Clinical significance of circulating CD33⁺CD11b⁺HLA-DR⁻ myeloid cells in Stage-IV melanoma patients treated with ipilimumab

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STATEMENT OF TRANSLATIONAL RELEVANCE

Despite increased survival rates of melanoma patients treated with ipilimumab, only a relative small proportion of patients 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 stage IV melanoma patients prior to 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. Additionally, elevated frequencies of CD33⁺CD11b⁺HLA-DR⁺ cells was also associated with CD247 down regulation 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 melanoma patients’ immune status prior to and during ipilimumab treatment. Furthermore, it provides the rationale to target these cells in combination with immune-based therapies to achieve improved clinical outcomes.
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 stage-IV melanoma patients treated with anti-CTLA4 (ipilimumab) therapy.

EXPERIMENTAL DESIGN:
Peripheral blood samples from 56 patients and 50 healthy donors (HD) 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 prior to anti-CTLA4 therapy correlate with the patients’ response and overall-survival (OS).

RESULTS:
Melanoma patients had significantly higher levels of circulating CD33\(^+\)CD11b\(^-\)HLA-DR\(^-\) MDSCs with suppressive phenotype when compared to HD. Low levels of MDSCs prior to 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 to 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 stage-IV melanoma patients treated with anti-CTLA4 therapy. This monitoring system may aid in the development of combinatorial modalities, targeting the suppressive environment in conjunction with ipilimumab, towards facilitating better disease outcomes.
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 U.S., 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 non-operable 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 advanced melanoma patients, which correlates with improved survival, tumor regression and disease stabilization (4). Despite the increased survival rates observed in melanoma patients treated with ipilimumab, only a relative small proportion of patients benefits from this treatment with an objective response rate of 10%-13% (4, 5). Furthermore, ipilimumab is not devoid of side effects, 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 (Tregs) 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). Both in mice and humans the expansion and activation of MDSCs is mediated by a complex network of pro-inflammatory cytokines, chemokines and growth factors, persistently secreted by normal or modified cells including tumor cells (12-14). In mice these cells are identified as GR1⁺CD11b⁺ cells and can be further sub divided into two main subsets with different phenotypic and biological properties, the monocytic MDSCs (Mo-MDSCs) characterized by CD11b⁺Ly6G⁻Ly6C^{high} and granulocytic like MDSCs (G-MDSCs) expressing CD11b⁺Ly6G⁺Ly6C^{low} (10, 12). In contrast to 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).

Based on 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^ monocyctic MDSC, but not Tregs could be used as prognostic biomarkers for predicting survival rates in advanced melanoma patients (9) and that increased levels of this unique monocyctic MDSC population correlates with lack of response in melanoma patients 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 the current study we evaluated the clinical significance of circulating CD33^+^CD11b^+^HLA-DR MDSCs as prognostic and predictive biomarkers in stage IV melanoma patients 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 melanoma patients’ immune status prior to and during ipilimumab treatment.
MATERIALS AND METHODS

Patients
Peripheral blood (3-5ml) was collected from 56 stage-IV melanoma patients 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. 50 healthy donors 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/ctcaev3.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 cryovials tubes. The cryovials tubes were first stored in an -80°C using Mr. Frosty containers (Thermo Scientific, 5100-0001) for 48h 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 15ml tubes containing preheated (37°C) medium (RPMI-1640). After one wash
whole blood samples were resuspended in PBSX1 (50-100μl whole blood in 200μl PBSX1) and stored in 4°C refrigerators for no more than 1h prior to 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) and anti-CD247 (H146); phycoerythrin (PE)-labeled anti-CD33 (WM53; Biolegend) and anti-CD56 (HCD56; Biolegend); APC-labeled anti-CD11b (ICRF44; Biolegend) and anti-CD3ε (OKT3; Biolegend); 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 and 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, CD3ε, CD247 antibodies, washed again and were resuspended with flow stain buffer. All samples were analyzed by FACSCalibur using Cell Quest software (BD).

**NO\(^\cdot\) and hROS measurements**

iNOS activity was evaluated by measuring intracellular NO\(^\cdot\) in HLA-DR\(^-\)CD33\(^+\)CD11b\(^+\) cells, by using the DAF-2DA reagent (NOS 200-1, Cell Technology Inc). hROS production in HLA-DR\(^-\)CD33\(^+\)CD11b\(^+\) cells was performed by using reactive oxygen species detection kit (APF 4011, Cell Technology Inc). In both cases, the detection of NO\(^\cdot\) 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 ± s.e.m. When comparing two groups, we determined statistical significance using two-tailed Student's \(t\) test. When more than two groups were investigated, we performed an analysis of variance (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 non-operable melanoma between July 2010 and November 2013. Patients were treated with 4 cycles of 3mg/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 (prior to the treatment) and after 3 weeks, when the second dose was given. Blood samples from melanoma patients and 50 healthy donors (HD) were compared as to the indicated immune parameters (Table 2).

Detection of MDSC frequencies in fresh vs. 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 studies, which used a Ficoll density gradient centrifugation step for peripheral blood mononuclear cells (PBMC) preparation, prior to 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 cells frequencies, blood samples from 15 HD were analyzed. Whole blood and matched Ficoll isolated PBMC from each of the tested donors were analyzed prior to and after freezing and thawing. Analyzing whole blood samples preserves the composition of lymphocytes, monocytes and granulocytes both in fresh (Figure 1A upper plots) and frozen (Figure 1A lower 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 (Figure 1B) and thus, does not reflect the original composition of immune cell populations and MDSC percentages in particular (Figure 1C). No significant changes were observed in CD33+CD11b+HLA-DR− MDCSs and CD3+ T cells frequencies in fresh vs. 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 cryopreserved/thawed Ficoll treated samples (Figure 1C-D). These results are supported by previous studies showing that PBMC isolation using Ficoll prior
to 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 stage IV melanoma patients**

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 to healthy subjects and assessed if they exhibit an increased suppressive phenotype, as we recently reported for colorectal cancer (CRC) patients (15). The percentage of MDSCs in the current study is shown as the CD33^+CD11b^+ (%) of gated HLA-DR^- cells, as previously shown by us and others in different malignancies including melanoma (15, 17, 18, 24) (Supplementary Figure 1A). Analysis of blood samples taken prior to the first ipilimumab treatment showed that the percentages of CD33^+CD11b^+HLA-DR^- MDSCs in the melanoma patients group was significantly higher (48.8±2.3, P<0.0001) as compared to HD (27.9±1.7) (Figure 2A). Despite our inability to reach a full age-matched distribution between HD and the melanoma patients (Table 2), there was no age-dependent alteration in MDSC frequencies in the melanoma patients group (Figure 2B), suggesting that age has no effect on MDSC numbers.

The observed increased MDSC levels in melanoma patients 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 (Figure 2C) and NO' (Figure 2D) in melanoma patients 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 to MDSCs from HD (NO': 12.67±0.57; hROS: 2.89±0.2).

The increased frequencies of activated MDSCs detected in the melanoma patients, suggest an immunosuppressive activity that leads to an impaired immunological status. We thus evaluated CD247 (TCRy 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
down regulated in various types of tumors, reflecting a suppressed patients’ immune status (26, 27). The results revealed that circulating CD3^+CD56^+ T-cells in melanoma patients displayed significantly reduced CD247 expression (77.1±3.5, P<0.0001) when compared to the HD group (100±3.5) (Figure 2E). It is important to note that the only TCR subunit affected by the inflammatory environment in the melanoma patients group was CD247. Evaluation of CD3ε showed no significant changes between the patients group (101.3±1.5) and HD (100±1.53) (Figure 2F). Moreover a significant inverse correlation (P<0.0015) was detected between MDSC levels and CD247 expression in melanoma patients. High levels of MDSCs correlated with low expression levels of CD247 and vice versa (Figure 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 sub stages according to the American Joint Committee on Cancer (AJCC) (28), based on the site of metastases and on lactate dehydrogenase (LDH) levels. The sub stages 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 sub stage due to its prognostic significance (28-30). Since stage IV sub classification 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 prior to 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 sub groups, when each group was evaluated separately (Figure 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) (Figure 3B). We then evaluated whether abnormal LDH serum levels (>480U/I) correlate with increased MDSC frequencies. Indeed, our results show a significant increase in the frequencies of circulating CD3^+CD11b^+HLA-DR^+ MDSCs in patients with abnormal LDH levels (50.6±3), relative to those found in patients with normal LDH (<480U/I, 37.4±3, P<0.006) (Figure 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 (Figure 3D), immunotherapy (Figure 3E) or radiotherapy (Figure 3F) prior to the ipilimumab treatments. No significant changes were also observed in MDSC frequencies when compared to the age (Figure 2B), tumor origin (Figure 3G) and gender (Figure 3H) within the described patients’ group. Moreover, when analyzing MDSC levels prior to ipilimumab therapy and after the 2nd treatment, no significant changes were observed in their frequencies (Figure 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 ipilimumab-treated patients**

Based on our results showing increased levels of circulating CD33⁺CD11b⁺HLA-DR⁺ MDSCs in stage IV melanoma patients 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 prior to the first ipilimumab treatment can distinguish between responders and non-responders. 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 non-responders were defined as PD (progressive disease), using the immune-related response criteria (irRC) (31).

Analysis of blood samples from melanoma patients prior to 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 to non-responding patients (48.6±2.7, n=42) (Figure 4A). In contrast to MDSC frequencies, LDH serum level, which is the only blood marker in advanced melanoma to be adopted by the AJCC staging (28), could not distinguish between the responder and non-responder populations (Figure 4B).

After identifying low frequencies of MDSCs associated with a clinical response to ipilimumab treatment, we next explored whether monitoring MDSC levels in melanoma patients prior to 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 then 1 year from therapy initiation, and patients that were considered as long-term survivors (regardless of their clinical response) and survived more then 1 year. When comparing these two groups to the percentage of MDSCs, significant association was observed (Figure 4C). Patients that their OS was less then one year had significantly higher levels of circulating MDSCs prior to 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 (Figure 4D).

Finally, we explored whether classifying the melanoma patients into two groups, one having high level (above $>55.5\%$) of circulating MDSCs and the other with low MDSC levels (below $<55.5\%$) prior to the first treatment, could provide a clue as to their survival benefit. The rational for this cut-off 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 to the group having less than 55.5% of these circulating cells (Figure 4E). Overall, our data show that low frequencies of circulating MDSCs detected prior to 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 prior to ipilimumab therapy is the only parameter associated with both clinical response and prolonged OS in stage IV melanoma patients. Analysis of the median OS (in months) of the 56 melanoma patients 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 to patients having low levels (15.6±1.9, $n=40$) (Figure 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 to patients with serum LDH under
480U/l (18.3±2.3 months, n=27) or stages M1a/b/c (17.2±2 months, n=35) (Figure 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 Figure 1B)
and M2 metastatic staging (Supplementary Figure 1C).

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 1). These patients did not have
increased MDSC levels (<55.5%), but their LDH serum levels were above >480U/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
Figure 1E). It is important to note that combination of MDSC and metastatic staging didn’t have
a synergistic effect when compared only to the metastatic parameter (Supplementary Figure 1C-
D). 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 (Figure 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 cancer patients undergoing anti-CTLA4 (ipilimumab) therapy. We analyzed peripheral blood samples of 56 stage IV melanoma patients receiving ipilimumab at different time points, prior to and following treatment and compared them to 50 healthy donors (HD). We demonstrate that increased frequencies of circulating MDSCs with suppressive features in stage IV melanoma patients, prior to ipilimumab treatment, correlate with non-responsiveness and minimal or no benefit in terms of survival, when compared to 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 to 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.

Based on 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 to HD (27.9±1.7). The increased frequencies of activated MDSCs detected in the melanoma patients, suggests an impaired immunological status. Indeed evaluation of CD247 (TCRζ chain) expression in T-cells, previously reported to be down regulated in
various types of tumors, revealed that circulating CD3⁺CD56⁻ T-cells in melanoma patients displayed significantly reduced CD247 expression (77.1±3.5, P<0.0001) when compared to the HD group (100±3.5). Furthermore, a significant invers correlation (P<0.0015) was detected between MDSC levels and CD247 expression in melanoma patients, 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 down regulated CD247 expression reflect an impaired immunological 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 down regulated in the course of chronic inflammation while the CD3⁺ or any other TCR subunit are unchanged (15, 26), and is in contrast to a normal T-cell activation process that leads to the down regulation of the entire TCR complex (26). Indeed, recent reports showed increased levels of pro-inflammatory cytokines in the serum of melanoma patients, 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 form patients but not from healthy donors 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 melanoma patients group. However, changes in the frequencies of MDSCs were detected when compared to 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, prior to ipilimumab therapy and after the 2nd 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<sup>+</sup> regulatory T-cells (39).

Although, ipilimumab treatment has been shown to increase OS in melanoma patients, only a relative small proportion of patients benefit from this treatment with an objective response rate of 10-13% (4, 5). In our study LDH serum levels could not distinguish between clinical responders and non-responders upon ipilimumab treatment, but were demonstrated to be a strong predictive factor for survival as previously shown by Kelderan et al (40). In contrast, analysis of blood samples from melanoma patients prior to the first ipilimumab treatment revealed significantly lower frequencies of circulating CD33<sup>-</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup> MDSCs in patients responding to the treatment (34.2±4.3, n=14, P<0.008) compared to 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 to patients displaying elevated MDSC levels, strengthening MDSCs also as prognostic markers for OS when measured prior to ipilimumab treatment. In parallel to our observations, it was recently shown that CD14<sup>-</sup>CD11b<sup>+</sup>HLA-DR<sup>-low</sup> monocyctic MDSC, but not Tregs could be used as prognostic biomarkers for predicting survival rates in advanced melanoma patients (9). Moreover, it was shown that circulating T-cells targeting specific melanoma-associated antigens (NY-ESO-1 or Melan-A) have also strong prognostic impact in melanoma patients (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<sup>-</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup> and CD14<sup>-</sup>CD11b<sup>+</sup>HLA-DR<sup>-low</sup> populations along side with genetic studies could predict better responses in advanced melanoma patients will necessitates 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 et al unpublished data). Along this strategy, anti-CD33 antibodies currently used for therapy of myeloid leukemia patients (43) could be used to deplete human CD33 positive MDSCs to increase immune system efficacy. Based on 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 prior to ipilimumab treatment or other immunotherapies such as PD1 blockade or adoptive cell therapies, the harmful inflammatory environment must be neutralized prior to 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 advanced melanoma patients. 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 towards optimizing personalized treatments, enabling a better disease outcome and life quality.
ACKNOWLEDGMENTS

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REFERENCES

FIGURE LEGEND:

Figure 1. Whole blood cryopreservation/thawing preserves MDSC frequencies. (A-B) Blood was drawn from 15 healthy donors (HD) to evaluate CD33⁺CD11b⁺HLA-DR⁻ MDSC and CD3⁺ T cell percentages prior to 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-D) Whole blood and matched Ficoll isolated PBMC were analyzed prior to 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.

Figure 2. CD33⁺CD11b⁺HLA-DR⁻ MDSC levels with high NO⁻ and hROS levels are increased in melanoma patients. 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 stage IV melanoma patients (n=56) prior to ipilimumab treatment using flow cytometry analysis (B) CD33⁺CD11b⁺HLA-DR⁻ MDSC percentages in melanoma patients were evaluated according to the age of the patients, numbers indicate years. ***P<0.0001 (t test); n.s- not significant. (C-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 stage IV melanoma patients (n=56) are presented. (E-F) Peripheral blood cells from HD (n=50) and melanoma-patients (n=56) were fixed, permeabilized and analyzed by flow cytometry for total expression (%) of CD247 (E) and CD3e (F), compared to 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 stage IV melanoma patients (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). Melanoma patients were divided into four sub groups M1a, M1b, M1c and M2 according to the
AJCC metastatic sub staging system in melanoma, and CD33⁺CD11b⁺HLA-DR⁺ MDSC percentages were evaluated according to this sub staging. (B) CD33⁺CD11b⁺HLA-DR⁺ MDSC percentages were evaluated in the melanoma patients 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<480U/I) and abnormal levels (LDH>480U/I) of LDH in the serum. LDH levels and MDSC percentages where evaluated in blood samples drawn at the same day from each patient, prior to the first ipilimumab treatment. (D-F) CD33⁺CD11b⁺HLA-DR⁺ MDSC percentages in melanoma patients receiving previous treatments such as chemotherapy (D), immunotherapy (E) and radiotherapy (F) were tested prior to 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 prior to (n=20) and after 2X treatments (n=20) with ipilimumab, compared to 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<480U/I) and elevated baseline (LDH>480U/I) serum LDH levels prior to the first ipilimumab treatment in patients responding or non-responding to the treatment. (C) CD33⁺CD11b⁺HLA-DR⁺ MDSC percentages in melanoma patients with minimal or no treatment benefit (<1 year) and patients with long-term overall survival (>1 year), was evaluated prior to the first ipilimumab treatment. (D) Correlation between MDSC percentages and overall survival (days) in stage IV melanoma patients (n=56). Each dot represents a patient **P<0.0022 (E) Kaplan-Meier survival curve of stage IV melanoma patients 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 long-rank test, P<0.003. (F-H) Survival rates of 56 stage IV melanoma patients according to parameters evaluated prior to 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 stage IV melanoma patients 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 to 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.
Figure 1

A

Gated on HLA-DR cells

Gated on lymphocytes

Fresh

Frozen

B

Gated on HLA-DR cells

Gated on lymphocytes

Fresh

Frozen

C

Gated on HLA-DR cells

D

Gated on lymphocytes

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Figure 2

A) Gated on HLA-DR+ cells

B) Gated on HLA-DR+ cells

C) HROS (MFI)

D) NO (MFI)

E) CD247 expression (%)

F) CD8 expression (%)

G) CD247 expression (%) vs. MDSCs (%)

* * * Online First on May 13, 2016; DOI: 10.1158/1078-0432.CCR-15-3104

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Figure 3

A. Gated on HLA-DR cells

B. Gated on HLA-DR cells

C. Gated on HLA-DR cells

D. Gated on HLA-DR cells

E. Gated on HLA-DR cells

F. Gated on HLA-DR cells

G. Gated on HLA-DR cells

H. Gated on HLA-DR cells

I. Gated on HLA-DR cells

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### Table 1

**Clinical details of melanoma patients**

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

SD stable disease; PR partial response; CR complete response; PD progressive disease; LDH lactate dehydrogenase; N.D not determined.
Table 2
Details of healthy and melanoma patients

<table>
<thead>
<tr>
<th>Details of patients and healthy donors</th>
<th>Numbers (%)</th>
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</thead>
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<tr>
<td>All patients (n=56)</td>
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<tr>
<td>Median age</td>
<td>60.7</td>
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<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24(42.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>32(57.1%)</td>
</tr>
<tr>
<td>Healthy Donors (n=50)</td>
<td></td>
</tr>
<tr>
<td>Median age</td>
<td>32.5</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31(62%)</td>
</tr>
<tr>
<td>Female</td>
<td>19(38%)</td>
</tr>
</tbody>
</table>

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

Clinical significance of circulating CD33+CD11b+HLA-DR-myeloid cells in Stage-IV melanoma patients treated with ipilimumab

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