

Research Article

Computational Algorithm-Driven Evaluation of Monocytic Myeloid-Derived Suppressor Cell Frequency for Prediction of Clinical Outcomes

Shigehisa Kitano¹, Michael A. Postow^{3,4}, Carly G.K. Ziegler³, Deborah Kuk³, Katherine S. Panageas³, Czrina Cortez^{3,5}, Teresa Rasalan^{2,3,5}, Mathew Adamow^{2,3}, Jianda Yuan^{2,3}, Philip Wong^{2,3}, Regoire Altan-Bonnet³, Jedd D. Wolchok^{2,3,4,5}, and Alexander M. Lesokhin^{3,4,5}

Abstract

Evaluation of myeloid-derived suppressor cells (MDSC), a cell type implicated in T-cell suppression, may inform immune status. However, a uniform methodology is necessary for prospective testing as a biomarker. We report the use of a computational algorithm-driven analysis of whole blood and cryopreserved samples for monocytic MDSC (m-MDSC) quantity that removes variables related to blood processing and user definitions. Applying these methods to samples from patients with melanoma identifies differing frequency distribution of m-MDSC relative to that in healthy donors. Patients with a pretreatment m-MDSC frequency outside a preliminary definition of healthy donor range (<14.9%) were significantly more likely to achieve prolonged overall survival following treatment with ipilimumab, an antibody that promotes T-cell activation and proliferation. m-MDSC frequencies were inversely correlated with peripheral CD8⁺ T-cell expansion following ipilimumab. Algorithm-driven analysis may enable not only development of a novel pretreatment biomarker for ipilimumab therapy, but also prospective validation of peripheral blood m-MDSCs as a biomarker in multiple disease settings. *Cancer Immunol Res*; 2(8); 812–21. ©2014 AACR.

Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of granulocyte- and monocyte-like cells that inhibit T-cell function (1, 2). Clinically significant MDSC accumulation has been observed in many challenges to the immune system in humans including chronic infection, transplant, and multiple malignancies (3–10). Diversity in phenotype and methods used for analysis creates challenges in prospectively and reproducibly defining the clinical import of this cellular subset. Monocytic MDSCs (m-MDSC) are frequently characterized as CD14⁺/HLA-DR^{low/-} cells in humans; however, HLA-DR expression is typically a broad distribution, making identification of a specific subset of cells susceptible to inter-user variability. Nevertheless, increased CD14⁺/HLA-DR^{low/-} cells in the peripheral

blood have been designated as m-MDSCs in individual datasets based on this cell population's ability to suppress lymphocyte function and are prognostic in patients with hematologic cancers (chronic lymphocytic leukemia and multiple myeloma), solid tumors (hepatocellular carcinoma, non-small cell lung cancer, melanoma, and others), chronic infection (HIV), cirrhosis, and allotransplantation (5, 8, 11–17).

In melanoma, m-MDSCs correlate with melanoma disease activity and are independently prognostic of overall survival (OS) in patients with stage IV disease (6, 18–20). Levels of m-MDSC inversely correlate with the presence of NY-ESO-1-specific T cells and seem to be increased in ipilimumab nonresponders (20, 21). This finding suggests a link between m-MDSC and antigen-specific immunity *in vivo* and provides additional rationale for routinely evaluating m-MDSCs as a biomarker in the context of immunotherapy clinical trials. However, a uniform methodology that corrects for artifacts introduced by cell processing, cryopreservation, and analysis needs to be developed to enable routine measurement of m-MDSCs for prospective testing as a biomarker (22).

Immunomodulatory therapy, which has emerged as a promising treatment approach for metastatic melanoma and other cancers, is an area in which biomarker development may enable selection of therapy for individuals more likely to achieve prolonged OS. Ipilimumab, an antibody that blocks the function of the immune inhibitory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4), was the first immunomodulatory antibody to gain regulatory approval as a cancer therapeutic based on two phase III studies demonstrating

Authors' Affiliations: ¹Department of Experimental Therapeutics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Tsukiji, Tokyo, Japan; ²Ludwig Center for Cancer Immunotherapy; ³Memorial Sloan-Kettering Cancer Center; ⁴Weill-Cornell Medical and Graduate Schools; and ⁵Ludwig Collaborative and Swim Across America Lab, New York, New York

Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

J.D. Wolchok and A.M. Lesokhin contributed equally to this article.

Corresponding Author: Alexander M. Lesokhin, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 212-639-3069; Fax: 646-227-7116; E-mail: lesokhia@mskcc.org

doi: 10.1158/2326-6066.CIR-14-0013

©2014 American Association for Cancer Research.

significant increases in OS in patients with metastatic melanoma (23, 24). However, only 20% to 30% of patients achieve long-term survival following therapy (25). This finding not only supports the need to define biomarkers in this context, but also to identify mechanisms of resistance that could lead to additional therapeutic targets for improved outcomes.

A number of biomarkers examining T-cell proliferation or activation and antigen-specific immunity have been assessed in the context of ipilimumab therapy. Gene expression profiling on tumor biopsies collected from 45 patients with melanoma before and after ipilimumab treatment showed that an immunologically active tumor microenvironment favors clinical response to ipilimumab (26, 27). In peripheral blood, sustained ICOS elevation in CD4⁺T cells, higher percentage of EOMES⁺ CD8⁺ T cells or Ki67⁺EOMES⁺CD8⁺ T cells, and an NY-ESO-1-specific CD8⁺ T-cell response in patients with NY-ESO-1-seropositive metastatic melanoma have all shown an association with clinical benefit and survival following ipilimumab therapy (28, 29).

Absolute lymphocyte count (ALC), the most clinically accessible biomarker, available through a routine complete blood count, has been shown to correlate with OS in several single-institution, noncontrolled studies (30). More recently, an analysis of almost 2,000 ipilimumab-treated patients in multiple studies, including randomized, controlled, and phase III studies, has demonstrated that an ALC increase is a specific pharmacodynamic biomarker of ipilimumab. In the absence of concomitant chemotherapy, the degree of this pharmacodynamic increase in lymphocyte count at the commercially available ipilimumab dose (3 mg/kg) is associated with OS (Postow et al.; submitted for publication), suggesting that ALC is worthy of further investigation in the context of risk-adapted clinical trial design.

We report the development of methods to enable uniform analysis of m-MDSCs that overcome issues related to blood processing and inter-user variability. This is achieved by deriving a measure of m-MDSCs using coefficient of variation (CV) to assess HLA-DR spread on CD14⁺CD11b⁺ cells and through the evaluation of stabilizers of HLA-DR levels in whole blood. We validate these methods by demonstrating that CD14⁺HLA-DR^{low/-} m-MDSC quantity derived from CV values is both inversely correlated with pharmacodynamics markers of ipilimumab function and also associated with OS among patients undergoing treatment with ipilimumab.

Materials and Methods

Patients

We identified 83 patients who were treated on a clinical study with ipilimumab between February 2008 and March 2012 and had cryopreserved peripheral blood samples in our tissue banks. Peripheral blood from healthy donor volunteers was obtained at the time of the current study and from samples in our institutional tissue bank. MDSC analyses were performed between December 2011 and March 2012. We excluded 4 and 11 samples in the 10-mg/kg and 3-mg/kg ipilimumab groups, respectively, because of an overnight delay between phlebotomy

and processing time, which validation studies confirmed affects levels of HLA-DR (Fig. 1E). Patients and healthy donors provided informed consent for the clinical studies and the collection of blood and tumor tissue on a correlative biospecimen protocol. Patients were treated with ipilimumab on Bristol-Myers Squibb studies CA184045 (NCT00495066) or CA184-087 (NCT00920907), with four doses of ipilimumab at 10 mg/kg or 3 mg/kg i.v. every 3 weeks during induction therapy, followed by maintenance ipilimumab at the same dose every 12 weeks, starting at week 24. Clinical benefit was determined by investigators at week 24 imaging based on interpretation of radiographic stable disease or better by modified World Health Organization (mWHO) or RECIST criteria. All studies were approved by the Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY) Institutional Review Board.

MDSC staining

Blood was collected and cryopreserved using BD Vacutainer CPT tubes (BD pharmingen) from patients with melanoma and healthy donors for the retrospective analysis. Samples were collected from patients and healthy donors in Cyto-Chex (Streck), Vacutainer CPT, or standard heparin vacutainer tubes for comparative analysis. Peripheral blood mononuclear cells (PBMC; 5×10^5) from patients with melanoma or healthy donors were washed with 2 mL FACS buffer (PBS containing 2% bovine serum albumin and 0.05 mmol/L EDTA). The following antibodies were then added for 30 minutes at 4°C: Lineage (CD3/CD16/CD19/CD20/CD56) cocktail FITC (BD Pharmingen), CD14-PerCP Cy5.5, CD11b-APC Cy7, CD33-PE-Cy7 (BD Pharmingen), and HLA-DR-ECD (Beckman Coulter). Isotype controls included the appropriate fluorochrome-conjugated mouse IgG1, IgG1k, IgG2a, or IgG2b k (BD Pharmingen; Beckman Coulter; R&D Systems). Whole blood samples were lysed for 10 minutes in BD Phosflow Lyse/Fix after staining (BD Pharmingen). Stained cells were detected using a LSR Fortessa with FACS Diva software (BD Biosciences). Analysis was carried out using FlowJo (TreeStar). m-MDSCs were quantified as described. Briefly, scale values for HLA-DR within a singlet, live, lineage-negative (Lin⁻; CD3, CD16, CD19, CD20, and CD56) cell population that expressed CD14⁺CD11b⁺ were exported from FlowJo and analyzed using code written in R software to derive the CV, a ratio of standard deviation (SD; σ) and geometric mean fluorescence intensity (GMFI). A %m-MDSC frequency defined as the %HLA-DR^{low/-} among CD14⁺CD11b⁺ cells was derived using a nomogram based on the 99th percentile CV_{HLA-DR} among CD14⁺CD11b⁺ cells from healthy donors. Absolute number of m-MDSC (/ μ L) in peripheral blood was estimated using the formula: (%m-MDSC) \times (number of monocytes (/ μ L) from a complete blood count on the same day.

T-cell suppression assay

A T-cell suppression assay was performed as described previously (31). Briefly, CD14⁺ PBMCs magnetically separated using MACS beads (Miltenyi Biotec) were cultured with 2×10^5 CFSE-labeled autologous CD14⁻ PBMCs in 96-well flat-bottom α -CD3-specific Ab-coated plates (OKT3, 100 μ L at 0.5 μ g/mL

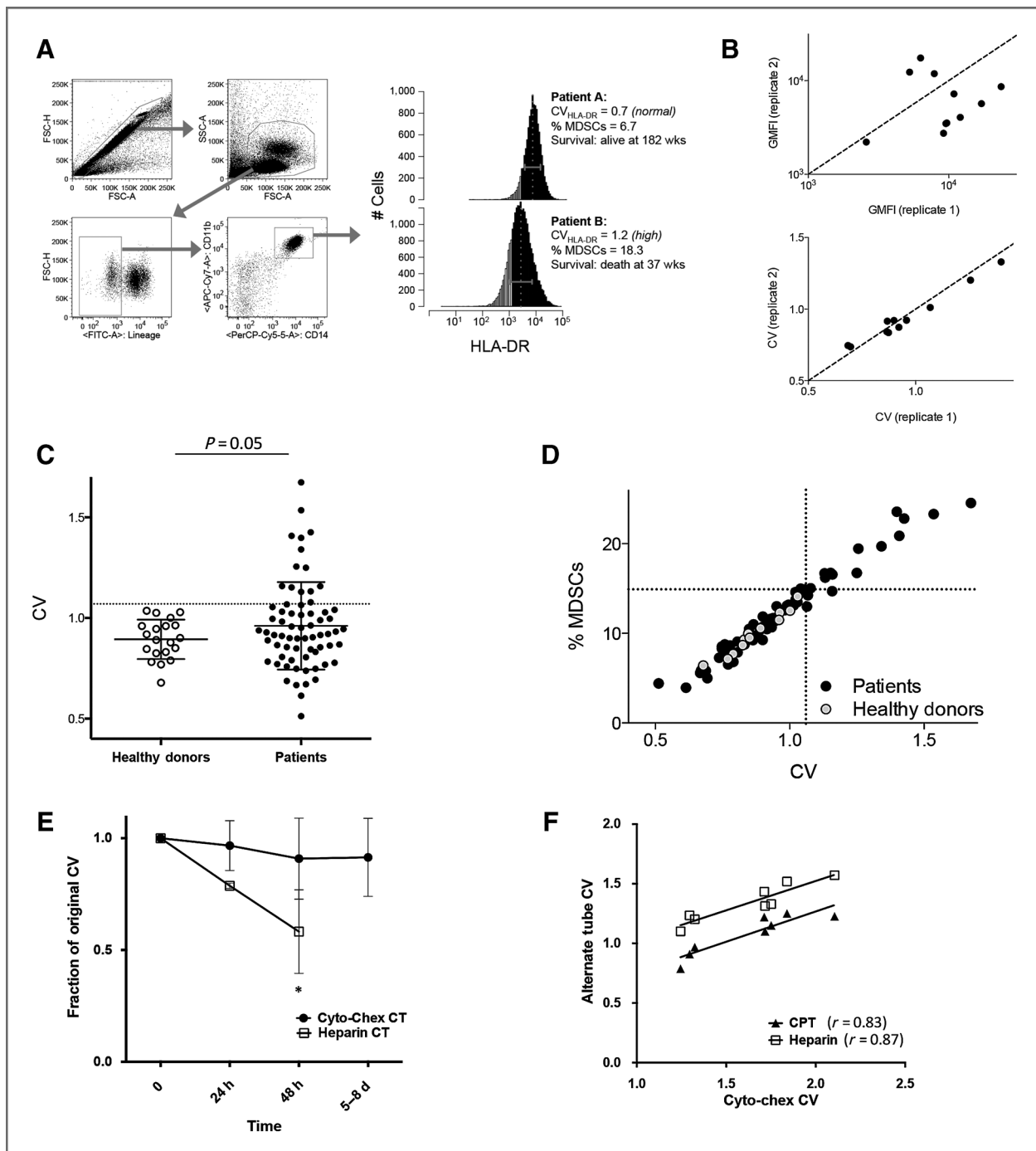


Figure 1. Analysis of MDSC frequency. PBMCs from patients with advanced melanoma and from healthy donors were stained with surface antibody and analyzed by multicolor flow cytometry. We defined monocytic myeloid cells based on the presence of CD14, CD11b in a CD3, CD16, CD19, CD20, and CD56 in a Lin⁻ population. Within this monocytic cell population, m-MDSCs were isolated on the basis of their low levels of HLA-DR expression. A, gating strategy to isolate myeloid-derived cells as CD14⁺CD11b⁺Lin⁻ cells. On the basis of the 99th percentile of healthy donor values, a cutoff for low expression of HLA-DR was set to isolate the population of m-MDSC (shaded in gray). B, m-MDSC composition by HLA-DR GMFI is subject to fluctuations in staining acquisition and sample handling. CV_{HLA-DR} represents a self-normalizing measurement and is stable among replicate measurements. C, comparison of CV for HLA expression within the myeloid compartment reveals a larger spread for patients pretreatment, compared with healthy donors and large differences in CV between patients (healthy donors vs. patients; $P < 0.05$). D, normogram plotting relationship between CV values and m-MDSC frequency. E, evaluation of whole blood collected in standard heparin or Cyto-Chex tubes ($n = 9$) for m-MDSC frequency and stored at room temperature for the specified interval between analysis and acquisition. Data are expressed as a percentage of total m-MDSCs present at baseline. *, $P = 0.002$. F, correlation between m-MDSC analysis of samples ($n = 8$) cryopreserved using BD Vacutainer CPT tubes, standard heparin tubes, and collected in Cyto-Chex tubes.

for 2 hours at 37°C) in RPMI-1640 medium supplemented with 10% FBS and IL2 (10 IU/mL; Roche). After 5 days, cells were harvested, stained with CD3-PECy7, CD4-ECD, and CD8-APC Cy7 (BD Pharmingen), and CFSE signal of gated CD8⁺ T cells (CD3⁺ CD4⁻) was measured by flow cytometry. The stimulation index (SI) was calculated by dividing the proliferation measured in the absence of m-MDSC by proliferation measured in the presence of m-MDSC, as previously described (31).

Statistical analysis

Patient characteristics were described using median and range for continuous variables and frequency and percentages for categorical variables. The primary endpoint for this retrospective analysis was OS, which is defined as the time from pretreatment %m-MDSC assessment to death or last follow-up. Landmark analysis from week 6 was also performed. Patients alive at last follow-up are censored. Maximally selected log-rank statistics was used to find a cutoff value for %m-MDSC. The Kaplan–Meier method and log-rank test were used to compare differences in survival for categorical variables. Univariate and multivariate Cox proportional hazards regression was used to assess the association between clinical variables and OS. A Student *t* test with Welch correction was used for comparisons of %m-MDSC frequency in the patient and healthy donor groups. Pearson correlation was used to evaluate for relationships between %m-MDSC and lymphocyte subsets.

Results

Measuring HLA-DR spread using a computational algorithm removes user bias and inter-replicate variability in m-MDSC assessment

Published reports of m-MDSC frequency have evaluated this cellular subset by gating on Lin⁻ CD14⁺ CD11b⁺ HLA-DR^{low/-} cells in the peripheral circulation. We similarly developed a flow cytometric strategy to define m-MDSC based on high abundance of CD14, CD11b, and low or absent HLA-DR expression in a CD3, CD16, CD19, CD20, CD56, Lin⁻ population (Supplementary Fig. S1). HLA-DR expression on myeloid cells displayed a wide continuous distribution rather than distinct populations. Log-rank tests based on different gating cutoffs resulted in a broad range of m-MDSC cutoff values and highly variable survival curves. Thus, selection of an accurate gate for a low or negative HLA-DR fraction is challenging and prone to user bias and experimental unreliability. However, we observed distinct spreads for the HLA-DR distribution between individual patients, suggesting that evaluating this parameter on CD11b⁺ CD14⁺ cells could serve as a measure of m-MDSC. Thus, we gated on CD11b⁺ CD14⁺ cells and measured HLA-DR GMFI, SD, and the CV, a ratio between GMFI and SD (Fig. 1A). Evaluating CV corrects for shifting GMFI due to staining protocol and nearly eliminates inter-replicate variability (Fig. 1B), enabling measurement of HLA-DR distribution on myeloid cells objectively and independently of staining fluctuations (32). Measurements across a cohort of healthy donors (*n* = 20) and patients with melanoma (*n* = 68) revealed a higher value of CV_{HLA-DR} among patients' myeloid cells (Fig. 1C). Furthermore,

we found a cohort of patient samples with CV_{HLA-DR} levels above the range for healthy donors (defined by the 99th percentile in CV values among healthy donors). For these patients, the higher CV value indicates a higher HLA-DR spread, representative of abnormal elevations in the number of m-MDSC (HLA-DR^{low} cells). To explicitly quantify the number of m-MDSCs, we use the upper limit of CVs for healthy donors (again, the 99th percentile, = X) as a "cutoff" and generate a nomogram to calculate an *ad hoc* quantitative measure of MDSC frequency (%m-MDSC). By translating the mean-normalized variance in the data to a concrete percentage of the population, we relate CV_{HLA-DR} to a classical immunophenotyping measurement (Fig. 1D).

Given the potential for changes in HLA-DR expression that may occur during blood processing or transport to significantly alter m-MDSC evaluation, we evaluated our methods in whole blood stored at room temperature as well as cryopreserved Ficoll purified PBMCs. We noted that CV_{HLA-DR} was significantly reduced as the interval between phlebotomy time and analysis increased: A 48-hour delay until processing demonstrated a nearly 50% reduction from baseline. Levels of CV_{HLA-DR} were, however, consistent over time in Cyto-Chex blood collection tubes even if whole blood was stored at room temperature before processing for up to 8 days after phlebotomy (Fig. 1E). Actual CV_{HLA-DR} values were different but clearly correlated between Cyto-Chex BCT, vacutainer CPT cell preparation tubes (*r* = 0.83), and standard heparin tubes (*r* = 0.87; Fig. 1F).

m-MDSCs occur with relatively higher frequency among patients with metastatic melanoma than in healthy donor controls

Using our CV_{HLA-DR}/m-MDSC conversion nomogram, we determined the relative frequency of m-MDSCs for 68 patients with melanoma treated with ipilimumab at either 10 mg/kg (*n* = 28) or 3 mg/kg (*n* = 40) for whom pretreatment and week 6 PBMC samples were processed the same day as phlebotomy and stored in our tissue repository. We again used healthy donors as controls. The baseline characteristics of the patients and healthy donors are described in Table 1. The overall median time from initial m-MDSC measurement to last recorded follow-up was 13.6 months (range, 0.66–63.9).

We found that the relative frequency of peripheral blood m-MDSCs was increased among patients with metastatic melanoma (*P* = 0.05) when compared with a group of healthy individuals (Fig. 2A). Pretreatment m-MDSC frequency did not differ significantly in our cohort between patients who were treated with different doses of ipilimumab (Fig. 2B).

Pretreatment m-MDSC quantity correlates with OS in patients treated with ipilimumab

To evaluate the hypothesis that lower frequency of m-MDSCs was associated with OS, we parsed our patients according to their %m-MDSC at baseline and after two doses of ipilimumab (week 6). On the basis of log-rank statistics within our ipilimumab-treated cohort, we defined 14.9% as the

Table 1. Patient and healthy donor characteristics

Characteristics	Ipilimumab 10 mg/kg	Ipilimumab 3 mg/kg	Healthy donors ^a
Number of patients	28	40	20
Median age (range)	62 (34–83)	60 (34–80)	38 (26–58)
Sex (%)			
Male	17 (61)	29 (73)	10 (50)
Female	11 (39)	11 (27)	7 (35)
Stage of disease (%)			
III (unresectable)	0	1	—
M1a	3	0	—
M1b	4	5	—
M1c	21	34	—
Median number of prior therapies (range)	1 (0–3)	1 (0–5)	—
Median LDH (range)	209 (113–968)	211 (117–816)	—
≥Upper limit of normal (% of available LDH)	13 (46)	28 (70)	—
<Upper limit of normal (% of available LDH)	15 (54)	12 (30)	—
MDSC frequency			
%HLA-DR ^{low/-} in Lin ⁻ CD14 ⁺ CD11b ⁺ (range) ^b	11.4 (3.9–24.5)	11.2 (5.8–20.9)	10.3 (6.4–14.3)
≥14.9 (%)	7 (25)	7 (18)	0 (0)
<14.9 (%)	21 (75)	33 (82)	20 (100)
Median baseline ALC (range)	1,250 (500–5,100)	1,100 (600–8,100)	—
≥1,000/μL (%)	19 (68)	25 (63)	—
<1,000/μL (%)	9 (32)	15 (37)	—

^aData for anonymously donated blood bank samples are unavailable.

^bBaseline values.

cutoff between "high" and "low" %m-MDSC. The distribution of m-MDSC frequencies among analyzed patients is summarized in Table 1.

Having less than 14.9% m-MDSC pretreatment was associated with improved OS among 68 patients treated with ipilimumab (Fig. 2C and Table 2) with a HR of 0.35 [95% confidence interval (CI), 0.18–0.70; $P = 0.003$]. When analyzed by individual dose groups, the difference was seen in patients treated at 10 mg/kg, but not at 3 mg/kg (Supplementary Table S1). We performed univariate (Table 2) and multivariate analyses (Table 3) to evaluate the impact of ALC, lactate dehydrogenase (LDH), and monocyte counts on survival in our patient cohort. %m-MDSC < 14.9% was correlated with superior OS on both univariate and multivariate analyses. Monocyte quantity was not predictive, suggesting that %m-MDSC represents a relative activation state within the monocyte compartment and is not a direct reflection of monocyte numbers.

At treatment week 6, the frequency of m-MDSCs correlates with OS in patients treated with ipilimumab

We also evaluated associations between %m-MDSC at week 6 and OS similarly to what has been evaluated previously for ALC (Table 2 and Supplementary Table S1; refs. 30, 33). %m-MDSC below 14.9% at week 6 was associated with superior OS (Fig. 2D) in patients receiving ipilimumab treatment with a HR of 0.38 (95% CI, 0.19–0.75; $P = 0.005$). As expected, ALC greater than or equal to 1,000 at week 6 was associated with improved OS in our cohort and

normal LDH (<250) at week 6 correlated with improved OS in patients treated with ipilimumab. To address potential confounding by ALC and LDH, a multivariate analysis was performed and week 6 %m-MDSC remained significantly associated with OS, even when accounting for both LDH and week 6 ALC (Table 3).

%m-MDSC is inversely correlated with CD8⁺ T-cell rise on therapy and suppresses T-cell proliferation *in vitro*

Ipilimumab has a specific pharmacodynamic effect on ALC, but data on the specific subset of cells affected are limited. Our group previously reported on a cohort of 35 patients treated with ipilimumab at 10 mg/kg in which the relationships between increases in CD8⁺ T cells, CD4⁺ T cells, and CD4⁺CD25⁺ regulatory T cells, and clinical outcome were analyzed. In this analysis, the majority of patients had increases in all three lymphocyte subsets, but only the mean increase in CD8⁺ T cells was significantly associated with clinical benefit (34).

Because m-MDSCs are defined by the ability to suppress CD8⁺ T-cell proliferation, we examined whether m-MDSC frequency affects T cells *in vivo* or *in vitro*. We first sought to explore whether relationships between ALC and m-MDSC were observed to be consistent with m-MDSC suppressive function *in vivo*. On the basis of the known biologic functions of m-MDSCs, we reasoned that a greater frequency of m-MDSCs would limit the T-cell proliferative response to ipilimumab. However, we did not find correlations between

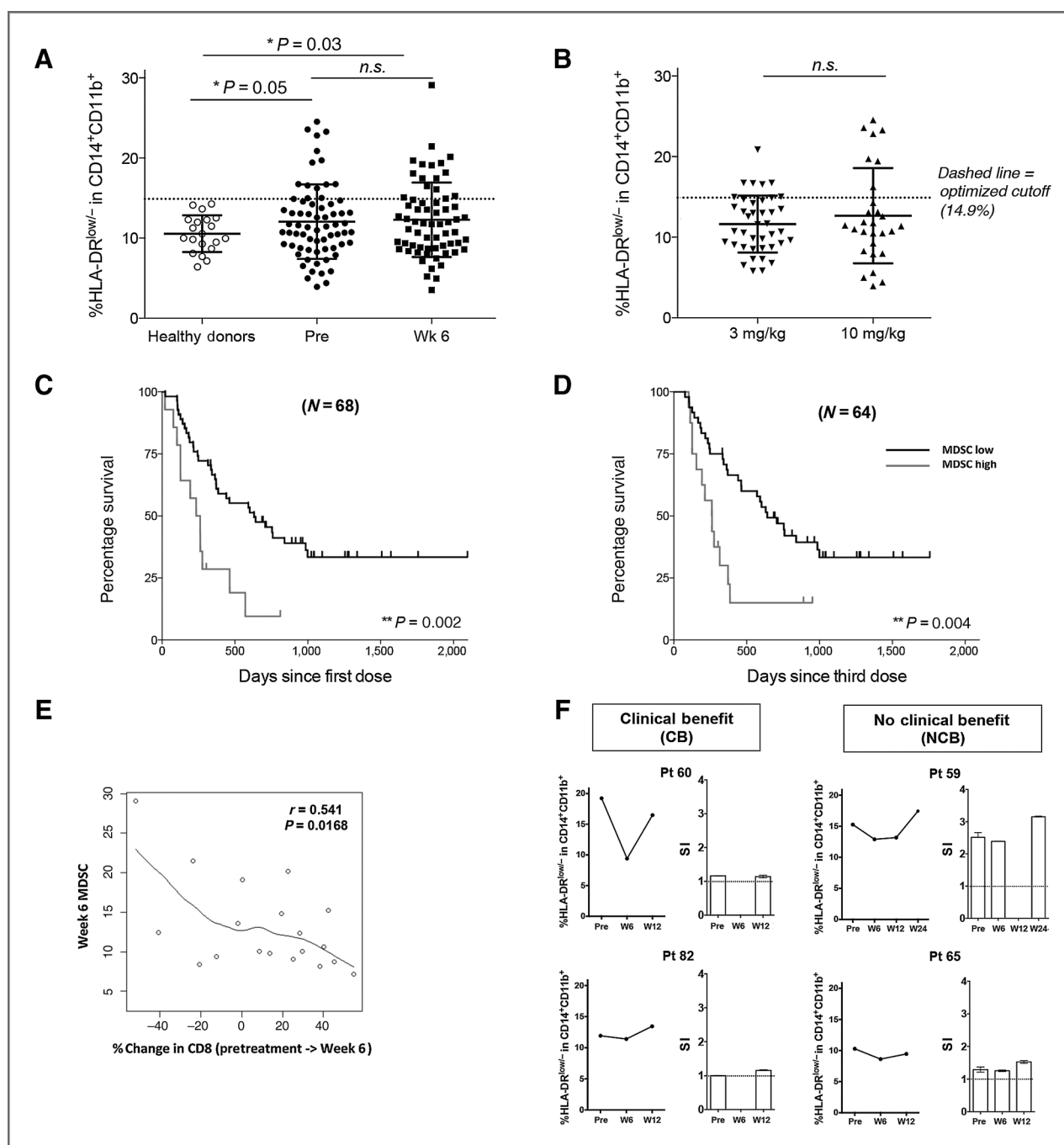


Figure 2. Functionally suppressive m-MDSCs are increased in patients with metastatic melanoma who are less likely to achieve prolonged OS following ipilimumab. **A**, PBMCs from patients with advanced melanoma and from healthy donors analyzed for %m-MDSC based on CV_{HLA-DR}. The frequency of m-MDSC in healthy donors ($n = 20$) and patients with melanoma analyzed at pretreatment baseline and week 6 (healthy donors vs. pretreatment, $P = 0.05$; healthy donors vs. week 6, $P = 0.03$). **B**, pretreatment values for subsets of patients treated with ipilimumab 10 mg/kg ($n = 28$) or 3 mg/kg ($n = 40$). **C**, OS based on m-MDSC quantity at pretreatment baseline. **D**, OS from 6 weeks after start of ipilimumab treatment. **E**, correlation between percentage change in CD8 T cells and week 6 m-MDSC frequency ($r = -0.541$; $P = 0.02$). Percentage change in CD8 T cells = [(wk 6 absolute CD8 – baseline absolute CD8)/(baseline absolute CD8)]. **F**, average SI graphed for 2 patients with melanoma with clinical benefit and 2 patients with melanoma with nonclinical benefit assessed at week 24. SI = (% proliferated CD3⁺ T cells in CD14-depleted PBMCs)/(% proliferated CD3⁺ T cells in CD14-PBMCs with CD14⁺ cells added back).

the percentage change in total ALC [(week 6 – pretreatment)/(pretreatment)] and pretreatment or week 6 m-MDSC frequency. Data on CD4⁺ and CD8⁺ T-cell subsets were available for 19

of the 40 patients treated with ipilimumab at 3 mg/kg. We observed a statistically significant inverse correlation only between percentage change in absolute CD8⁺ T-cell number

Table 2. Univariate analysis of relationship between m-MDSC and OS at pretreatment baseline and week 6 after ipilimumab

	Ipilimumab treated					
	Pretreatment			Week 6		
	<i>n</i>	HR (95% CI)	<i>P</i>	<i>n</i>	HR (95% CI)	<i>P</i>
MDSC < 14.9%	68	0.35 (0.18–0.70)	0.002	64	0.38 (0.19–0.75)	0.004
ALC ≥ 1,000 cells/μL	68	0.73 (0.41–1.33)	0.303	64	0.22 (0.11–0.45)	<0.001
LDH < 250	68	0.33 (0.18–0.59)	<0.001	65	0.37 (0.20–0.68)	0.001
Monocytes < 300 cells/μL	68	0.70 (0.25–1.95)	0.495	64	1.77 (0.69–4.51)	0.233

and m-MDSC frequency at week 6 ($r = -0.54$; $P = 0.0168$; Fig. 2E), and no correlation was observed between that and percentage change in CD4⁺ T-cell numbers on therapy.

We next assessed for suppressive function by measuring T-cell proliferation in PBMCs in the presence or absence of CD14⁺ cells. We inferred that suppressive function was present if enhanced proliferation was observed among PBMCs stimulated with anti-CD3 and IL2 in the absence of CD14-expressing cells (Supplementary Fig. S2). Proliferation of CD3⁺ T cells was increased to a greater extent in the absence of CD14-expressing cells only in PBMC samples taken from patients who did not achieve clinical benefit as measured at week 24 imaging (Fig. 2F). These data suggest that higher frequency of m-MDSCs in patients with inferior outcomes is correlated with diminished T-cell proliferation *in vitro*.

Discussion

We developed an objective methodology to evaluate m-MDSC frequency in the peripheral blood of patients with metastatic melanoma receiving immunotherapy with ipilimumab at our center. In our single institution cohort, we found that patients with metastatic melanoma have a greater frequency of m-MDSC than a group of healthy donors. An m-MDSC quantity before treatment and at week 6 that was outside the healthy donor range that we defined was significantly associated with inferior OS, independent of LDH (at baseline and week 6) and ALC (at week 6) in a multivariate model. Our observations suggest that m-MDSC frequency is a novel prognostic indicator of OS in patients with metastatic melanoma treated with ipilimumab.

The CV-based cutoff presented here represents an objective methodology for determining m-MDSC composition independent of fluorescence variability in FACS analysis. The cutoff level derived here was consistent with a level greater than the 99th percentile of a preliminary cohort of healthy donor m-MDSCs, suggesting that our method enables distinction of normal versus abnormal CV_{HLA-DR} and m-MDSC evaluation in a prospective fashion. Thus, we suggest that using healthy donors as a calibration can lead to an easily implementable, automated, and objective tool for monitoring the frequency of m-MDSCs within patients' blood samples, and distinguish between "normal" and "high" ranges. However, it is important to note the preliminary nature of our healthy donor range and that further study of the effects of age, gender, body mass index, and nonmalignant comorbid conditions on CV_{HLA-DR} are necessary to propose a "cutoff" value capable of prospectively segregating patients with melanoma more or less likely to benefit from ipilimumab.

As the CV is defined as the ratio of the SD to the mean, we obtained a metric independent of nonbiologically meaningful fluctuations in sample handling, FACS protocol, and fluorescence intensity. Although using "non-normalized" metrics for HLA-DR^{low/-} populations (GMFI and SD; Supplementary Fig. S3) replicates the reported observations, the survival-based cutoffs determined here by GMFI or SD do not represent universal standards, and would be expected to be unstable differentiating factors with subsequent validation. Using CV effectively captures either the decreasing GMFI and/or increasing SD of the HLA-DR fluorescence intensity characteristic of cellular populations with higher numbers of m-MDSCs and eliminates replicate variability. By establishing a protocol in

Table 3. Multivariate analysis of relationship between m-MDSC and OS at pretreatment baseline and week 6 after ipilimumab treatment

	Ipilimumab					
	Pretreatment			Week 6		
	<i>n</i>	HR (95% CI)	<i>P</i>	<i>n</i>	HR (95% CI)	<i>P</i>
MDSC ≤ 14.9%	68	0.47 (0.23–0.94)	0.033	63	0.38 (0.18–0.81)	0.012
ALC ≥ 1,000 cells/μL	—	—	—	63	0.21 (0.10–0.46)	<0.001
LDH < 250	68	0.38 (0.21–0.69)	0.002	63	0.29 (0.15–0.56)	<0.001

which healthy donor CV provides the necessary threshold for a "normal" CV range, we can achieve a robust identification of patients with high m-MDSC composition.

ALC rise on therapy has been associated with improved OS following ipilimumab therapy. In a small cohort of patients at our center treated with ipilimumab at 10 mg/kg, we found that CD4⁺ lymphocytes, CD4⁺CD25⁺ regulatory T cells, and CD8⁺ lymphocytes increased with therapy. However, increases in the absolute number of CD8⁺ lymphocytes were significantly greater among patients who achieved clinical benefit from ipilimumab when compared with patients who did not benefit (34). In the current analysis, we observed inverse correlations between percentage change in CD8⁺ T cells with m-MDSC frequency *in vivo*. These findings are consistent with an *in vitro* suppression assay in which higher frequencies of m-MDSCs were associated with greater suppressive activity. We propose that in patients with melanoma receiving ipilimumab, the pharmacodynamic effects on lymphocyte subset increase and the quantity of m-MDSCs are interrelated. Evaluations of m-MDSC quantity and changes in T-cell subsets are worthy of further study as pharmacodynamic markers of therapeutic efficacy and are perhaps sufficient to guide risk-adapted clinical trials. Furthermore, taken together, the observations reported here suggest the hypothesis that m-MDSC suppression of lymphocytes may be limiting the therapeutic benefit of ipilimumab. A larger cohort of patients will need to be studied to confirm our findings and to assess whether escalating ipilimumab dose or combination therapies, including m-MDSC-directed therapies, can modulate CD8⁺ and m-MDSC interactions.

In our study, we developed an objective method to evaluate pretreatment Lin⁻CD14⁺HLA-DR^{low/-} m-MDSC frequency building on the phenotype reported in the literature by other research groups (5, 6, 21). Similar to results from Gros and colleagues (19) and Meyer and colleagues (21), we found a greater frequency of these cells in the peripheral blood of patients with melanoma in comparison with healthy donors. These cells coexpress CD11b (Supplementary Fig. S1) and in most cases also coexpress CD33 (data not shown). Similar to findings by other authors, we also found that disease course paralleled m-MDSC frequency, that is, patients without radiographic benefit following ipilimumab tended to have increasing frequencies of m-MDSCs over time (data not shown). Nevertheless, the prognostic significance of m-MDSC frequency in our analysis was independent of LDH, a known prognostic marker associated with disease burden in patients with melanoma (35).

Clinically significant m-MDSC accumulation characterized with diverse myeloid phenotypic markers has been observed in a number of malignancies in humans (4–10). Young and colleagues measured CD34⁺ natural suppressor cells and found that excess of CD34⁺ cells at the tumor site was associated with relapse of head and neck cancer (4). Solito and colleagues (36) and Gabitass and colleagues (37) have reported that the quantity of m-MDSCs with an immature myeloid phenotype (Lin⁻, HLA-DR⁻, CD11b⁺, and CD33⁺) is a prognostic marker in breast and colorectal cancer or gastric, esophageal, and pancreatic cancer, respectively. In renal cell

carcinoma, Zea and colleagues have described CD33⁺, CD15⁺ granulocytic MDSCs (10), whereas in melanoma, both Filipazzi and colleagues and Poschke and colleagues have found that m-MDSC function is within the monocytic CD14⁺, HLA-DR^{low/-} cell population (5,6). Our report adds to this emerging literature with the first description of statistically significant associations between m-MDSC accumulation, survival outcomes, and specific lymphocyte parameters following an immunomodulatory antibody therapy.

In summary, we have developed a method that enables accurate measurement of Lin-CD14⁺HLA-DR^{low/-} m-MDSCs independent of technical variables related to sample processing time and flow cytometry. Using this method, we demonstrate for the first time that higher pretreatment quantities of Lin-CD14⁺HLA-DR^{low/-} m-MDSCs are associated with inferior OS in patients with metastatic melanoma treated with ipilimumab. Inverse correlations between CD8⁺ T-cell increases and m-MDSC frequency in patients treated with ipilimumab suggest a role for m-MDSC-mediated lymphocyte suppression in OS following ipilimumab therapy. Further prospective studies are needed to validate m-MDSC measurement as a prognostic biomarker for melanoma and other disease states. Uniform methods of analysis, along with the use of Cyto-Chex tubes, make it possible for similar studies to proceed across multiple laboratories.

Disclosure of Potential Conflicts of Interest

M.A. Postow reports receiving a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member for the same. J.D. Wolchok reports receiving a commercial research grant and other commercial research support from Bristol-Myers Squibb and is a consultant/advisory board member for the same. A.M. Lesokhin reports receiving a commercial research grant from Bristol-Myers-Squibb and is a consultant/advisory board member for Bristol-Myers Squibb and Efranat, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Kitano, M.A. Postow, J. Yuan, J.D. Wolchok, A.M. Lesokhin

Development of methodology: S. Kitano, K.S. Panageas, M. Adamow, J. Yuan, P. Wong, J.D. Wolchok, A.M. Lesokhin

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kitano, M.A. Postow, T. Rasalan, M. Adamow, P. Wong, J.D. Wolchok, A.M. Lesokhin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kitano, M.A. Postow, C.G.K. Ziegler, D. Kuk, K.S. Panageas, M. Adamow, J. Yuan, G. Altan-Bonnet, J.D. Wolchok, A.M. Lesokhin

Writing, review, and/or revision of the manuscript: S. Kitano, M.A. Postow, D. Kuk, K.S. Panageas, J. Yuan, G. Altan-Bonnet, J.D. Wolchok, A.M. Lesokhin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.A. Postow, C. Cortez, A.M. Lesokhin
Study supervision: J.D. Wolchok, A.M. Lesokhin

Grant Support

This work was supported by grants from the NIH (R01 CA056821 to J.D. Wolchok and a core grant P30 CA008748 supporting the biostatistics core), the American Cancer Society (MRSG-11-054-01-LIB to A.M. Lesokhin), the Melanoma Research Alliance (to A.M. Lesokhin and J.D. Wolchok), Swim Across America (to J.D. Wolchok), the Cancer Research Institute (to J.D. Wolchok), and the Lita Annenberg Hazen Foundation (to J.D. Wolchok).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 20, 2014; revised April 22, 2014; accepted May 12, 2014; published OnlineFirst May 20, 2014.

References

- Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;9:162–74.
- Poschke I, Kiessling R. On the armament and appearances of human myeloid-derived suppressor cells. *Clin Immunol* 2012;144:250–68.
- Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 2012;12:253–68.
- Young MR, Wright MA, Lozano Y, Prechel MM, Benefield J, Leonetti JP, et al. Increased recurrence and metastasis in patients whose primary head and neck squamous cell carcinomas secreted granulocyte-macrophage colony-stimulating factor and contained CD34⁺ natural suppressor cells. *Int J Cancer* 1997;74:69–74.
- 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.
- Poschke I, Mouggiakakos 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.
- 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.
- Chikamatsu K, Sakakura K, Toyoda M, Takahashi K, Yamamoto T, Masuyama K. Immunosuppressive activity of CD14⁺HLA-DR⁻ cells in squamous cell carcinoma of the head and neck. *Cancer Sci* 2012;103:976–83.
- 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.
- 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.
- Qin A, Cai W, Pan T, Wu K, Yang Q, Wang N, et al. Expansion of monocytic myeloid-derived suppressor cells dampens T cell function in HIV-1-seropositive individuals. *J Virol* 2013;87:1477–90.
- Mouggiakakos D, Jitschin R, von Bahr L, Poschke I, Gary R, Sundberg B, et al. Immunosuppressive CD14⁺HLA-DR^{low/neg} IDO⁺ myeloid cells in patients following allogeneic hematopoietic stem cell transplantation. *Leukemia* 2013;27:377–88.
- Luan Y, Mosheir E, Menon MC, Wilson D, Woytovich C, Ochando J, et al. Monocytic myeloid-derived suppressor cells accumulate in renal transplant patients and mediate CD4(+) Foxp3(+) treg expansion. *Am J Transplant* 2013;13:3123–31.
- Huang A, Zhang B, Wang B, Zhang F, Fan KX, Guo YJ. Increased CD14(+)HLA-DR(-/low) myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to chemotherapy in non-small cell lung cancer patients. *Cancer Immunol Immunother* 2013;62:1439–51.
- Arihara F, Mizukoshi E, Kitahara M, Takata Y, Arai K, Yamashita T, et al. Increase in CD14⁺HLA-DR^{-/low} myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis. *Cancer Immunol Immunother* 2013;62:1421–30.
- Gustafson MP, Abraham RS, Lin Y, Wu W, Gastineau DA, Zent CS, et al. Association of an increased frequency of CD14⁺HLA-DR^{lo/neg} monocytes with decreased time to progression in chronic lymphocytic leukaemia (CLL). *Br J Haematol* 2012;156:674–6.
- 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.
- Postow MA, Callahan MK, Barker CA, Yamada Y, Yuan J, Kitano S, et al. Immunologic correlates of the abscopal effect in a patient with melanoma. *N Engl J Med* 2012;366:925–31.
- Gros A, Turcotte S, Wunderlich JR, Ahmadzadeh M, 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.
- Weide B, Martens A, Zelba H, Stutz C, Derhovanessian E, Di Giacomo AM, et al. 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. *Clin Cancer Res* 2014;20:1601–9.
- Meyer C, Cagnon L, Costa-Nunes CM, Baumgaertner P, Montandon N, Leyvraz L, et al. Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. *Cancer Immunol Immunother* 2014;63:247–57.
- Institute of Medicine. Evaluation of biomarkers and surrogate endpoints in chronic disease. Washington, DC: The National Academies Press; 2010.
- 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.
- Robert C, Thomas L, Bondarenko I, O'Day S, MD JW, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011;364:2517–26.
- Wolchok JD, Weber JS, Maio M, Neyns B, Harmankaya K, Chin K, et al. Four-year survival rates for patients with metastatic melanoma who received ipilimumab in phase II clinical trials. *Ann Oncol* 2013;24:2174–80.
- Ji RR, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol Immunother* 2012;61:1019–31.
- Hamid O, Schmidt H, Nissán A, Ridolfi L, Aamdal S, Hansson J, et al. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. *J Transl Med* 2011;9:204.
- Carthon BC, Wolchok JD, Yuan J, Kamat A, Ng Tang DS, Sun J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. *Clin Cancer Res* 2010;16:2861–71.
- Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, et al. Integrated NY-ESO-1 antibody and CD8⁺ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A* 2011;108:16723–8.
- Ku GY, Yuan J, Page DB, Schroeder SE, Panageas KS, Carvajal RD, et al. Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. *Cancer* 2010;116:1767–75.
- Mandrizzato 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.
- Feinermaier O, Veiga J, Dorfman JR, Germain RN, Altan-Bonnet G. Variability and robustness in T cell activation from regulated heterogeneity in protein levels. *Science* 2008;321:1081–4.
- Wolchok JD, Neyns B, Linette G, Negrier S, Lutzky J, Thomas L, et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol* 2010;11:155–64.
- Yang A, Kendle RF, Ginsberg BA, Roman RA, Heine AI, Pogoriler E, et al. CTLA-4 blockade with ipilimumab increases peripheral CD8⁺ T cells: correlation with clinical outcomes. *J Clin Oncol* 28:15s, 2010 (suppl; abstr 2555).
- Deichmann M, Benner A, Bock M, Jackel A, Uhl K, Waldmann V, et al. S100-Beta, melanoma-inhibiting activity, and lactate dehydrogenase discriminate progressive from nonprogressive American

- Joint Committee on Cancer stage IV melanoma. *J Clin Oncol* 1999; 17:1891–6.
36. 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.
37. Gabitass RF, Annels NE, Stocken DD, Pandha HA, Middleton GW. Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. *Cancer Immunol Immunother* 2011;60: 1419–30.

Cancer Immunology Research

Computational Algorithm-Driven Evaluation of Monocytic Myeloid-Derived Suppressor Cell Frequency for Prediction of Clinical Outcomes

Shigehisa Kitano, Michael A. Postow, Carly G.K. Ziegler, et al.

Cancer Immunol Res 2014;2:812-821. Published OnlineFirst May 20, 2014.

Updated version	Access the most recent version of this article at: doi: 10.1158/2326-6066.CIR-14-0013
Supplementary Material	Access the most recent supplemental material at: http://cancerimmunolres.aacrjournals.org/content/suppl/2014/06/03/2326-6066.CIR-14-0013.DC1

Cited articles	This article cites 35 articles, 13 of which you can access for free at: http://cancerimmunolres.aacrjournals.org/content/2/8/812.full#ref-list-1
-----------------------	--

Citing articles	This article has been cited by 15 HighWire-hosted articles. Access the articles at: http://cancerimmunolres.aacrjournals.org/content/2/8/812.full#related-urls
------------------------	--

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
----------------------	--

Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
-----------------------------------	--

Permissions	To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/2/8/812 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.
--------------------	--