

Myeloid-derived suppressor cells predict survival of advanced melanoma patients: comparison with regulatory T cells and NY-ESO-1- or Melan-A-specific T cells

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Running head: Prognostic impact of MDSCs and Tregs in stage IV melanoma

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Statement of translational relevance

The level of myeloid-derived suppressor cells (MDSC) in the peripheral blood is strongly associated with survival of melanoma patients with distant metastasis. After further validation, the MDSC level may represent a candidate to serve as a prognostic factor for stratification/classification purposes and for patient counselling. 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 regarding outcome of immunotherapies and to investigate MDSC-depleting strategies in the therapeutic setting, especially in combination with vaccination or T-cell-transfer approaches.

Abstract

PURPOSE:

To analyze the prognostic relevance and relative impact of circulating myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) compared to functional tumor- antigen-specific T cells in melanoma patients 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 vs. 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 advanced melanoma patients. Our findings provide a rationale to investigate MDSC-depleting strategies in the therapeutic setting especially in combination with vaccination or T-cell-transfer approaches.

Introduction

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

Myeloid-derived suppressor cells (MDSCs) 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 (Tregs) are essential for maintaining self-tolerance and are assumed to hamper anti-tumor immune responses (23). Tregs are overrepresented in the blood of patients with metastatic melanoma (4, 24-27), and specifically recognise 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 non-specific 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 melanoma patients has only been analyzed in small patient cohorts thus far and was recently reviewed by Jacobs *et al* (31).

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

Patients and Methods

Patients

Cryopreserved peripheral blood mononuclear cells (PBMCs) 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 two investigators (A. M. and H. Z.). PBMCs were thawed using a step two procedure by incubation for 1-2 min in a 37°C water bath, followed by addition of equal amounts of 4°C cold RPMI 1640 (Life Technologies GmbH, Darmstadt, Germany), before and after incubation for 5 min 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, Leverkusen, Germany), and dead cells were labeled for exclusion with ethidium monoazide (EMA, Invitrogen, Karlsruhe, Germany).

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, Toronto, Canada) and CD14/Pe-Cy7 (BioLegend, Fellbach, Germany). 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, Berlin, Germany). After culture for 12 days, T cells were re-stimulated at a ratio of 1:2 with autologous, fluorescent-labelled PBMCs (5 µM CFSE; Invitrogen; replaced after February 2012 by 10µM Cell Proliferation Dye eFluor 450; eBioscience, San Diego, USA) 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, Bergisch Gladbach, Germany), 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 one of the six analyzed cytokines: The cytokine-producing cell population had to be clearly distinguishable from non-producing cells and the ratio between the peptide-pulsed and unpulsed samples had to be greater than two. Samples were measured immediately using an LSR II and FACSDiva software (both BD Biosciences). Data were analyzed using FlowJo

software (Tree Star Inc, Ashland, USA). A detailed illustration of the gating strategy for MDSCs and Tregs is presented in Supplemental Fig. 1. 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 non-visceral 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 due to melanoma have been considered, whereas deaths due to other causes were regarded as censored events. Estimates of cumulative survival probabilities according to Kaplan-Meier were described together with 95% confidence intervals (95%-CIs) and compared using log rank tests. Median survival times (MST) are 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 non-significant 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 hazard ratios (HR) 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 Chi square and Fisher's 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, Chicago, Illinois, USA).

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 median survival time (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 (IQR) 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 (Figure 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 one-year survival rate was highest for M1a/M1b patients (62.8%). The largest differences in one-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 $\leq 11\%$ MDSCs vs. 30.4% for $> 11\%$ MDSCs). The largest 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 to those with $\leq 11\%$ ($p=0.011$). 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 Supplemental Table 1.

Analyzing both cohorts in combination, the MST of patients with a lower frequency of MDSCs was 13 months vs. 8 months for the others ($p<0.001$), while differences in prognosis according to the level of Tregs were not observed (Supplemental Figure 1). The independent impact of the MDSCs frequency on survival is illustrated after stratification into different combinations of M-category and MDSCs levels (Figure 2).

The association between specific T cells and MDSCs

NY-ESO-1-specific T cells were more frequently observed in patients with low compared to 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 Interferon- γ (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 $p=0.565$, respectively).

Discussion

The strongest associations with favorable survival of advanced melanoma patients in this study were the presence of NY-ESO-specific T cells ($p < 0.001$) and a low frequency of MDSCs with the $CD14^+CD11b^+HLA-DR^{-/low}$ phenotype ($p = 0.011$). In addition, the prognostic relevance of the M-category was likewise evident in our patients but less significant compared to the two 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 two 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 pre-existing 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 ten 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). On the other hand, co-culture 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 re-stimulation.

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 which are in line with our findings. Filipazzi *et al.* analyzed stage II/III melanoma patients 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 to 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 *et al.*, who reported a strong association

with outcome after multi-peptide 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 (Supplemental Table 2). In two 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 pre-treatment 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 four weeks before blood draw to the others. Moreover, no differences were observed between 99 pretreated patients irrespective of the kind of therapy compared to 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 regarding 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 inter-observer variability has to be assumed. Compared to the detection of NY-ESO-1-specific T cells the analysis of MDSCs alone was less powerful to predict survival of melanoma patients with distant metastases. Nevertheless, prognosis prediction was considerably improved by the analysis of MDSCs compared to the classification based on the M-category alone. Moreover, the analysis of MDSCs can be performed *ex vivo* without the need for *in vitro* pre-sensitization 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 anti-cancer 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 *et al.* (5), who analyzed 14 melanoma patients. 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 *et al.* even observed higher blood levels of Tregs in patients with clinical response after high-dose IL-2/vaccine treatment compared to 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 melanoma patients.

In conclusion, circulating CD14⁺CD11b⁺HLA-DR^{-/low} MDSCs have strong prognostic impact in melanoma patients 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.

References

1. Neuman HB, Patel A, Ishill N, Hanlon C, Brady MS, Halpern AC, et al. A single-institution validation of the AJCC staging system for stage IV melanoma. *Ann Surg Oncol* 2008;15:2034-41.
2. Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* 2001;19:3635-48.
3. Weide B, Zelba H, Derhovanessian E, Pflugfelder A, Eigentler TK, Di Giacomo AM, et al. Functional T cells targeting NY-ESO-1 or Melan-A are predictive for survival of patients with distant melanoma metastasis. *J Clin Oncol*. 2012;30:1835-41.
4. McCarter MD, Baumgartner J, Escobar GA, Richter D, Lewis K, Robinson W, et al. Immunosuppressive dendritic and regulatory T cells are upregulated in melanoma patients. *Ann Surg Oncol* 2007;14:2854-60.
5. Baumgartner JM, Gonzalez R, Lewis KD, Robinson WA, Richter DA, Palmer BE, et al. Increased survival from stage IV melanoma associated with fewer regulatory T Cells. *J Surg Res* 2009;154:13-20.
6. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. *Cancer Res* 2010;70:4335-45.
7. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with

modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol* 2007;25:2546-53.

8. Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* 1999;17:2105-16.

9. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-23.

10. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni V, Mocellin S, et al. IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. *J Immunol* 2009;182:6562-8.

11. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res* 2005;65:3044-8.

12. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;9:162-74.

13. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. 2012;12:253-68.

14. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother* 2009;58:49-59.

15. Eruslanov E, Neuberger M, Daurkin I, Perrin GQ, Algood C, Dahm P, et al. Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer. *Int J Cancer* 2012;130:1109-19.
16. Yuan XK, Zhao XK, Xia YC, Zhu X, Xiao P. Increased circulating immunosuppressive CD14(+)HLA-DR(-/low) cells correlate with clinical cancer stage and pathological grade in patients with bladder carcinoma. *J Int Med Res* 2011;39:1381-91.
17. Schilling B, Sucker A, Griewank K, Zhao F, Weide B, Gorgens A, et al. Vemurafenib reverses immunosuppression by myeloid derived suppressor cells. *Int J Cancer* 2013;133:1653-63.
18. Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, et al. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* 2011;118:2254-65.
19. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Muropeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med* 2012;10.
20. Kalathil S, Lugade AA, Miller A, Iyer R, Thanavala Y. Higher Frequencies of GARP+CTLA-4+Foxp3+ T Regulatory Cells and Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma Patients Are Associated with Impaired T-Cell Functionality. *Cancer Res* 2013;73:2435-44.
21. Filipazzi P, Pilla L, Mariani L, Patuzzo R, Castelli C, Camisaschi C, et al. Limited Induction of Tumor-cross-reactive T Cells without a Measurable Clinical Benefit in Early Melanoma Patients Vaccinated with Human Leukocyte Antigen-Class I-Modified Peptides. *Clin Cancer Res* 2012;18:6485-96.

22. Gros A, Turcotte S, Ahmadzadeh M, Wunderlich JR, Dudley ME, Rosenberg SA. Myeloid cells obtained from the blood but not from the tumor can suppress T cell proliferation in patients with melanoma. *Clin Cancer Res* 2012; 18:5212-23.
23. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;133:775-87.
24. Nicholaou T, Ebert LM, Davis ID, McArthur GA, Jackson H, Dimopoulos N, et al. Regulatory T-cell-mediated attenuation of T-cell responses to the NY-ESO-1 ISCOMATRIX vaccine in patients with advanced malignant melanoma. *Clin Cancer Res* 2009;15:2166-73.
25. Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 2004;173:1444-53.
26. Cesana GC, DeRaffele G, Cohen S, Moroziewicz D, Mitcham J, Stoutenburg J, et al. Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncol* 2006;24:1169-77.
27. Gray CP, Arosio P, Hersey P. Association of increased levels of heavy-chain ferritin with increased CD4+ CD25+ regulatory T-cell levels in patients with melanoma. *Clin Cancer Res* 2003;9:2551-9.
28. Vence L, Palucka AK, Fay JW, Ito T, Liu YJ, Banchereau J, et al. Circulating tumor antigen-specific regulatory T cells in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 2007;104:20884-9.

29. Jandus C, Bioley G, Speiser DE, Romero P. Selective accumulation of differentiated FOXP3(+) CD4 (+) T cells in metastatic tumor lesions from melanoma patients compared to peripheral blood. *Cancer Immunol Immunother* 2008;57:1795-805.
30. Ahmadzadeh M, Felipe-Silva A, Heemskerk B, Powell DJ, Jr., Wunderlich JR, Merino MJ, et al. FOXP3 expression accurately defines the population of intratumoral regulatory T cells that selectively accumulate in metastatic melanoma lesions. *Blood* 2008;112:4953-60.
31. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol* 2012;13:e32-e42.
32. Filipazzi P, Huber V, Rivoltini L. Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. *Cancer Immunol Immunother* 2012;61:255-63.
33. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-61.
34. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 2009;27:6199-206.
35. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J Immunol* 2010;184:3106-16.

36. Nagaraj S, Nelson A, Youn JI, Cheng P, Quiceno D, Gabrilovich DI. Antigen-specific CD4(+) T cells regulate function of myeloid-derived suppressor cells in cancer via retrograde MHC class II signaling. *Cancer Res* 2012;72:928-38.
37. Poschke I, Mao Y, Adamson L, Salazar-Onfray F, Masucci G, Kiessling R. Myeloid-derived suppressor cells impair the quality of dendritic cell vaccines. *Cancer Immunol Immunother* 2012;61:827-38.
38. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 2007;13:828-35.
39. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, et al. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc Natl Acad Sci U S A* 2005;102:4185-90.
40. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* 2006;203:2691-702.
41. Mao Y, Poschke I, Wennerberg E, Pico de Coana Y, Egyhazi Brage S, Schultz I, et al. Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. *Cancer Res* 2013;73:3877-87.
42. Ahmadzadeh M, Rosenberg SA. IL-2 administration increases CD4+ CD25(hi) Foxp3+ regulatory T cells in cancer patients. *Blood* 2006;107:2409-14.
43. Berntsen A, Brimnes MK, thor Straten P, Svane IM. Increase of circulating CD4+CD25highFoxp3+ regulatory T cells in patients with metastatic renal cell carcinoma during treatment with dendritic cell vaccination and low-dose interleukin-2. *J Immunother* 2010;33:425-34.

44. Veltman JD, Lambers ME, van Nimwegen M, Hendriks RW, Hoogsteden HC, Aerts JG, et al. COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cells in mesothelioma. Celecoxib influences MDSC function. *BMC Cancer* 2010;10:464.:464.
45. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood* 2011;118:5498-505.
46. Alhamarneh O, Agada F, Madden L, Stafford N, Greenman J. Serum IL10 and circulating CD4(+) CD25(high) regulatory T cell numbers as predictors of clinical outcome and survival in patients with head and neck squamous cell carcinoma. *Head Neck* 2011;33:415-23.
47. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942-9.
48. Griffiths RW, Elkord E, Gilham DE, Ramani V, Clarke N, Stern PL, et al. Frequency of regulatory T cells in renal cell carcinoma patients and investigation of correlation with survival. *Cancer Immunol Immunother* 2007;56:1743-53.
49. Schwartzentruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med* 2011;364:2119-27.

Tables

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

Prognostic Factor	n	%	% Dead	1 Year survival rate [95%-CI^a] (%)	2 Year survival rate [95%-CI^a] (%)	p^b
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 years	46	48.9	78.3	39.4 [24.8; 53.9]	18.8 [6.0; 31.5]	
> 55 years	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 95%-CI = 95% confidence interval; ^b p-values are results of log rank tests excluding cases with missing values. Tregs - regulatory T cells; MDSCs – myeloid-derived suppressor cells.

Table 2: Cox models for disease-specific death

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

^a 95% CI = 95% confidence interval; ^b 1 patient had an unknown M-category and was excluded. Significant findings in the final models are highlighted by bold letters. Model 1 was adjusted for the confounding effects of Melan-A-specific T cells and CD14⁺CD11b⁺HLA-DR^{-low} MDSCs (MDSCs) and no confounding or significant interactions were detected in Model 2.

Legends to figures

Fig 1. Kaplan-Meier survival curves of non-resectable stage IV patients according to the frequency of circulating $CD14^+CD11b^+HLA-DR^{-/low}$ myeloid-derived suppressor cells (MDSCs) (A), $CD4^+CD25^+FoxP3^+$ Tregs (B), the presence of NY-ESO-1-specific T cells (C), and according to the M-category (D).

Fig 2. Kaplan-Meier survival curves according to the frequency of circulating myeloid-derived suppressor cells (MDSCs) and M-category in combination demonstrating the independent impact of both factors.

Fig 3. Correlation between myeloid-derived suppressor cells (MDSCs) and antigen-specific T cells. NY-ESO-1- and Melan-A-specific T cells were more frequently observed in patients with low compared to 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 regarding $CD8^+$ compared to $CD4^+$ T cells, if patients are analyzed for the presence of Interferon- γ -releasing NY-ESO-1- or Melan-A-specific T cells (C).

Figure 1

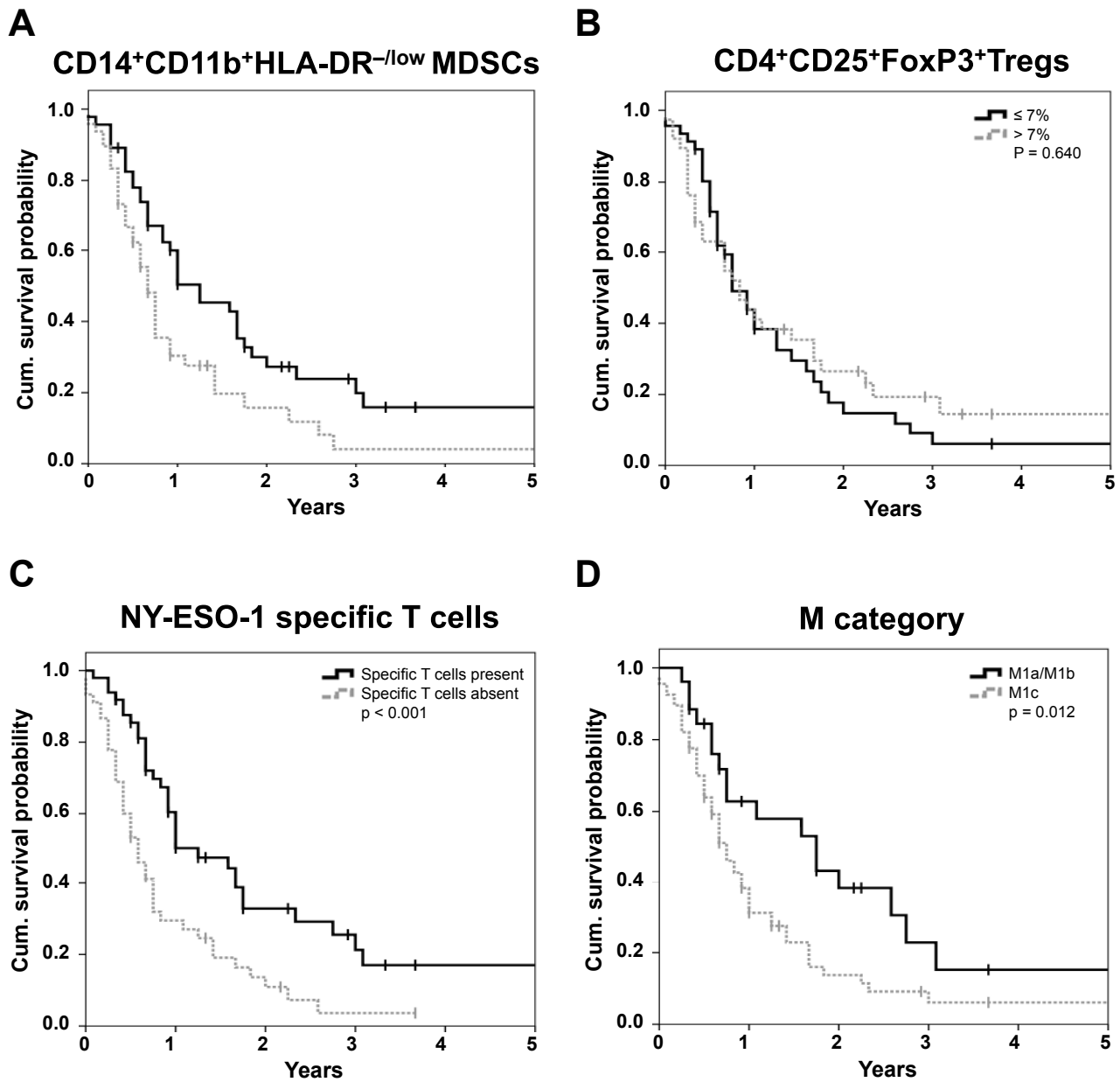


Figure 2

M category and MDSCs combined

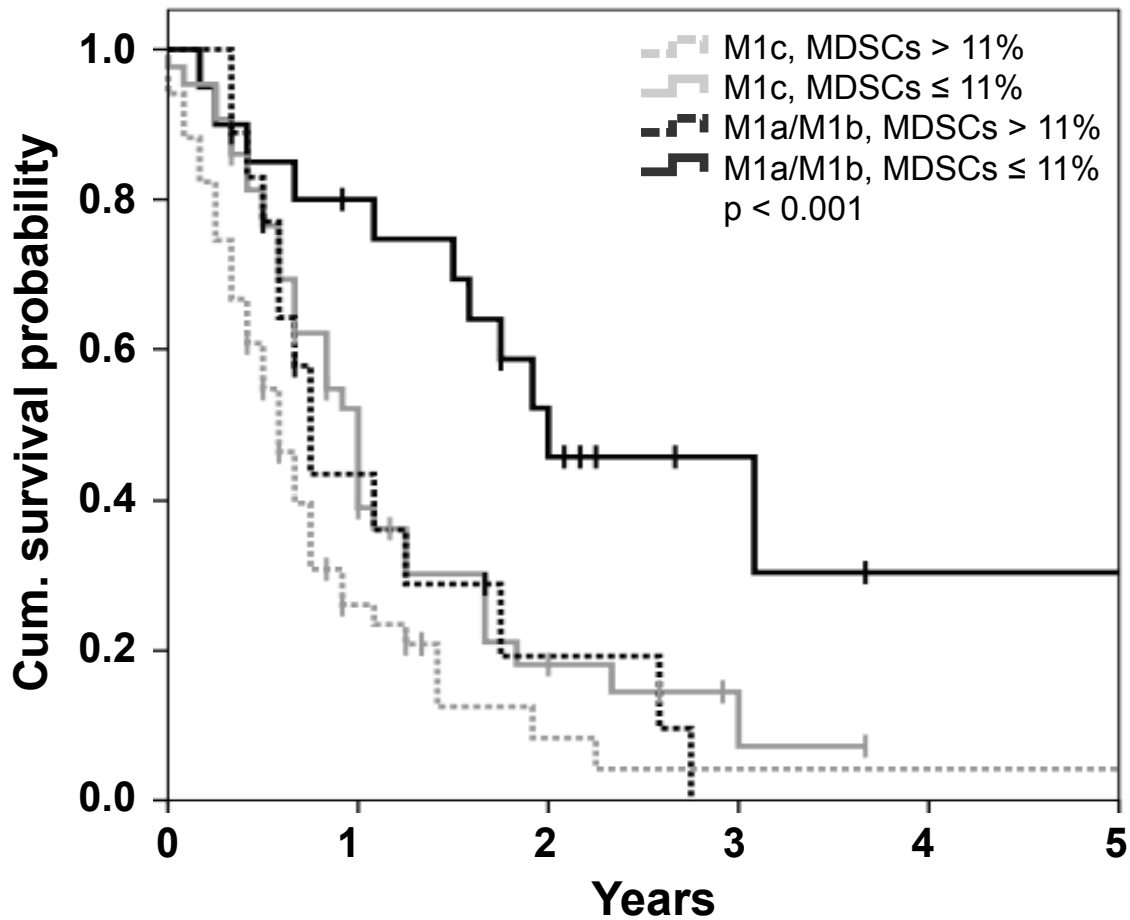
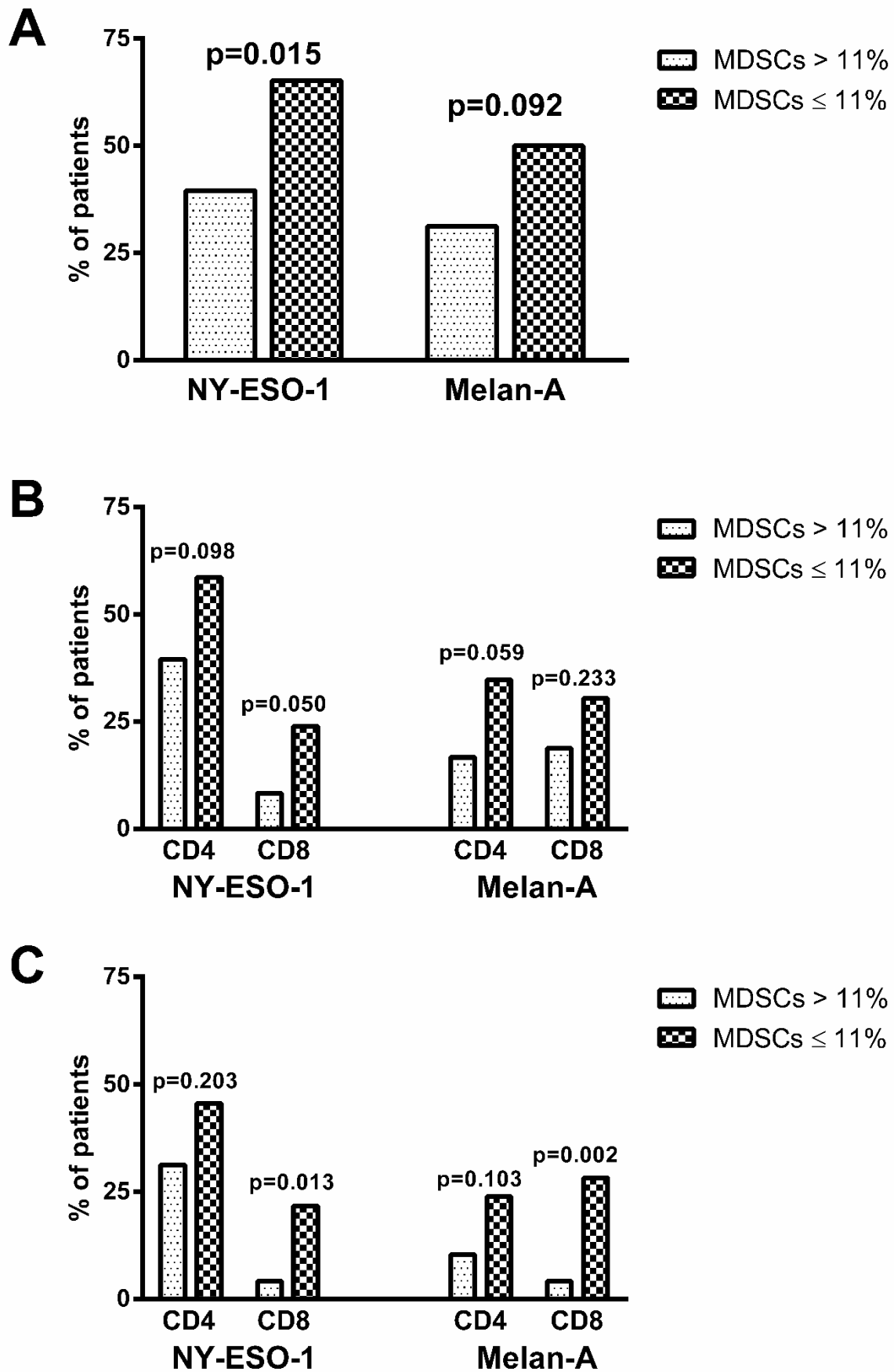


Figure 3



Clinical Cancer Research

Myeloid-derived suppressor cells predict survival of advanced melanoma patients: comparison with regulatory T cells and NY-ESO-1- or Melan-A-specific T cells

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