PerCP (BD Biosciences), TNF/FITC (BioLegend), IL-10/PE (eBioscience). Antigen-specific T cells were defined as being present if the following criteria were met for at least 1 of the 6 analyzed cytokines. The cytokine-producing cell population had to be clearly distinguishable from nonproducing cells and the ratio between the peptide-pulsed and -unpulsed samples had to be greater than 2. Samples were measured immediately using an LSR II and FACSDiva software (both BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.). A detailed illustration of the gating strategy for MDSCs and Tregs is presented in Supplementary Fig. S1. The gating strategy and data interpretation for the analysis of antigen-specific T cells was performed as described in detail elsewhere (3).

Statistics

The percentage of MDSCs and Tregs and age at the date of blood draw was dichotomized using the integral number next to the median of its distribution. In addition, gender and the AJCC M-category (M1a or b vs. M1c) and the presence or absence of NY-ESO-1- and Melan-A–specific T cells was considered in the analysis.

The M1a category includes patients with distant metastasis limited to nonvisceral soft-tissue and normal LDH. Patients with visceral metastasis are aligned to categories M1b in case of lung metastases and normal LDH or to M1c in case of visceral metastases other than lung and/or elevated LDH (34).

Follow-up time was defined from the date of blood draw of the analyzed sample to the date of last follow-up or death. Disease-specific survival probabilities have been calculated and only deaths because of melanoma have been considered, whereas deaths because of other causes were regarded as censored events. Estimates of cumulative survival probabilities according to Kaplan–Meier were described together with 95% confidence intervals (95% CI) and compared using log-rank tests. Median survival times (MST) are

Figure 1. Kaplan–Meier survival curves of nonresectable stage IV patients according to the frequency of circulating CD14$^+$ CD11b$^+$HLA-DR$^{-/low}$ MDSC (A), CD4$^+$CD25$^+$FoxP3$^+$ Tregs (B), the presence of NY-ESO-1–specific T cells (C), and according to the M-category (D).
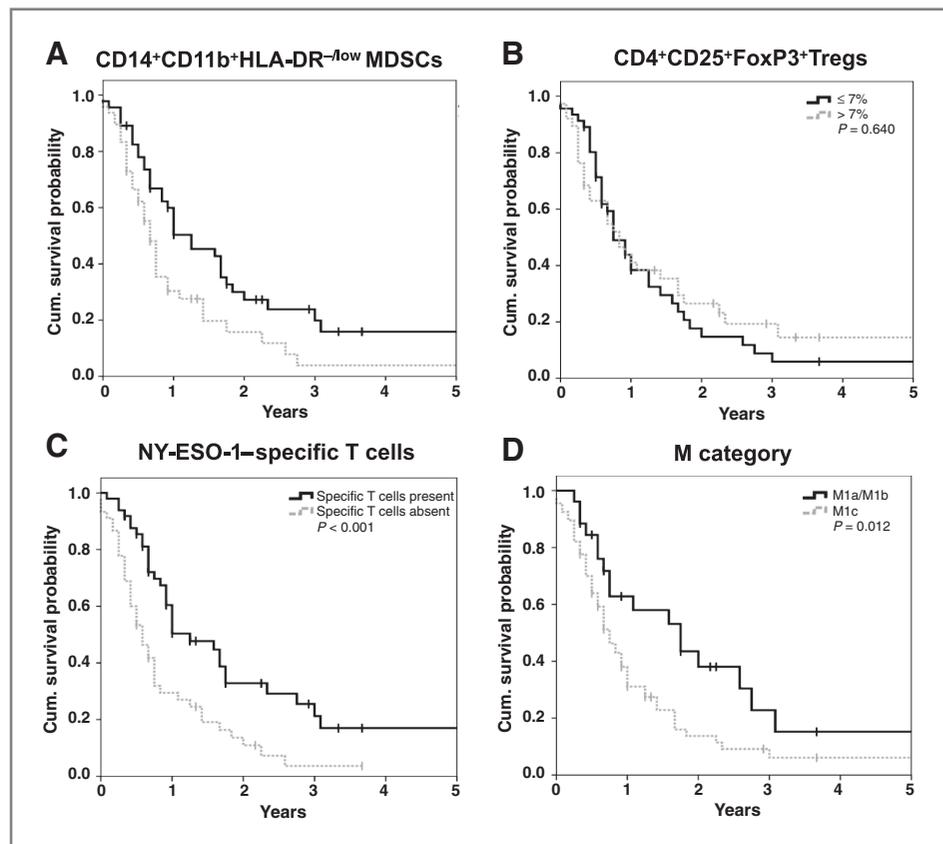
presented. Multivariable Cox proportional hazard analyses were used to determine the independent effects of prognostic factors. Patients with missing data in variables analyzed in the given Cox regression model were excluded. Models were established using backward and forward stepwise procedures. Remaining nonsignificant factors were assessed for potential confounding effects. Changes in the estimates of factors in a model by more than 5% were taken as indicative for confounding. Results of the Cox model were described by means of HRs together with 95% CIs, and P values were based on the Wald test. Associations between presence of antigen-specific T cells and frequencies of MDSCs were calculated by the χ^2 and Fisher exact tests. Throughout the analysis, P values less than 0.05 were considered statistically significant. All analyses were carried out using SPSS Version 21 (IBM SPSS).

Results

Patients

For the most detailed investigations, 94 patients were included with enough PBMCs available for the full spectrum of analyses including the detection of NY-ESO-1- and Melan-A–specific T cells (cohort 1). The MST was 10 months. Median follow-up was 15 months for patients who were alive at the last follow-up, and 8 months for

patients who died. Of 93 patients with known M-category, 67 were assigned to the M-category M1c (72%) and 13 (14%) each to M1a and M1b. Median age was 56 years with an interquartile range of 48 to 69 years, and 63.8% were male. NY-ESO-1- or Melan-A–specific T cells were detectable in 52.1% and 39.4% of patients, respectively. The median percentage of MDSCs and Tregs was 11.3% and 6.7%, respectively.

Survival analysis

Kaplan–Meier analysis of 94 patients (Table 1) showed that the presence of functional NY-ESO-1–specific T cells (P < 0.001), a low frequency of MDSCs (P = 0.011), and the M categories M1a/M1b (P = 0.012) were associated with prolonged survival. No differences in disease outcome were observed according to the frequency of Tregs (Fig. 1). A trend for a more favorable outcome was evident in patients with detectable Melan-A–specific T cells (P = 0.101) but no associations with prognosis were found for age or gender. In univariate analysis, the 1-year survival rate was highest for M1a/M1b patients (62.8%). The largest differences in 1-year survival rates were seen according to whether NY-ESO-1–specific T cells were present or absent (60.4% vs. 29.5%) and similarly according to the frequency of MDSCs (59.9% for ≤11% MDSCs vs. 30.4% for >11% MDSCs). The largest

Table 1. Patient characteristics and analysis of overall survival in cohort 1

Prognostic factor	n	%	% Dead	1-year survival rate (95% CI) (%)		2-year survival rate (95% CI) (%)		P^a
All patients	94	100.0	76.6	45.4	(35.0–55.9)	23.3	(13.7–32.8)	
Gender								0.861
Male	60	63.8	78.3	42.6	(29.7–55.5)	22.9	(11.0–34.7)	
Female	34	36.2	73.5	50.7	(33.3–68.1)	23.4	(7.5–39.3)	
Age								0.274
≤55 y	46	48.9	78.3	39.4	(24.8–53.9)	18.8	(6.0–31.5)	
>55 y	48	51.1	75.0	51.3	(36.6–66.0)	27.7	(13.8–41.6)	
M-category								0.012
M1a or M1b	26	28.0	65.4	62.8	(43.4–82.2)	43.5	(22.8–64.2)	
M1c	67	72.0	82.1	38.0	(26.0–50.0)	13.7	(4.1–23.3)	
Missing data	1							
NY-ESO-1–specific T cells								<0.001
Present	49	52.1	65.3	60.4	(46.1–74.7)	32.8	(17.8–47.8)	
Absent	45	47.9	88.9	29.5	(15.8–43.1)	13.6	(2.9–24.4)	
Melan-A–specific T cells								0.101
Present	37	39.4	73.0	55.9	(39.7–72.1)	32.6	(16.2–49.1)	
Absent	57	60.6	78.9	38.2	(24.8–51.5)	16.9	(5.9–27.9)	
Tregs								0.64
≤7%	46	54.8	80.4	43.8	(28.7–59.0)	17.7	(5.2–30.2)	
>7%	38	45.2	78.9	43.8	(27.8–59.8)	26.5	(12.0–41.0)	
Missing data	10							
MDSCs								0.011
≤11%	48	51.1	79.2	30.4	(16.4–44.3)	15.8	(3.0–28.6)	
>11%	46	48.9	73.9	59.9	(45.6–74.3)	30.0	(15.9–44.0)	

[a]P values are results of log-rank tests excluding cases with missing values.

Table 2. Cox models for disease-specific death

Prognostic factor	N^a	% Dead	Model 1		Model 2	
			HR (95% CI)	P	HR (95% CI)	P
M-category						
M1a or M1b	26	65.4%	1	**0.006**	1	**0.019**
M1c	67	82.1%	**2.2 (1.3–4.0)**		**1.9 (1.1–3.4)**	
NY-ESO-1–specific T cells					Not considered	
Present	48	66.7%	1	**0.041**		
Absent	45	88.9%	**1.7 (1.02–2.9)**			
Melan-A–specific T cells					Not considered	
Present	38	73.7%	1	0.067		
Absent	55	80.0%	1.6 (0.97–2.8)			
MDSCs						
≤11%	45	75.6%	1	0.199	1	**0.029**
>11%	48	79.2%	1.4 (0.84–1.3)		**1.7 (1.1–2.7)**	

NOTE: Significant findings in the final models are shown in bold. Model 1 was adjusted for the confounding effects of Melan-A–specific T cells and $CD14^+CD11b^+HLA-DR^{-/low}$ MDSCs and no confounding or significant interactions were detected in model 2.
^{a}One patient had an unknown M-category and was excluded.

differences in long-term outcome were seen with the presence or absence of NY-ESO-1–specific T cells corresponding to 17% vs. 3.4% survival at 5 years.

The M-category represented the most powerful predictor of survival with an increased risk of death for M1c patients (HR = 2.2; P = 0.006) according to multivariable Cox proportional hazards analysis. In addition, the absence of NY-ESO-1–specific T cells independently worsened the prognosis (HR = 1.7; P = 0.041). The frequency of MDSCs did not add independent prognostic information in this model (Table 2, model 1).

Next, we performed Cox proportional hazards analysis without consideration of NY-ESO-1- and Melan-A–specific T cells (Table 2, model 2). In this second model, a high level of MDSCs was independently associated with impaired overall survival (HR = 1.7; P = 0.029) in addition to the predictive function of the M-category (HR = 1.9; P = 0.019 for M1c).

The association of the frequency of MDSCs with survival was validated in a second confirmatory cohort of 39 patients. Prognosis was very similar to that observed in the first cohort confirming the worse overall survival of patients with a frequency of MDSCs >11% compared with those with ≤11% (P = 0.011). A trend was observed for the M-category (P = 0.103). No apparent association was observed for gender (P = 0.394), age (P = 0.733), or Tregs (P = 0.917). The patient's characteristics and the detailed survival analysis of cohort 2 can be found in Supplementary Table S1.

Analyzing both cohorts in combination, the MST of patients with a lower frequency of MDSCs was 13 months versus 8 months for the others (P < 0.001), whereas differences in prognosis according to the level of Tregs were not observed (Supplementary Fig. S1). The independent impact of the MDSCs frequency on survival is illustrated after

stratification into different combinations of M-category and MDSCs levels (Fig. 2).

The association between specific T cells and MDSCs

NY-ESO-1–specific T cells were more frequently observed in patients with low frequencies of MDSCs compared with those with high frequencies of MDSCs (65.2% vs. 39.6%; P = 0.015) and similar albeit not significant results (50.0% vs. 31.3%; P = 0.092) were observed for Melan-A–specific T cells (Fig. 3A). The same trend was observed when $CD4^+$ and $CD8^+$ T cells were analyzed separately (Fig. 3B). In the subgroup of
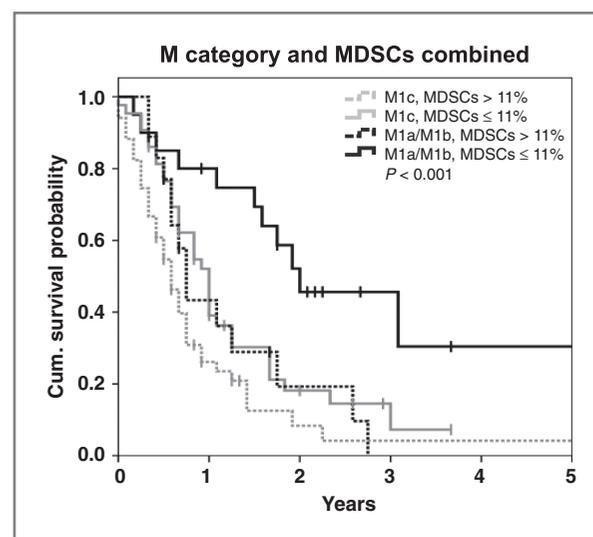

Figure 2. Kaplan–Meier survival curves according to the frequency of circulating MDSCs and M-category in combination demonstrating the independent impact of both factors.
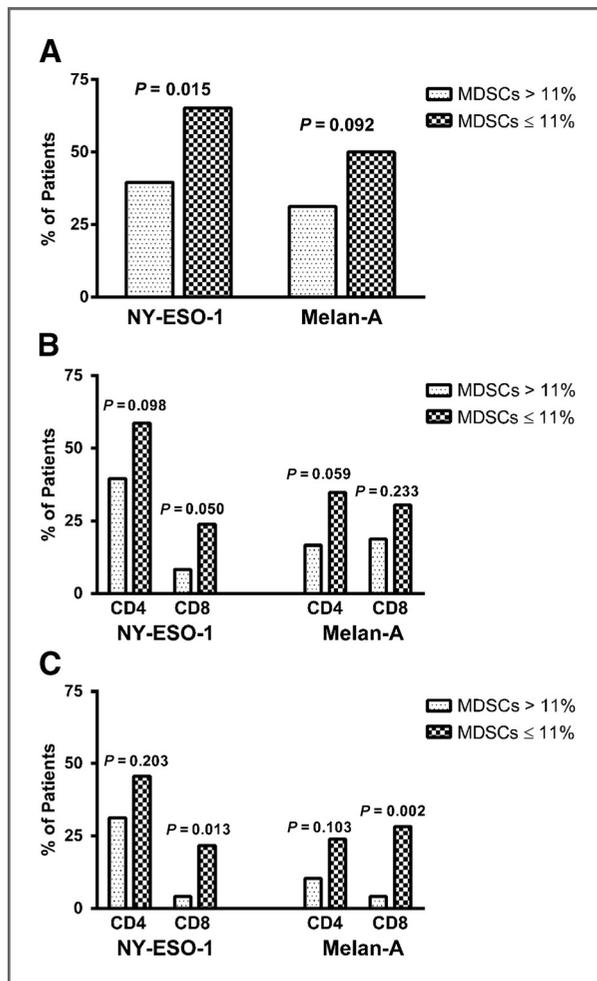
Figure 3. Correlation between MDSCs and antigen-specific T cells. NY-ESO-1- and Melan-A–specific T cells were more frequently observed in patients with low frequencies of MDSCs compared with those with high frequencies of MDSCs (A). The same trend was observed when CD4$^+$ and CD8$^+$ T cells were analyzed separately (B). The correlation was stronger for CD8$^+$ compared with CD4$^+$ T cells, if patients are analyzed for the presence of IFN-γ–releasing NY-ESO-1- or Melan-A–specific T cells (C).

IFN-γ–releasing T cells, the differences in the detection rate according the MDSCs frequencies were more evident in CD8$^+$ T cells than CD4$^+$ T cells (Fig. 3C). No association was found between the M category and the frequencies of MDSCs, neither in patients of cohort 1 nor in the entire population ($P = 1.000$ and 0.565, respectively).

Discussion

The strongest associations with favorable survival of patients with advanced melanoma in this study were the presence of NY-ESO–specific T cells ($P < 0.001$) and a low frequency of MDSCs with the CD14$^+$CD11b$^+$HLA-DR$^{-/\text{low}}$ phenotype ($P = 0.011$). In addition, the prognostic relevance of the M-category was likewise evident in our patients but less significant compared with the 2 immune cell subsets in univariate analysis ($P = 0.012$). The presence of NY-ESO-1–specific T cells predicted

favorable survival independent of the M-category and was the strongest factor to indicate the chance for long-term survival, as we already reported before (3). The frequency of MDSCs did not add independent prognostic information according to Cox regression analysis if analyzed in addition to NY-ESO-1–specific T cells. In contrast, the MDSCs blood level predicted survival independently of the AJCC M-category in patients with distant metastasis if NY-ESO-1 and Melan-A–specific T cells were not considered. We found a high degree of correlation between high levels of MDSCs and the absence of functional antigen-specific T cells, suggesting a causal interaction between these immune cell subsets.

It was shown before that MDSCs are responsible for induction of antigen-specific T-cell tolerance (12, 35–37), but the present study is the first to demonstrate directly the triple-correlation between MDSCs, antigen-specific T cells, and clinical outcome.

There are 2 possible interpretations of the inverse correlation between MDSCs and the detection of antigen-specific T cells observed in our study. The first interpretation is that differences in the detection rate of antigen-specific T cells in our assays reflect true *in vivo* differences in the frequency of preexisting specific memory T cells. In this case, the assumed interaction between MDSCs and the detection of specific T cells takes place *in vivo*. The second possibility is that MDSCs, which are present during the expansion period of our assay impact the proliferation of antigen-specific T cells *in vitro* depending on the MDSC frequency. If present at high concentrations, MDSCs would exert their suppressive effects on T cells during the expansion period making it less likely that T cells proliferate to detectable levels. We did not obtain conclusive results in additional MDSC-depletion experiments to investigate the impact of MDSCs in the expansion phase of our assay to detect antigen-specific T cells in 10 patients with available PBMCs (data not shown). Studies supporting both possibilities can be found in the literature, and an *in vivo* correlation was demonstrated in different mouse models (36, 38–40). However, coculture experiments show that MDSCs can effectively suppress not only CD3$^+$ T-cell proliferation in general (22, 41) but also CD4$^+$ and CD8$^+$ T cells (6, 17) and can affect the quality of antigen presenting DCs *in vitro* (37). Clarification in future studies is clearly required, because MDSCs might influence the results of broadly used *in vitro* assays analyzing specific T-cell responses after restimulation.

The prognostic impact of MDSCs was not restricted to patients with limited stage IV disease, because this study was driven by 72% of patients with visceral metastases other than lung, and/or with elevated LDH. The association of a high frequency of these cells with poor patient survival as shown in our study supports the hypothesis that MDSCs have a clinically relevant immunosuppressive function, at least in malignant melanoma (32). There are other recent studies that are in line with our findings. Filipazzi and colleagues analyzed patients with stage II/III melanoma with no evidence of disease and found a trend for better disease-free survival in patients with low amounts of CD14$^+$

CD11b$^+$HLA-DR$^{-/low}$ MDSCs compared with those with a high frequency of these cells ($P = 0.08$), but their patient cohort ($n = 33$) was very small (21). A negative impact of this particular MDSCs population on survival was also observed in a completely different setting by Walter and colleagues, who reported a strong association with outcome after multipeptide vaccination and survival in patients with renal cell cancer (19).

Generally, we did not observe substantial differences in the frequencies of Tregs or MDSCs according to prior therapies (Supplementary Table S2). In 2 patients who received systemic IL-2–based therapies within 4 weeks before blood draw for this study, unusually high frequencies of Tregs were observed. An increase of Tregs after IL-2 has been described before (42, 43). Therefore, we cannot rule out that the high levels were related to the treatment. Nevertheless, the lack of association between Tregs and survival remained basically unchanged if these patients were not considered in univariate analysis ($P = 0.509$). There was no pretreatment with fludarabine or cyclophosphamide and no other meaningful differences were observed in the frequencies of Tregs or MDSCs for other systemic treatments comparing patients treated within 4 weeks before blood draw to the others. Moreover, no differences were observed between 99 pretreated patients irrespective of the kind of therapy compared with those 34 without any prior systemic therapy. Based on these data, the impact of prior treatments in general, as well as the timing of blood draw seems to be limited about treatment-induced changes in frequency of these immune-cell subsets. Nevertheless, the treatment-related short-term effects need to be analyzed in subsequent studies.

Our findings highlight the role of MDSCs to serve as a prognostic marker, whereas the applicability of the detection of antigen-specific T cells to serve as a prognostic marker is limited. This is because 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, an initial *in vitro* stimulation period is applied to ensure expansion of memory T cells to a measurable extent. This requires the availability of fully equipped laboratories able to perform T-cell culturing and more sophisticated flow cytometry. A large number of PBMCs is also required and the minimum amount of blood draw is 25 mL to ensure sufficient cells to perform the analysis of antigen-specific T cells. The complex protocol requirements and data analysis require the involvement of specially trained and experienced personal but nevertheless a high degree of interobserver variability has to be assumed. Compared with the detection of NY-ESO-1-specific T cells, the analysis of MDSCs alone was less powerful to predict survival of patients with melanoma with distant metastases. Nevertheless, prognosis prediction was considerably improved by the analysis of MDSCs compared with the classification based on the M-category alone. Moreover, the analysis of MDSCs can be performed *ex vivo* without the need for *in vitro* presensitization and in contrast to the detection of antigen-specific T cells, only

PBMCs corresponding to 5 mL peripheral blood are needed. Therefore, the feasibility of analyzing the frequency of MDSCs in clinical practice can be assumed.

The threshold of the MDSCs frequency was established based on the median value measured among all cohort 1 patients (11.3%), but was adapted to the next integral number (11%) to allow reasonable clinical usage and further validation.

The high clinical relevance of specific T-cell immunity is again highlighted by our study and further provides a rationale to pursue vaccination and T-cell transfer strategies targeting NY-ESO-1, at least in melanoma. Our findings also provide a rationale to investigate MDSC-depleting strategies in the therapeutic setting especially in the frame of specific immunotherapies, such as anticancer vaccination or adoptive T-cell transfer. Another strategy in addition to their depletion might be to block the suppressive effects of MDSCs. This can be achieved by inhibition of the cyclooxygenase-2 (COX-2)/prostaglandin E2 pathway (39, 41, 44, 45) or after application of phosphodiesterase inhibitors (40).

In contrast to MDSCs, the level of circulating Tregs was not associated with prognosis in our patients. This finding is in contrast to an initial report by Baumgartner and colleagues (5), who analyzed 14 patients with melanoma. No prognostic relevance has been reported in patients with head and neck cancer (46), but poor survival was described in patients who had high levels of circulating Tregs in ovarian (47) and renal cell cancer (48). Schwartzentruber and colleagues even observed higher blood levels of Tregs in patients with clinical response after high-dose IL-2/vaccine treatment compared with those with progressive disease (49). Summarizing the prognostic role of circulating Tregs, in contrast to other malignancies, there is no compelling clinical evidence either in the literature, or based on our own data reported here, to suggest an important unfavorable prognostic impact of their cell frequencies in the peripheral blood of patients with melanoma.

In conclusion, circulating CD14$^+$CD11b$^+$HLA-DR$^{-/low}$ MDSCs have strong prognostic impact in patients with melanoma with distant metastasis and are inversely correlated with the presence of functional antigen-specific T cells. A frequency greater than 11% was independently associated with poor survival and was as important as the M-category in predicting outcome according to Cox regression analysis. The role of MDSCs to serve as a prognostic marker in stage IV melanoma needs to be confirmed in independent studies. Our findings warrant further investigation of MDSCs-depleting strategies in the therapeutic setting especially in combination with vaccination or T-cell transfer approaches.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

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