

Clinical Significance of Circulating CD33⁺ CD11b⁺HLA-DR⁻ Myeloid Cells in Patients with Stage IV Melanoma Treated with Ipilimumab

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Abstract

Purpose: High levels of circulating myeloid-derived suppressor cells (MDSCs) in various cancer types, including melanoma, were shown to correlate with poor survival. We investigated whether frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs could be used as immune system monitoring biomarkers to predict response and survival of patients with stage IV melanoma treated with anti-CTLA4 (ipilimumab) therapy.

Experimental Design: Peripheral blood samples from 56 patients and 50 healthy donors (HDs) were analyzed for CD33⁺CD11b⁺HLA-DR⁻ MDSC percentage, NO⁻, and hROS levels by flow cytometry. We determined whether MDSC levels and suppressive features detected before anti-CTLA4 therapy correlate with the patients' response and overall survival (OS).

Results: Patients with melanoma had significantly higher levels of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs with suppressive phenotype when compared with HDs. Low levels of MDSCs

before CTLA-4 therapy correlated with an objective clinical response, long-term survival, increased CD247 expression in T cells, and an improved clinical status. No predictive impact was observed for lactate dehydrogenase (LDH). Kaplan-Meier and log-rank tests performed on the 56 patients showed that the presence of more than 55.5% of circulating CD33⁺CD11b⁺ out of the HLA-DR⁻ cells, were associated with significant short OS ($P < 0.003$), a median of 6.5 months, in comparison with the group showing lower MDSC frequencies, with a median survival of 15.6 months.

Conclusions: Our study suggests the use of CD33⁺CD11b⁺HLA-DR⁻ cells as a predictive and prognostic biomarker in patients with stage IV melanoma treated with anti-CTLA4 therapy. This monitoring system may aid in the development of combinatorial modalities, targeting the suppressive environment in conjunction with ipilimumab, toward facilitating better disease outcomes. *Clin Cancer Res*; 22(23); 5661–72. ©2016 AACR.

Introduction

Melanoma, a form of skin malignancy that originates in melanocytes, is the most deadly form of skin cancer and the fifth leading type of cancer in the United States, representing 4.6% of all new cancer cases (1). Until recently, patients with advanced or metastatic melanoma were faced with poor prognosis and therapeutic options, with a median survival time of 6 to 9 months (2). In 2011, the fully humanized anti-CTLA4 antibody ipilimumab (Yervoy) was approved by the FDA for the treatment of nonoperable and metastatic melanoma (3). Ipilimumab treatment is an immune-based therapy that blocks the CTLA-4 molecule, which is

responsible for preventing overwhelmed activation and consequently, tissue damage by activated T cells. Treatment with ipilimumab increases T-cell activation in patients with advanced melanoma, which correlates with improved survival, tumor regression, and disease stabilization (4). Despite the increased survival rates observed in patients with melanoma treated with ipilimumab, only a relative small proportion of patients benefits from this treatment with an objective response rate of 10% to 13% (4, 5). Furthermore, ipilimumab is not devoid of side effects, as it could lead to inflammatory colitis, hepatotoxicity, and a variety of endocrinopathies (6).

An important feature of melanoma is the induction of a strong chronic inflammatory environment, detected in the advanced stages of the disease, which is accompanied by an increased production of inflammatory factors and accumulation of suppressive immune cells such as regulatory T cells (Treg) and MDSCs (7–9).

Common to MDSCs from different chronic diseases, including melanoma, are their myeloid origin, immature state, and their remarkable ability to suppress both the innate and adaptive immune responses, mainly by the production of NO⁻ and hROS (10, 11). In both mice and humans, the expansion and activation of MDSCs is mediated by a complex network of proinflammatory cytokines, chemokines, and growth factors, persistently secreted by normal or modified cells, including tumor cells (12–14). In mice, these cells are identified as

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Translational Relevance

Despite increased survival rates of patients with melanoma treated with ipilimumab, only a relative small proportion of these individuals benefit from this treatment. Thus, clinical determinants of response are needed. Here, we report that elevated frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ myeloid cells with increased NO⁻ and hROS production detected in the peripheral blood of patients with stage IV melanoma before therapy, correlate with poor responsiveness, disease severity, and minimal or no benefit in terms of survival. In contrast, responders and long-term survivors had significantly low frequencies of these cells. In addition, elevated frequencies of CD33⁺CD11b⁺HLA-DR⁻ cells was also associated with CD247 downregulation in T cells, suggesting a systemic immunosuppression mediated by these cells. Our study highlights the potential use of CD33⁺CD11b⁺HLA-DR⁻ as biomarkers for evaluating/monitoring the immune status of patients with melanoma before and during ipilimumab treatment. Furthermore, it provides the rationale to target these cells in combination with immune-based therapies to achieve improved clinical outcomes.

Gr1⁺CD11b⁺ cells and can be further subdivided into two main subsets with different phenotypic and biologic properties, the monocytic MDSCs (Mo-MDSC) characterized by CD11b⁺Ly6G⁻Ly6C^{high} and granulocytic like MDSCs (G-MDSC) expressing CD11b⁺Ly6G⁺Ly6C^{low} (10, 12). In contrast with mice, human MDSCs lack the Gr1-like antigen and are still being characterized both biologically and phenotypically. However, as previously shown by others and us, the use of CD33⁺CD11b⁺HLA-DR⁻ to define MDSCs in various types of cancer has become very common (11, 15–18). Within this CD33⁺ population, the CD14⁺CD15^{low/-}CD11b⁺ MDSCs share characteristic similar to the murine monocytic MDSCs, whereas the CD14⁻CD15⁺CD11b⁺ MDSCs resemble the murine granulocytic subtype (11, 12).

On the basis of our understanding that MDSCs interfere with immune responses under the settings of chronic inflammation, we hypothesized that high levels of MDSCs with suppressive features may lead to T-cell dysfunction and failure to respond to a given immunotherapy. Indeed, recent studies have shown that circulating CD14⁺CD11b⁺HLA-DR^{-/low} monocytic MDSC, but not Tregs could be used as prognostic biomarkers for predicting survival rates in patients with advanced melanoma (9) and that increased levels of this unique monocytic MDSC population correlates with lack of response in patients with melanoma treated with anti-CTLA4 therapy (19, 20). However, whether high levels of the global population of MDSCs, CD33⁺CD11b⁺HLA-DR⁻, containing both the granulocytic and monocytic subpopulations also correlate with lack of response and short-term survival in patients treated with anti-CTLA4 remains an open question.

In this study, we evaluated the clinical significance of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs as prognostic and predictive biomarkers in patients with stage IV melanoma subjected to anti-CTLA4 therapy using whole-blood samples. We found that elevated levels of these cells correlate with no

clinical responses, short-term survival and parameters reflecting disease severity, suggesting the use of these cells as biomarkers for evaluating/monitoring the immune status of patients with melanoma before and during ipilimumab treatment.

Materials and Methods

Patients

Peripheral blood (3–5 mL) was collected from 56 patients with stage IV melanoma subjected to ipilimumab (anti-CTLA4) treatment and analyzed under the approval of the Institutional Ethical Committee (MOH registration number 920051034). The blood samples were taken from all patients at the same day of the first ipilimumab treatment and after 3 weeks, when the second dose was given. Fifty healthy donors (HDS) were used as controls. In this study, clinical parameters were acquired from the medical records of patients under the care of Prof. Michal Lotem at the Oncology department, Hadassah University Medical Center Ein-Kerem, Jerusalem. Assessment of response was based on radiologic tumor assessments, performed in all patients at baseline and at week 12, and in patients in whom the disease had not progressed, every 12 weeks. We defined response according to the modified World Health Organization criteria as the sum of the products of bidimensional measurements of target lesions; a complete response is defined by the disappearance of all known lesions, a partial response by a decrease of at least 50% from baseline in the sum of the products of the diameters of index lesions, stable disease by failure to meet the criteria for either partial response or progressive disease, and progressive disease by a 25% increase in an existing lesion or the development of a new lesion. Adverse events were graded with the use of the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0 (http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcae3.pdf).

Cryopreservation and thawing procedure of human blood samples

Peripheral blood was drawn into heparinized tubes and was immediately cryopreserved until use. For cryopreservation, whole blood was mixed with 20% DMSO/80% FCS (freezing medium) at a 1:1 ratio and was transferred into cryovial tubes. The cryovial tubes were first stored in an –80°C using Mr. Frosty containers (Thermo Scientific; 5100-0001) for 48 hours and then moved into liquid N₂ containers. For sample analysis, cryovials were removed from the liquid nitrogen containers and transferred to a 37°C water bath for 5 seconds. Whole-blood samples were then thawed into 15-mL tubes containing preheated (37°C) medium (RPMI 1640). After one wash, whole-blood samples were resuspended in PBSX1 (50–100 µL whole blood in 200 µL PBSX1) and stored in 4°C refrigerators for no more than 1 hour before staining, flow-cytometry analysis, or functional assays.

Flow cytometry and antibodies

The antibodies used for labeling of samples were FITC-labeled anti-HLA-DR (L243; Biolegend), anti-CD247 (H146), phycoerythrin (PE)-labeled anti-CD33 (WM53; Biolegend), anti-CD56 (HCD56; Biolegend), APC-labeled anti-CD11b (ICRF44; Biolegend), anti-CD3ε (OKT3; Biolegend), and Pacific blue-labeled anti-HLA-DR (L243; Biolegend). For

human MDSCs staining, whole-blood samples were stained with anti-CD11b, CD33, and HLA-DR mAb, fix/Lyse solution (eBioscience) was then added. Next, the samples were washed and resuspended in flow stain buffer. For CD247 intracellular detection, whole-blood samples were washed, fixed with 2% paraformaldehyde, washed, and permeabilized with permeabilization buffer (PB-0.1% saponin and 1% human serum). Samples were washed, incubated with anti-CD56, CD3e, CD247 antibodies, washed again, and were resuspended with flow stain buffer. All samples were analyzed by FACSCalibur using Cell Quest software (BD Biosciences).

NO⁻ and hROS measurements

iNOS activity was evaluated by measuring intracellular NO⁻ in HLA-DR⁻CD33⁺CD11b⁺ cells, by using the DAF-2DA reagent (NOS 200-1; Cell Signaling Technology Inc.). hROS production in HLA-DR⁻CD33⁺CD11b⁺ cells was performed by using reactive oxygen species detection kit (APF 4011, Cell Signaling Technology Inc.). In both cases, the detection of NO⁻ and hROS was performed according to the manufacturer's instructions and was determined by flow cytometry analysis following staining.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.04. Averaged values are presented as the mean \pm SEM. When comparing two groups, we determined statistical significance using the two-tailed Student *t* test. When more than two groups were investigated, we performed an ANOVA. Overall survival (OS) was defined from the date of the first anti-CTLA4 treatment until the date of death or last follow-up. Survival analyses were assessed according to the Kaplan–Meier method with 95% confidence intervals and compared using log-rank tests. Only deaths due to melanoma were considered, other death causes were regarded as censored events. Patients with missing data in variables analyzed were excluded. Multivariable analysis was based on Cox proportional hazards regression analysis and was used to determine the independent effects of prognostic factor. *P* values of less than <0.05 were considered statistically significant.

Results

Patients

In this study, we analyzed whole-blood samples from 56 patients, >20 years of age with stage IV nonoperable melanoma between July 2010 and November 2013. Patients were treated with four cycles of 3 mg/kg ipilimumab every 3 weeks and had at least one first line of treatment before receiving ipilimumab (Table 1). Blood samples from all patients were withdrawn on the same day of the first ipilimumab treatment (before the treatment) and after 3 weeks, when the second dose was given. Blood samples from patients with melanoma and 50 HDs were compared as with the indicated immune parameters (Table 2).

Detection of MDSC frequencies in fresh versus cryopreserved/thawed whole-blood samples

In the course of our analysis, we used a flow cytometry test to detect CD33⁺CD11b⁺HLA-DR⁻ MDSC frequencies in whole-blood frozen samples; unlike many of the published

Table 1. Clinical details of patients with melanoma

Details of patients with melanoma treated with ipilimumab	Numbers (%)
Response to ipilimumab	
SD/PR/CR	14 (25%)
PD	42 (75%)
Previous treatments	
Chemotherapy	30 (53.5%)
Radiotherapy	7 (12.5%)
Immunotherapy	18 (32%)
Tumor origin	
Skin	37 (66%)
Ocular	14 (25%)
Mucosal	5 (8.9%)
Stage at study entry	
M1a	7 (12.5%)
M1b	12 (21.4%)
M1c	16 (28.5%)
M2	21 (37.5%)
LDH at study entry (U/l)	
<480 U/l	28 (50%)
>480 U/l	21 (37.5%)
N.D.	7 (12.5%)

Abbreviations: SD, stable disease; PR, partial response; CR, complete response; PD, progressive disease; LDH, lactate dehydrogenase; N.D., not determined.

studies, which used a Ficoll density gradient centrifugation step for peripheral blood mononuclear cells (PBMCs) preparation, before freezing the samples and evaluating MDSC frequencies (9, 17, 19–21). To determine the effect of cryopreservation/thawing and Ficoll centrifugation on MDSCs as well as on T-cell frequency, blood samples from 15 HD were analyzed. Whole blood and matched Ficoll-isolated PBMCs from each of the tested donors were analyzed before and after freezing and thawing. Analyzing whole-blood samples preserves the composition of lymphocytes, monocytes and granulocytes both in fresh (Fig. 1A, top plots) and frozen (Fig. 1A, bottom plots) samples and reflects the original distribution of immune cell populations in the tested sample. In contrast, Ficoll density gradient centrifugation leads to changes in the distribution of the leukocytes populations (Fig. 1B) and thus, does not reflect the original composition of immune cell populations and MDSC percentages in particular (Fig. 1C). No significant changes were observed in CD33⁺CD11b⁺HLA-DR⁻ MDSCs and CD3⁺ T cells frequencies in fresh versus cryopreserved/thawed whole-blood samples. In contrast, the percentage of these two cell types was significantly changed (MDSC, *P* < 0.0005; T cells, *P* < 0.0052) between fresh and

Table 2. Details of healthy and patients with melanoma

Details of patients and HDs	Numbers (%)
All patients (<i>n</i> = 56)	
Median age	60.7
Gender	
Male	24 (42.8%)
Female	32 (57.1%)
HDs (<i>n</i> = 50)	
Median age	32.5
Gender	
Male	31 (62%)
Female	19 (38%)

NOTE: All patients (*n* = 56) were diagnosed as stage IV and were treated with repeated cycles of 3 mg/kg ipilimumab every 21 days.

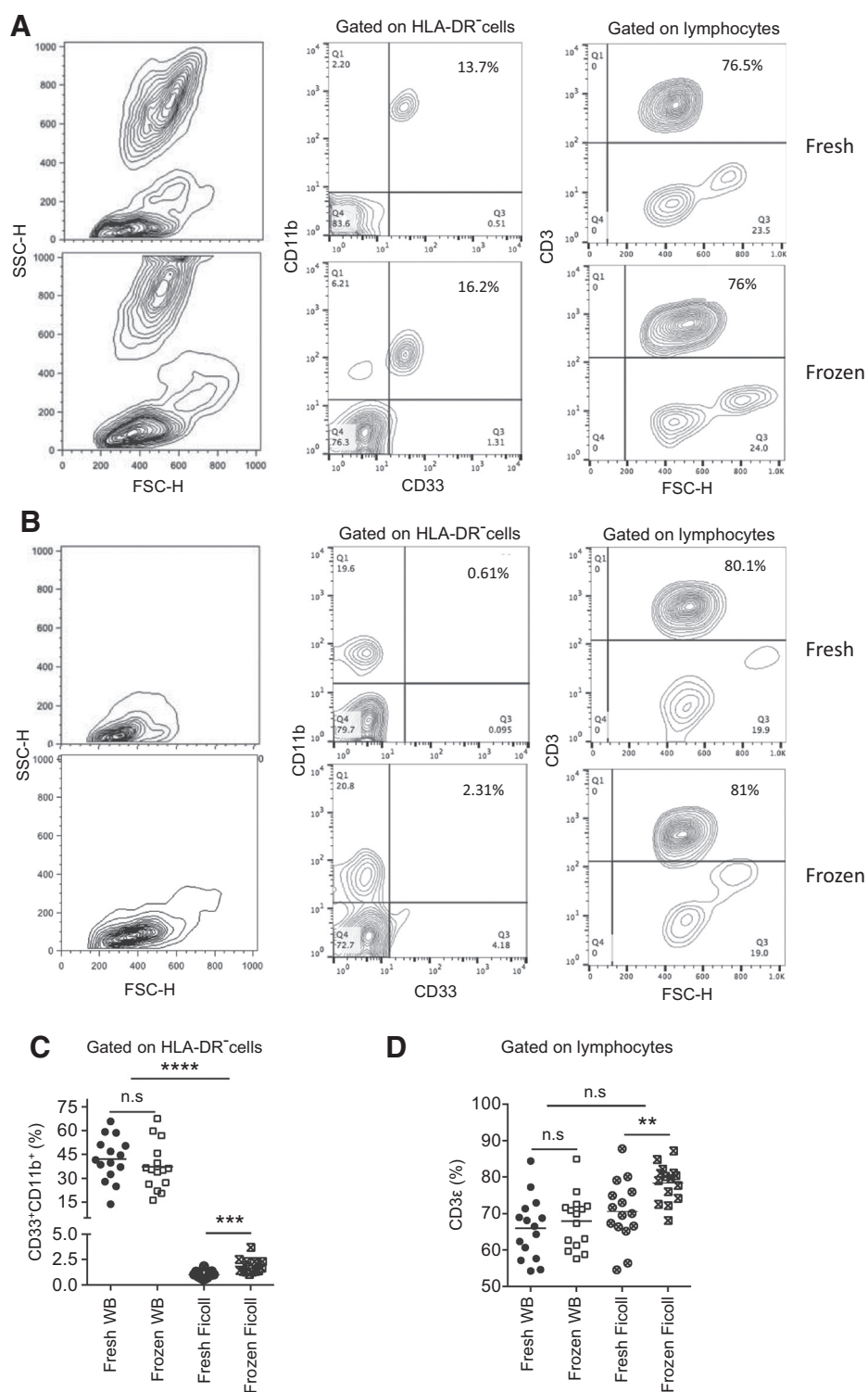


Figure 1. Whole-blood cryopreservation/thawing preserves MDSC frequencies. **A** and **B**, blood was drawn from 15 HDs to evaluate CD33⁺CD11b⁺HLA-DR⁻ MDSC and CD3⁺ T-cell percentages before and after freezing and thawing, using whole blood and matched Ficoll-treated samples. Representative plots showing the differences in distribution of immune populations, MDSC and T-cell percentages between fresh and frozen whole blood samples (**A**) and matched samples after Ficoll density gradient centrifugation (**B**). MDSCs are presented by the percentages of CD33⁺CD11b⁺ of gated HLA-DR⁻ cells. **C** and **D**, whole blood and matched Ficoll-isolated PBMCs were analyzed before and after freezing/thawing for the percentages of CD33⁺CD11b⁺HLA-DR⁻ MDSCs (**C**) and CD3⁺ T cells (**D**) in HD (*n* = 15); **, *P* < 0.0052; ***, *P* < 0.0005; ****, *P* < 0.0001 (*t* test); n.s., not significant.

cryopreserved/thawed Ficoll treated samples (Fig. 1C-D). These results are supported by previous studies showing that PBMC isolation using Ficoll before and after freezing, significantly changes the frequencies of both CD33⁺CD11b⁺HLA-DR⁻ and CD15⁺LIN⁻HLA-DR⁻ MDSC populations after sample are thawed (22, 23).

Elevated frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ myeloid cells in patients with stage IV melanoma

Before evaluating CD33⁺CD11b⁺HLA-DR⁻ MDSCs as potential biomarkers for anti-CTLA4 therapy, we assessed whether this population is enriched in the patient group as compared with healthy subjects and assessed if they exhibit an increased

suppressive phenotype, as we recently reported for patients with colorectal cancer (CRC) (15). The percentage of MDSCs in this study is shown as the CD33⁺CD11b⁺ (%) of gated HLA-DR⁻ cells, as previously shown by us and others in different malignancies, including melanoma (Supplementary Fig. S1A; refs. 15, 17, 18, 24). Analysis of blood samples taken before the first ipilimumab treatment showed that the percentages of CD33⁺CD11b⁺HLA-DR⁻ MDSCs in the group of patients with melanoma was significantly higher (48.8 ± 2.3 , $P < 0.0001$) as compared with HD (27.9 ± 1.7 ; Fig. 2A). Despite our inability to reach a full age-matched distribution between HD and the patients with melanoma (Table 2), there was no age-dependent alteration in MDSC frequencies in the group of patients with melanoma (Fig. 2B), suggesting that age has no effect on MDSC numbers.

The observed increased MDSC levels in patients with melanoma alongside with the inflammatory characteristics of advanced melanoma (7, 25) suggest that MDSC-suppressive features and activity could also be affected. To test the suppressive features of MDSCs, we assessed their ability to produce NO⁻ and hROS. High levels of these mediators have been reported as impairing the immune function of both the adaptive and innate immune system compartments (10), the first being essential for the success of ipilimumab treatment. Analysis of MDSCs and their ability to produce hROS (Fig. 2C) and NO⁻ (Fig. 2D) in patients with melanoma revealed that their suppressive activity (NO⁻: 16.59 ± 0.6 , $P < 0.0001$; hROS: 4.05 ± 0.24 , $P < 0.0011$) was significantly higher as compared with MDSCs from HD (NO⁻: 12.67 ± 0.57 ; hROS: 2.89 ± 0.2).

The increased frequencies of activated MDSCs detected in the patients with melanoma, suggest an immunosuppressive activity that leads to an impaired immunologic status. We thus evaluated CD247 (TCR ζ chain) expression in T cells, which is a key component responsible for the initiation of effector immune responses mediated by these cells, and previously reported to be downregulated in various types of tumors, reflecting a suppressed patients' immune status (26, 27). The results revealed that circulating CD3⁺CD56⁻ T cells in patients with melanoma displayed significantly reduced CD247 expression (77.1 ± 3.5 , $P < 0.0001$) when compared with the HD group (100 ± 3.5 ; Fig. 2E). It is important to note that the only TCR subunit affected by the inflammatory environment in the group of patients with melanoma was CD247. Evaluation of CD3 ϵ showed no significant changes between the patients group (101.3 ± 1.5) and HD (100 ± 1.53 ; Fig. 2F). Moreover, a significant inverse correlation ($P < 0.0015$) was detected between MDSC levels and CD247 expression in patients with melanoma. High levels of MDSCs correlated with low expression levels of CD247 and vice versa (Fig. 2G), proposing a linkage between high levels of circulating MDSCs and impaired immune status of T cells.

Changes in MDSC frequencies correlate only with metastatic stage and LDH severity

Metastatic melanoma can be classified into substages according to the American Joint Committee on Cancer (AJCC; ref. 28), based on the site of metastases and on lactate dehydrogenase (LDH) levels. The substages are divided into four groups: metastatic spread to soft tissues and lymph nodes (M1a), lung (M1b), parenchymal organs (M1c), and M2, representing any organ involvement associated with abnormal LDH levels. LDH

is a prognostic serum marker associated with increased tumor burden in late-stage malignant melanoma, and has been included in the AJCC staging system as a separate substage due to its prognostic significance (28–30). Because stage IV subclassification correlates with disease severity and survival rates (28), we set out to explore the impact on MDSC levels. Flow-cytometry analysis of the blood samples before the ipilimumab treatments detected a significant elevation in MDSC percentages only in the M2 group (53.6 ± 3.4 , $n = 21$) relative to the other subgroups, when each group was evaluated separately (Fig. 3A). We therefore divided the patients into two groups, M1a/b/c and M2. Analysis of these two groups showed again a significant elevation in MDSC percentages in the M2 group (53.8 ± 3.4 , $P < 0.002$) relative to the M1a/b/c group (39.1 ± 2.7 ; Fig. 3B). We then evaluated whether abnormal LDH serum levels (>480 U/l) correlate with increased MDSC frequencies. Indeed, our results show a significant increase in the frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs in patients with abnormal LDH levels (50.6 ± 3), relative to those found in patients with normal LDH (<480 U/l, 37.4 ± 3 , $P < 0.006$; Fig. 3C).

Finally, we addressed the question of whether other parameters such as age, gender, primary tumor site, and previous treatments given before initiating the first ipilimumab session could also affect MDSC frequencies. We observed no significant changes in CD33⁺CD11b⁺HLA-DR⁻ MDSC levels in patients that were treated either with chemotherapy (Fig. 3D), immunotherapy (Fig. 3E), or radiotherapy (Fig. 3F) before the ipilimumab treatments. No significant changes were also observed in MDSC frequencies when compared with the age (Fig. 2B), tumor origin (Fig. 3G), and gender (Fig. 3H) within the described patients' group. Moreover, when analyzing MDSC levels before ipilimumab therapy and after the second treatment, no significant changes were observed in their frequencies (Fig. 3I). Taken together, these results indicate that MDSC levels are changed only by parameters that reflect disease severity.

MDSC levels predict responsiveness and survival of patients treated with ipilimumab

On the basis of our results showing increased levels of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs in patients with stage IV melanoma treated with ipilimumab, and changes in this cell population according to disease severity parameters (metastases and LDH), we assessed whether monitoring the frequencies of this cell population before the first ipilimumab treatment can distinguish between responders and nonresponders. Assessment of response was performed at week 12, and patients with no disease progression, were assessed every 12 weeks. Patients with an objective tumor response were defined as: CR (complete responders), PR (partial responders), or SD (stable disease), whereas nonresponders were defined as PD (progressive disease), using the immune-related response criteria (irRC; ref. 31).

Analysis of blood samples from patients with melanoma before the first ipilimumab treatment revealed significantly lower frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs in patients responding to the treatment (34.2 ± 4.3 , $n = 14$, $P < 0.008$) compared with nonresponding patients (48.6 ± 2.7 , $n = 42$; Fig. 4A). In contrast with MDSC frequencies, LDH serum level, which is the only blood marker

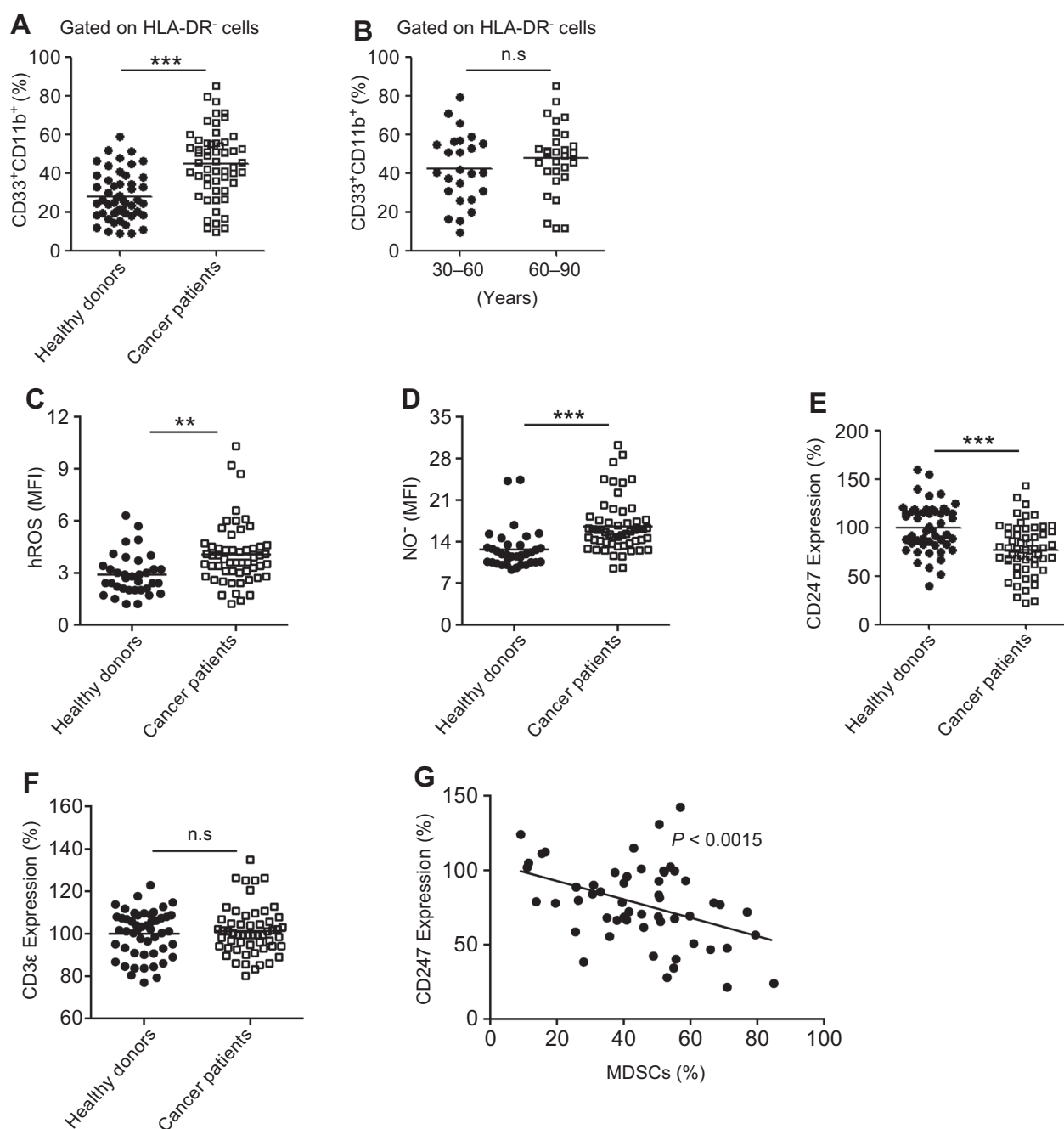


Figure 2.

CD33⁺CD11b⁺HLA-DR⁻ MDSC levels with high NO⁻ and hROS levels are increased in patients with melanoma. MDSCs are presented by the percentages of CD33⁺CD11b⁺ of gated HLA-DR⁻ cells. **A**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages were detected in peripheral blood of HD ($n = 50$) and patients with stage IV melanoma ($n = 56$) before ipilimumab treatment using flow cytometry analysis. **B**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages in patients with melanoma were evaluated according to the age of the patients, numbers indicate years. **C** and **D**, Intracellular concentrations of hROS (**C**) and NO⁻ (**D**) were measured, and are shown by mean fluorescence intensity (MFI), in CD33⁺CD11b⁺HLA-DR⁻ gated cells. Results from HD ($n = 35$) and patients with stage IV ($n = 56$) are presented. **E** and **F**, Peripheral blood cells from HD ($n = 50$) and patients with melanoma ($n = 56$) were fixed, permeabilized, and analyzed by flow cytometry for total expression (%) of CD247 (**E**) and CD3ε (**F**), compared with their expression in HD, set as 100%. CD247 plots are of gated CD3⁺CD56⁻ T cells. **G**, Correlation between MDSC percentages and CD247 expression (%) in patients with stage IV melanoma ($n = 56$). Each dot represents the levels of both MDSCs and CD247 within the same patient. **, $P < 0.0011$; ***, $P < 0.0001$ (t test); n.s., not significant.

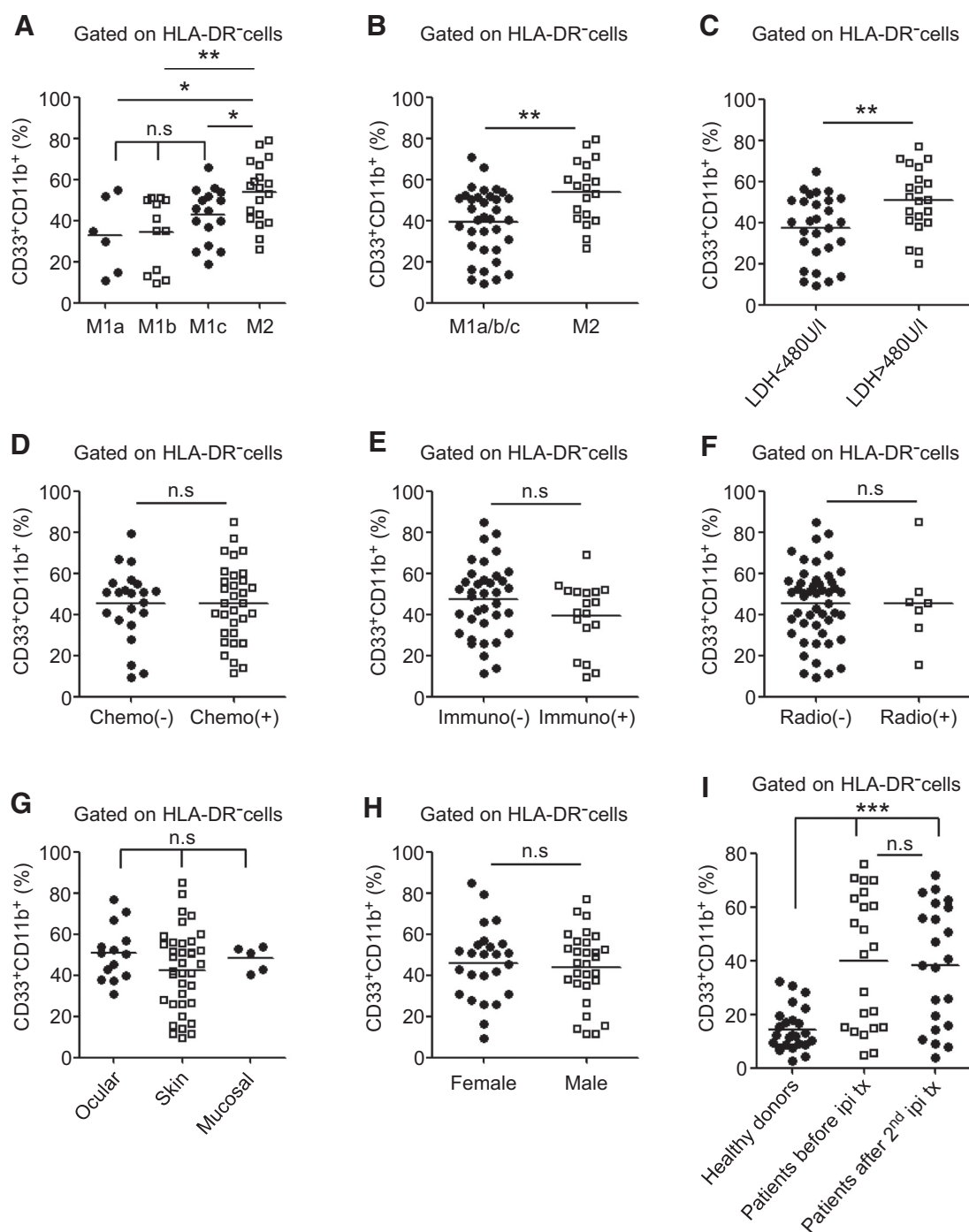


Figure 3.

MDSC percentage correlates with LDH levels and metastatic severity. **A**, Patients with melanoma were divided into four subgroups M1a, M1b, M1c, and M2 according to the AJCC metastatic staging system in melanoma, and CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages were evaluated according to this staging. **B**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages were evaluated in the patients with melanoma divided into two groups, M1a/b/c and M2. **C**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages were evaluated in the patient group according to the normal baseline (LDH < 480 U/l) and abnormal levels (LDH > 480 U/l) of LDH in the serum. LDH levels and MDSC percentages were evaluated in blood samples drawn at the same day from each patient, before the first ipilimumab (ipi) treatment. **D** and **F**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages in patients with melanoma receiving previous treatments such as chemotherapy (**D**), immunotherapy (**E**) and radiotherapy (**F**) were tested before the first ipilimumab treatment. **G**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages in the melanoma patient group were evaluated according to the origin of the tumor; ocular, skin, or mucosal. **H**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages in the melanoma patient group were evaluated according to their gender. **I**, CD33⁺CD11b⁺HLA-DR⁻ MDSC frequencies displayed before ($n = 20$) and after two treatments ($n = 20$) with ipilimumab, compared with HD ($n = 25$). *, $P < 0.0034$; **, $P < 0.006$; ***, $P < 0.0001$ (t test); n.s., not significant; LDH, lactate dehydrogenase; ipi tx, ipilimumab treatment. The percentage of MDSCs shown here are of gated HLA-DR⁻ cells (**A-I**).

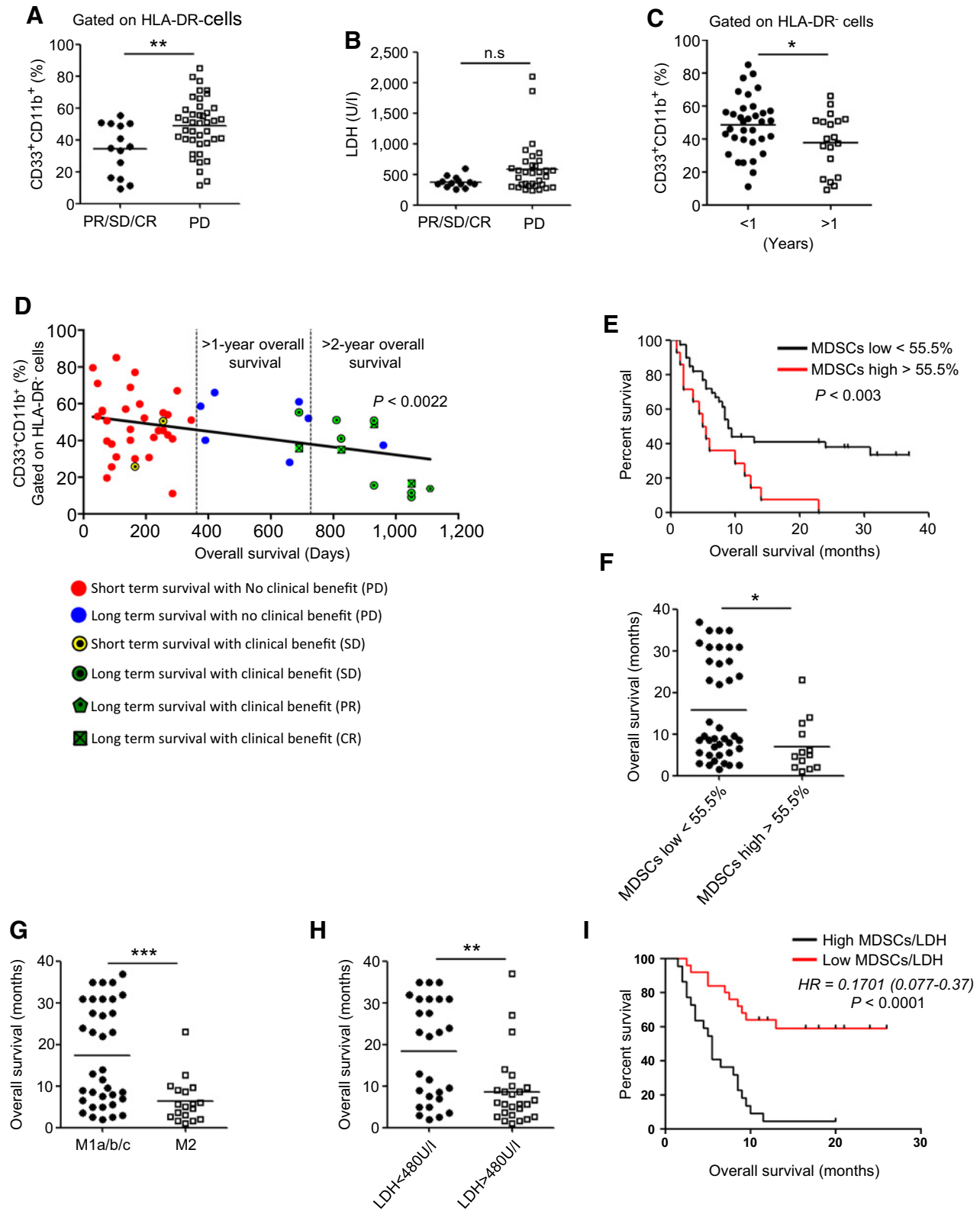


Figure 4. Reduced MDSC levels are associated with long-term survival and clinical response to ipilimumab therapy. **A**, CD33⁺CD11b⁺HLA-DR⁻ MDSCs in ipilimumab-treated patients responding (PR/SD/CR) or non-responding (PD) to the treatment are presented, following assessment by flow-cytometry analysis. MDSC levels are of gated HLA-DR⁻ cells. **B**, Normal baseline (LDH < 480 U/l) and elevated baseline (LDH > 480 U/l) serum LDH levels before the first ipilimumab treatment in patients responding or non-responding to the treatment. (Continued on the following page.)

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in advanced melanoma to be adopted by the AJCC staging (28), could not distinguish between the responder and non-responder populations (Fig. 4B).

After identifying low frequencies of MDSCs associated with a clinical response to ipilimumab treatment, we next explored whether monitoring MDSC levels in patients with melanoma before the first treatment could provide a clue as to their OS. We first divided the patients into two groups: patients that had minimal or no benefit and survived less than 1 year from therapy initiation, and patients that were considered as long-term survivors (regardless of their clinical response) and survived more than 1 year. When comparing these two groups to the percentage of MDSCs, significant association was observed (Fig. 4C). Patients with an OS of less than 1 year had significantly higher levels of circulating MDSCs before therapy. Moreover, when looking at the correlation between OS and MDSC frequencies, a significant inverse correlation ($P < 0.0022$) was detected between MDSC and OS. High levels of MDSCs correlated with short OS and in most cases with minimal or no benefit from the therapy, whereas, low levels of MDSCs were associated with long-term OS and patients having some kind of a clinical benefit (Fig. 4D).

Finally, we explored whether classifying the patients with melanoma into two groups, one having high level (above >55.5%) of circulating MDSCs and the other with low MDSC levels (below <55.5%) before the first treatment, could provide a clue as to their survival benefit. The rationale for this cutoff in our study was the fact that 55.5% was the highest point for MDSCs in the responder group. Kaplan–Meier analysis of 56 patients showed that the presence of more than 55.5% of circulating MDSCs was associated with significant short OS ($P < 0.003$), in comparison with the group having less than 55.5% of these circulating cells (Fig. 4E). Overall, our data show that low frequencies of circulating MDSCs detected before the first ipilimumab treatment associates with clinical response and prolonged survival.

Improved survival prediction by monitoring a combination of biomarkers

Our results have shown that low percentages of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs detected before ipilimumab therapy is the only parameter associated with both clinical response and prolonged OS in patients with stage IV melanoma. Analysis of the median OS (in months) of the 56 patients with melanoma in our study, relating to the metastatic severity, LDH and MDSC levels, showed decreased median OS in patients having abnormality in one of these parameters. Patients with high levels of circulating MDSCs (>55.5%) had a significantly reduced median survival rate (6.9 ± 1.7 months, $n = 15$, $P < 0.016$) in comparison with patients having low levels (15.6 ± 1.9 , $n = 40$; Fig. 4F).

Similarly, a significant low median OS in patients with serum LDH above 480U/l (8.5 ± 1.6 months, $n = 26$, $P < 0.0014$) or M2 metastatic staging (6.3 ± 1.2 months, $n = 20$, $P < 0.0006$) was observed, when compared with patients with serum LDH under 480 U/l (18.3 ± 2.3 months, $n = 27$) or stages M1a/b/c (17.2 ± 2 months, $n = 35$; Fig. 4G–H). Moreover, Kaplan–Meier analysis based only on LDH level or metastatic staging, showed a significant decrease in OS within patients having abnormal LDH (Supplementary Fig. S1B) and M2 metastatic staging (Supplementary Fig. S1C).

Further analysis of the 14 responding patients in this study showed a median OS of 27.21 ± 2.5 months. However, within this group, two patients, number 6 and 12, had short OS of 5.5 and 8.5 months, respectively (Supplementary Table S1). These patients did not have increased MDSC levels (<55.5%), but their LDH serum levels were above >480 U/l. No doubt, that a combination of high metastatic burden, LDH, and MDSC count could increase the identification of patients that will have long-term OS and will benefit more from the treatment. Indeed when evaluating different combinations of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs, LDH and metastatic severity, a synergistic effect in terms of OS is observed (Supplementary Fig. S1E). It is important to note that combination of MDSC and metastatic staging did not have a synergistic effect when compared only with the metastatic parameter (Supplementary Fig. S1C and S1D). Multivariable analysis for the different prognostic parameters showed that the best combinations for the identification of patients that will benefit more from the treatment are MDSC and LDH (Fig. 4I). However, it is important to note that of the two parameters; MDSCs and serum LDH, the former represents the only parameter that could be used by itself as a diagnostic marker for evaluating the patient's likelihood to respond to immunotherapy, as well as to benefit long-term survival following ipilimumab treatment. Overall, our results show that identification of patients that will benefit from ipilimumab treatment is feasible by monitoring MDSC levels or by combining the MDSC/LDH parameters, before therapy initiation.

Discussion

In the present study, we evaluated the clinical relevance of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs in patients with cancer undergoing anti-CTLA4 (ipilimumab) therapy. We analyzed peripheral blood samples of 56 patients with stage IV melanoma receiving ipilimumab at different time points, before and following treatment and compared them with 50 HDs. We demonstrate that increased frequencies of circulating MDSCs with suppressive features in patients with stage IV melanoma before ipilimumab treatment, correlate with

(Continued.) **C**, CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages in patients with melanoma with minimal or no treatment benefit (<1 year) and patients with long-term OS (>1 year), was evaluated before the first ipilimumab treatment. **D**, correlation between MDSC percentages and OS (days) in patients with stage IV melanoma ($n = 56$). Each dot represents a patient; **, $P < 0.0022$. **E**, Kaplan–Meier survival curve of patients with stage IV melanoma treated with ipilimumab according to the frequencies of high (>55.5%) and low (<55.5%) circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs. Comparison of survival curves was done using the log-rank test, $P < 0.003$. **F–H**, survival rates of 56 patients with stage IV melanoma according to parameters evaluated before the first ipilimumab treatment. Numbers indicate survival in months based on high and low MDSC percentages (**F**), metastatic severity (**G**), and LDH serum levels (U/l; **H**). **I**, Kaplan–Meier survival curve of patients with stage IV melanoma treated with ipilimumab according to the following combination: frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs <55.5%, and LDH<480U/l levels (low MDSCs/LDH), compared with high frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs > 55.5% and LDH>480U/l levels (high MDSCs/LDH). Comparison of survival curves was done using the log-rank test, $P < 0.0001$. *, $P < 0.035$; **, $P < 0.008$; ***, $P < 0.0006$ (t test); n.s., not significant. PR, partial response; SD, stable disease; CR, complete response; PD, progressive disease.

nonresponsiveness and minimal or no benefit in terms of survival, when compared with patients having low frequencies of this cell population.

In the course of our study, we used a flow-cytometry test applied on whole-blood fresh and frozen samples, unlike many published studies, which used a Ficoll density gradient centrifugation step for PBMC preparation, before freezing the samples and evaluating MDSC frequencies (9, 17, 19–21). We found that in contrast to blood samples separated by Ficoll density gradient (fresh or cryopreserved/thawed), only cryopreservation/thawing of the whole-blood sample preserves the levels of CD33⁺CD11b⁺HLA-DR⁻ MDSCs as detected in fresh samples. These results are supported by previous studies showing that fresh or cryopreserved/thawed PBMCs, isolated on Ficoll density gradient, showed significantly modified frequencies of both CD33⁺CD11b⁺HLA-DR⁻ and CD15⁺LIN⁻HLA-DR⁻ MDSC populations as compared with their profile in the whole fresh blood sample (22, 23). It is important to note that in the process of the Ficoll density gradient separation step, due to the distribution of the different leukocyte populations, cells such as granulocytic MDSCs are neglected (missed) and only PBMCs containing the monocytic MDSCs are taken. Therefore, the original composition of immune cell populations and MDSC percentages in particular, that contain both the granulocytic and monocytic fractions, is distorted.

On basis of our analyses, significantly higher levels of circulating MDSCs (48.8 ± 2.3 , $P < 0.0001$) with increased ability to produce both NO⁻ and hROS were detected in the patients' blood samples as compared with HD (27.9 ± 1.7). The increased frequencies of activated MDSCs detected in the patients with melanoma, suggest an impaired immunologic status. Indeed evaluation of CD247 (TCR ζ chain) expression in T cells, previously reported to be downregulated in various types of tumors, revealed that circulating CD3⁺CD56⁻ T cells in patients with melanoma displayed significantly reduced CD247 expression (77.1 ± 3.5 , $P < 0.0001$) when compared with the HD group (100 ± 3.5). Furthermore, a significant inverse correlation ($P < 0.0015$) was detected between MDSC levels and CD247 expression in patients with melanoma, pointing at the link between high levels of circulating MDSCs and an impaired immune status of T cells. Such a relation was previously validated by numerous studies, including ours in various tumors (15, 27, 32, 33), suggesting that elevated MDSC levels and downregulated CD247 expression reflect an impaired immunologic status in these patients, due to a developing chronic inflammation (7, 25). This is supported by our results showing that the CD247 is sole TCR molecule to be downregulated in the course of chronic inflammation while the CD3 or any other TCR subunit are unchanged (15, 26), and is in contrast with a normal T-cell activation process that leads to the downregulation of the entire TCR complex (26). Indeed, recent reports showed increased levels of proinflammatory cytokines in the serum of patients with melanoma, generating an immunosuppressive environment enriched by MDSCs and Tregs (7, 19, 34, 35). Additional studies in renal cell carcinoma (36) and melanoma (17) patients support our observations as they show that CD33⁺ MDSCs isolated from patients but not from HDs are able to suppress antigen specific T-cell responses *in vitro*, a phenomenon that is reversible upon inhibition of hROS production or maturation of these cells into antigen-presenting precursors.

When evaluating the effect of various parameters on MDSC frequencies in the tested patients, we did not detect any gender, tumor origin, and age-dependent alterations in MDSC levels in the group of patients with melanoma. However, changes in the frequencies of MDSCs were detected when compared with LDH serum levels and metastatic severity, two parameters that are known to reflect disease severity in melanoma (28). Moreover, when analyzing MDSC levels at several time points, before ipilimumab therapy and after the second treatment, no significant changes were observed in their frequencies, suggesting that MDSCs are not affected by this treatment. Our observation is further supported by a recently published study (20), showing that ipilimumab treatment has no effect on the frequencies of circulating monocytic like Lin⁻CD14⁺HLA-DR⁻ MDSCs. These observations in conjunction with previous studies showing that CD4⁺ and CD8⁺ T cells are affected by this treatment (37) suggest that most probably, activated T cells and Tregs that constantly express high levels of CTLA-4 on their cell surface (38) are the most promising candidates to be directly targeted upon ipilimumab treatment. Indeed, it has been shown that ipilimumab therapy in melanoma mediates both *in vivo* and *ex vivo* depletion of Foxp3⁺ Treg cells (39).

Although ipilimumab treatment has been shown to increase OS in patients with melanoma, only a relative small proportion of patients benefit from this treatment with an objective response rate of 10% to 13% (4, 5). In our study, LDH serum levels could not distinguish between clinical responders and nonresponders upon ipilimumab treatment, but were demonstrated to be a strong predictive factor for survival as previously shown by Kelderman and colleagues (40). In contrast, analysis of blood samples from patients with melanoma before the first ipilimumab treatment revealed significantly lower frequencies of circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs in patients responding to the treatment (34.2 ± 4.3 , $n = 14$, $P < 0.008$) compared with non-responding patients (48.6 ± 2.7 , $n = 42$), suggesting that MDSCs could be used as predictive biomarkers to be measured before ipilimumab treatment to estimate likelihood to benefit from this therapy. However, concluding about the predictive impact of MDSCs will necessitate further evaluation using larger cohorts and additional clinical situations. The mean OS within patients having low MDSC levels before treatment was significantly ($P < 0.003$) higher as compared with patients displaying elevated MDSC levels, strengthening MDSCs also as prognostic markers for OS when measured before ipilimumab treatment. In parallel to our observations, it was recently shown that CD14⁺CD11b⁺HLA-DR^{-/low} monocytic MDSC, but not Tregs could be used as prognostic biomarkers for predicting survival rates in patients with advanced melanoma (9). Moreover, it was shown that circulating T cells targeting specific melanoma-associated antigens (NY-ESO-1 or Melan-A) have also a strong prognostic impact on patients with melanoma (9). In addition to MDSCs and other cell populations effecting the efficacy of ipilimumab therapy, genetic studies of whole-exome sequencing (WES) and RNA sequencing that were performed on bulk tumor tissues taken from patients treated with anti-CTLA4, showed that high mutational load and neoantigens presented by the tumor cells, correlate with response to this therapy (41, 42). Whether combined use of both CD33⁺CD11b⁺HLA-DR⁻ and CD14⁺CD11b⁺HLA-DR^{-/low} populations alongside with genetic studies could predict better

responses in patients with advanced melanoma will necessitate further studies.

MDSCs, in addition to their use as biomarkers for the evaluation of the patients' immune status, could also serve as targets. In mice, depletion of MDSCs by anti-Gr1 antibodies leads to immune system recuperation that increases efficacy of immune-based therapies (13) as well as to tumor regression (Sade-Feldman and colleagues; unpublished data). Along this strategy, anti-CD33 antibodies currently used for therapy of patients with myeloid leukemia (43) could be used to deplete human CD33 positive MDSCs to increase immune system efficacy. On basis of our results, we propose that if high levels of circulating MDSCs with elevated suppressive features are detected alone or in conjunction with high tumor burden before ipilimumab treatment or other immunotherapies such as PD1 blockade or adoptive cell therapies, the harmful inflammatory environment must be neutralized before or in conjunction with the treatment. This strategy is expected to improve disease outcome.

To conclude, the results presented herein demonstrate for the first time that circulating CD33⁺CD11b⁺HLA-DR⁻ MDSCs with suppressive features are associated with long-term survival and objective clinical response to ipilimumab treatment in patients with advanced melanoma. Furthermore, future studies should include additional analysis of the phenotypic properties of these cells and the mechanisms underlying their immune suppressive effect. Collectively, our findings may lead to a judicious use of ipilimumab when combined with immune monitoring system toward optimizing personalized treatments, enabling a better disease outcome and life quality.

Disclosure of Potential Conflicts of Interest

M. Lotem is a consultant/advisory board member for MSD. No potential conflicts of interest were disclosed by the other authors.

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