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Cancer Research

Immature Immunosuppressive CD14$^+$HLA-DR$^{-/low}$ Cells in Melanoma Patients Are Stat3^{hi} and Overexpress CD80, CD83, and DC-Sign

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Abstract

Myeloid-derived suppressor cells (MDSC) have emerged as key immune modulators in various tumor models and human malignancies, but their characteristics in humans remain to be unequivocally defined. In this study, we have examined circulating CD14$^+$HLA-DR$^{-/low}$ MDSC in 34 advanced malignant melanoma (MM) patients. Their frequency is significantly increased and associated with disease activity. Contrary to the common notion that MDSC are a heterogeneous population of exclusively immature cells, we find the coexpression of markers associated with mature phenotype. We show for the first time the overexpression of CD80, CD83, and DC-Sign in human MDSC. Further, increased levels of signal transducer and activator of transcription 3 (Stat3), an important regulator in MDSC development and function, were noted in MM-MDSC. Stat3 was altered toward an active, phosphorylated state in the HLA-DR$^-$ population of CD14$^+$ cells and was more reactive to activating stimuli in patients. Importantly, inhibition of Stat3 abolished their suppressive activity almost completely. The described MM-MDSC use arginase in conjunction with other yet undefined mechanisms to suppress CD4$^+$ and CD8$^+$ T cells. Several observations suggest a redox imbalance in MDSC and indicate an important role of Stat3-dependent oxidative stress in MDSC-mediated T-cell suppression. These results emphasize the diversity of MDSC in human cancer and provide potential targets for therapeutic interventions. *Cancer Res; 70(11); 4335–45. ©2010 AACR.*

Introduction

Myeloid-derived suppressor cells (MDSC) have emerged as key immune modulators ablating lymphocyte responses in tumor-bearing animals and cancer patients. Increased numbers of MDSC have been observed in various solid and hematologic malignancies (1–4).

MDSC are potent suppressors of T-cell activation and function both *in vitro* and *in vivo* exerting their suppressive activity through a broad range of mechanisms, including nutrient starvation (5), generation of reactive oxygen and nitrogen species (ROS and RNOS; refs. 6, 7), and induction of regulatory T cells (T_{reg}; ref. 1). Several key molecules, such as arginase 1 (Arg1), inducible nitric oxide synthase (iNOS), and transforming growth factor (TGF) β mediate these suppressive modalities (3). MDSC depletion or blocking of their suppressive pathways delays tumor development and growth in mouse models, proving that MDSC-mediated suppression is reversible (8–10).

In mice, MDSC are defined as a heterogeneous population of immature myeloid cells that express CD11b, Gr1, and IL-4Rα, and bear resemblance to monocytes or granulocytes depending on the tumor model studied (4, 7). Increasing evidence suggests that they are recruited from the bone marrow by tumor-derived factors, such as vascular endothelial growth factor (VEGF), interleukin (IL) 6, prostaglandin E2, IL-1β, stem cell factor, macrophage- and granulocyte macrophage colony–stimulating factors (M-CSF and GM-CSF). MDSC are thought to be activated upon exposure to IFN γ, IL-4, IL-13, TGFβ, and toll-like receptor ligands (reviewed in ref. 11).

In cancer patients, MDSC have been mainly characterized as Lin$^-$HLA-DR$^-$CD33$^+$ cells that increase with disease progression and tumor burden (2, 12–15). However, CD15$^+$Arg1$^+$ granulocytes with MDSC characteristics have been described in renal cell carcinoma (16, 17) and CD14$^+$HLA-DR$^-$ cells sharing multiple MDSC features were recently identified in the blood of patients with hepatocellular (18), ovarian carcinoma (19), and melanoma (20, 21), supporting the concept of MDSC heterogeneity and plasticity. Similar to mouse models, human MDSC have been reported to use Arg1, ROS/RNOS, and TGFβ as well as T_{reg} induction to mediate systemic T-cell

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suppression (15, 16, 18, 20, 22, 23). The exact signals triggering the accumulation of MDSC in cancer patients remain elusive. A plethora of potential MDSC-inducing factors is generated by human tumors (24, 25). These findings suggest that MDSC populations in cancer patients are diverse and can differ in phenotype and function, probably depending on factors secreted by the tumor.

Understanding the MDSC-mediated systemic immune suppression could be exploited in cancer therapy, treatment of autoimmune diseases, or management of side effects from bone marrow transplantation.

Here, we report a detailed characterization of $CD14^+HLA\text{-}DR^{-/low}$ MDSC derived from the blood of patients with advanced malignant melanoma (MM). We find that MM-MDSC display aberrant expression of surface markers associated with mature myeloid cells, high levels of oxidative stress that correlate with increased signal transducers and activators of transcription 3 (Stat3) activation, and strong T-cell suppressive activity that is at least partially mediated by Arg1 and ROS production and that can be blocked by preventing cell-to-cell contact or Stat3 signaling.

Materials and Methods

Donor recruitment and blood sample preparation

This study was approved by the Karolinska Institute review board (KI 00-425 and KI 20010305,01-50) and informed donor consent was obtained in accordance with the Declaration of Helsinki. Peripheral blood was collected from 34 patients with MM stage III to IV, and 21 healthy donors with similar gender and age distribution. Patient characteristics are detailed in Table 1. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Hypaque, GE) within 2 hours of sample collection.

Antibodies and flow cytometry

Antibody details are provided in Supplementary Table S1. PBMCs were stained according to the manufacturer's recommendations. Before intracellular staining of phosphorylated Stat3 (pStat3), whole blood was fixed with BD PermBuffer III (BD Biosciences) and permeabilized in 90% methanol. For proliferation assays, responder cells were labeled with 2.5 μmol/L carboxyfluorescein succinimidyl ester (Sigma-Aldrich).

Cells were analyzed using an LSRII flow cytometer (BD Biosciences) and FlowJo analysis software (Treestar).

MDSC isolation and depletion

$CD14^+HLA\text{-}DR^-$ MDSC were immunomagnetically enriched from PBMC: first, $HLA\text{-}DR^+$ cells (control population) were removed by positive selection. Subsequently, $CD14^+HLA\text{-}DR^-$ cells (>80% purity) were isolated from the $HLA\text{-}DR^-$ cell fraction with anti-CD14 microbeads. For coculture experiments, autologous T cells were isolated from PBMC using Pan T-cell Isolation-kit II. In selected assays, CD14-depleted PBMC were generated using anti-CD14 microbeads. All microbeads were purchased from Miltenyi Biotec.

Table 1. Clinicopathologic characteristics of patients (n = 34)

Age (y; median, range)	62, 28–81	
Gender (n and %)		
Male	24	71%
Female	10	29%
AJCC stage at time of sampling		
III	11	32%
IV	23	68%

Abbreviations: n, number of patients; AJCC, American Joint Committee on Cancer.

Proliferation and cytokine secretion assays

To determine the suppressive capacity of MDSC on a per cell basis, MDSC or $HLA\text{-}DR^+$ controls were cocultured with autologous T cells for 5 days. T cells (50,000/well) were stimulated using 1.5 anti-CD2/-CD3/-CD28 beads (Miltenyi Biotec) per T cell and 25,000 (1:2), 12,500 (1:4), 6,250 (1:8), or 0 MDSC/control cells were added. As cells for Transwell (Sigma-Adrich) and Stat3 inhibition assays were isolated from frozen PBMCs, these cocultures were performed at MDSC/T cell ratios 1:1 or 1:2 to compensate for a slight loss in suppressiveness observed after freeze thawing.

To further study the potential suppressive mechanisms used by MM-MDSC, total or CD14-depleted PBMCs (100,000/well) were cultured for 5 days in the presence of activating beads. Inhibitors of candidate suppressive molecules were added in the following final concentrations: 100 IU/mL catalase (Sigma-Aldrich), 200 IU/mL superoxide-dismutase (Sigma-Aldrich), 500 μmol/L NG-monomethyl-L-arginine-acetate (Sigma-Aldrich), 500 μmol/L N(ω)-hydroxy-nor-L-arginine (Calbiochem), 10 μg/mL neutralizing pan-specific anti-human TGFβ antibody (R&D Systems), or 10 μmol/L AG490 (Sigma-Aldrich). T-cell proliferation and IFN-γ production were assessed by flow cytometry.

Oxidative stress measurements

The intracellular oxidation levels were determined by using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein-diacetate-acetyl-ester (Invitrogen), which is metabolized to fluorescent (FL-1) 2′,7′-dichlorofluorescein (DCF) upon oxidation. Briefly, PBMCs were incubated with 0.25 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein-diacetate-acetyl-ester for 10 minutes at 37°C, washed twice in ice-cold PBS, stained for surface markers, and the mean fluorescence intensity (MFI) of intracellularly retained DCF was determined by flow cytometry.

Quantitative PCR

Total RNA from $CD14^+HLA\text{-}DR^{-/low}$ cells was extracted using the RNeasy mini kit (Qiagen) and reverse transcribed to cDNA (iScript cDNA synthesis kit, Bio-Rad). Messenger RNA was quantified by real-time PCR (SYBR Green Supermix, iCycler, Bio-Rad) using the $2^{-\Delta\Delta C_T}$ method, in which C_T represents the threshold cycle (ABI 7500 Prism Detection

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System, Applied Biosystems). Relative gene expression was determined by normalizing the gene expression of each target gene to β-actin. Primer sequences for all gene-specific amplifications are shown in Supplementary Table S2.

Statistical analysis

Based on the distribution level, differences in means and correlation analyses were evaluated with parametric (two-tailed Student's or paired *t* test and Pearson's test) or non-parametric (Mann-Whitney U or Wilcoxon and Spearman's ρ test) tests. All analyses were performed at a significance level of 5% ($P \leq 0.05$) using Statistica version 7.0 (Statsoft, Inc.) and SPSS version 16.0 (SPSS, Inc.).

Results

CD14$^+$HLA-DR$^{-/low}$ cells are increased in the peripheral blood of patients with advanced MM

The frequency of CD14$^+$HLA-DR$^{-/low}$ cells was significantly increased in MM-PBMCs (Fig. 1A). The majority (54%; range, 9–100%) of circulating CD14$^+$ monocytes in MM patients had low or undetectable HLA-DR expression, whereas in healthy donors, only 36% (range, 3–59%) displayed this phenotype (representative staining shown in Fig. 1B). No difference in the percentages of Lin$^-$HLA-DR$^-$CD33$^+$ could be detected between patients and healthy donors (Supplementary Fig. S1).

Overall frequencies of monocytes and T cells did not differ significantly (Supplementary Fig. S2). We could not detect any correlation of CD14$^+$HLA-DR$^{-/low}$ cells to T_{reg} frequencies in concert with their failure to induce T_{reg} *in vitro* (data not shown).

A patient subgroup without evidence of macroscopic disease (verified by body imaging) at time of sample collection had CD14$^+$HLA-DR$^{-/low}$ frequencies comparable with healthy donors and significantly lower than observed in patients with clinically detectable active disease (Fig. 1C). From five patients, a second blood sample was collected within 2.5 to 9 months and MDSC frequencies were found to be associated with disease course (Fig. 1D).

No significant association between MDSC frequencies and disease stage could be noted potentially due to the high proportion of stage IV patients in our cohort.

Melanoma MDSC express increased levels of CD80, CD83, and DC-Sign

In humans, MDSC isolation and phenotyping are often difficult due to the lack of specific and unique markers.

We studied MM-MDSC regarding phenotypic markers linked to myeloid cell differentiation and maturation. Interestingly, CD80, CD83, and DC-Sign, markers associated with

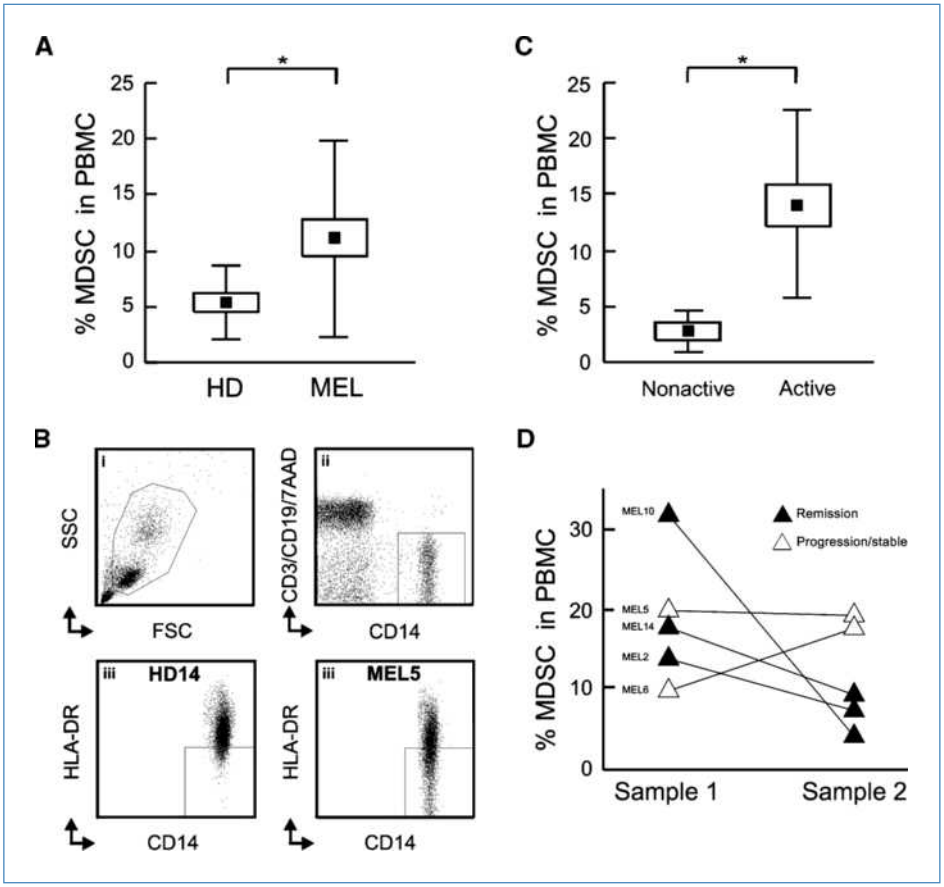

Figure 1. MDSC are increased in PBMCs of patients with melanoma. Flow cytometry shows that CD14$^+$ HLA-DR$^{-/low}$ cell frequencies (A) are increased in MM (MEL; $n = 31$) versus healthy donor (HD; $n = 15$) PBMCs, with (B) decreased HLA-DR expression on MM (bottom right) versus healthy donor (bottom left) CD14$^+$ cells; top, gating strategy from (i) PBMCs (top left) to (ii) CD14$^+$(CD3$^-$CD19$^-$7AAD$^-$) cells (top right). C, higher MDSC frequencies are observed in patients with active ($n = 25$) versus nonactive ($n = 7$) disease. D, correlation of MDSC with disease course in patients with tumor reduction or progression. Points, mean; bars, SEM; *, $P < 0.05$.

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mature or differentiated cells such as dendritic cells and macrophages, were more strongly expressed on $CD14^+HLA\text{-}DR^{-/low}$ cells in patients than healthy donors and even CD86 and Ilt3 expression showed a similar trend (Fig. 2A).

Short-term cytokine-free culture led to an increase of CD80, CD83, DC-Sign, Ilt3, CD1a, and HLA-DR expression (Supplementary Fig. S3), but not to a loss of CD14 expression (data not shown), indicating no clear lineage-specific differentiation despite expression of several dendritic cell-markers.

Because membrane-bound and soluble CD83 splice variants (CD83c) can both exert immunosuppressive function (26–28), we determined the expression of CD83c in $CD14^+HLA\text{-}DR^{-/low}$ cells by quantitative PCR and found significantly lower CD83c gene expression in MM-MDSC (Fig. 2B).

IL-4Rα, but not S100A9, is differentially expressed by MM-MDSC

The IL-4 receptor α subunit (IL-4Rα or CD124), which comprises part of the receptor for IL-4 and IL-13, has been postulated as a potential marker for MDSC in mouse models and patients with cancer, including melanoma (29, 30). IL-4Rα was expressed significantly higher in MM $CD14^+HLA\text{-}DR^{-/low}$ cells in comparison with healthy donors (Fig. 2C), in line with our observations[1] that IL-4Rα can be detected on $CD14^+HLA\text{-}DR^{-/low}$ cells of some patients and seems to be upregulated at the tumor site.

S100A9, a calcium-binding protein exerting leukocyte chemoattraction during inflammation, is expressed by mouse $CD11b^+Gr1^+$ cells and contributes to the differentiation arrest and accumulation of MDSC in tumor-bearing animals (31, 32). $S100A9^+$ myeloid progenitors have even been detected in human colon cancer tissue (33).

S100A9 was expressed on over 90% of monocytes with no substantial difference in S100A9 expression between MM and healthy donor $CD14^+HLA\text{-}DR^{-/low}$ cells or between $HLA\text{-}DR^{-/low}$ $CD14^+$ and $HLA\text{-}DR^+CD14^+$ cells in MM-PBMCs (Fig. 2D).

MM-MDSC are potent suppressors of T-cell proliferation and IFNγ production

$CD14^+HLA\text{-}DR^{-/low}$ cells from MM, but not from healthy donors, significantly decreased $CD4^+$ and $CD8^+$ T-cell proliferation and IFNγ production (Supplementary Fig. S4; Fig. 3A and B). At the physiologically relevant MDSC/T-cell ratio of 1:4 (mean ratio in MM patient blood, 1:3.45), T-cell proliferation and IFNγ production were reduced by 40%. In contrast, healthy donor cells at this ratio were not suppressive and even slightly stimulated IFNγ production.

These results show that MM-MDSC not only occur at significantly increased frequencies but also seem to be more suppressive on a per cell basis compared with healthy donor cells of the same phenotype.

Interestingly, we noticed that $CD14^+HLA\text{-}DR^{-/low}$ cells of some healthy donors did suppress autologous T-cell proliferation and/or IFNγ production. However, it should be noted that the tested coculture conditions were selected to mimic MDSC/T-cell ratios in patients and may have limited relevance in healthy donors, considering their lower MDSC/T-cell ratios. Moreover, we could not exclude natural immunemodulatory events such as minor infections or allergies in healthy donors.

Suppression of T-cell proliferation by MM-MDSC is dependent on Arg1 and oxidative stress, and requires cell contact

To delineate the suppressive mechanisms used by MM-MDSC, we analyzed the expression of candidate molecules in $CD14^+HLA\text{-}DR^{-/low}$ cells by quantitative PCR. Arg1 was expressed at significantly higher levels in patients than in healthy donors (Fig. 4A), whereas levels of cyclooxygenase 2 and iNOS transcription were significantly lower in MM compared with healthy donors. No difference in indoleamine 2,3-dioxygenase, IL-10, and TGFβ expression was observed.

To explore the relative contribution of candidate molecules, MM-PBMCs were cultured with various "salvage" agents, including the ROS-inactivating enzymes catalase and superoxide-dismutase, the iNOS inhibitor NG-monomethyl-L-arginine-acetate, the arginase inhibitor N(ω)-hydroxy-nor-L-arginine (nor-NOHA), and a neutralizing anti-TGFβ antibody. In keeping with the quantitative PCR results, inhibition of arginase consistently improved T-cell proliferation, whereas only marginal or inconsistent improvements could be observed after incubation with catalase, superoxide-dismutase, NG-monomethyl-L-arginine-acetate, and TGFβ-blocking antibody (Fig. 4B). Proliferation of healthy donor T cells was not affected by the aforementioned substances (Supplementary Fig. S5). None of the tested substances could restore IFNγ levels (Fig. 4B). However, the depletion of $CD14^+$ cells from patient PBMCs improved IFNγ production, suggesting the existence of other undefined immunosuppressive mechanisms.

Despite large interpatient variation, we frequently observed a concomitant salvage effect of catalase and nor-NOHA in individual patients. A correlation analysis revealed a strong connection between arginase- and ROS-mediated suppression (Fig. 4C).

Because we detected the differential expression of several cell surface molecules on MM compared with healthy donor $CD14^+HLA\text{-}DR^{-/low}$ cells (Fig. 2A), we investigated the need for cell-cell contact during MDSC-mediated suppression. When MDSC and T cells were cocultured in separate chambers of a Transwell plate, T-cell proliferation and IFNγ production was only marginally reduced. The significant difference between suppression exerted in normal compared with Transwell assays suggests that suppression either relies on direct cell-to-cell contact or requires close proximity between MDSC and T cells (Fig. 4D). Additional experiments using CD80, CD83, and DC-Sign blocking antibodies did not reveal consistent improvement of T-cell proliferation or function (data not shown), suggesting the involvement of as of yet unidentified molecules.

[1] Unpublished observations.

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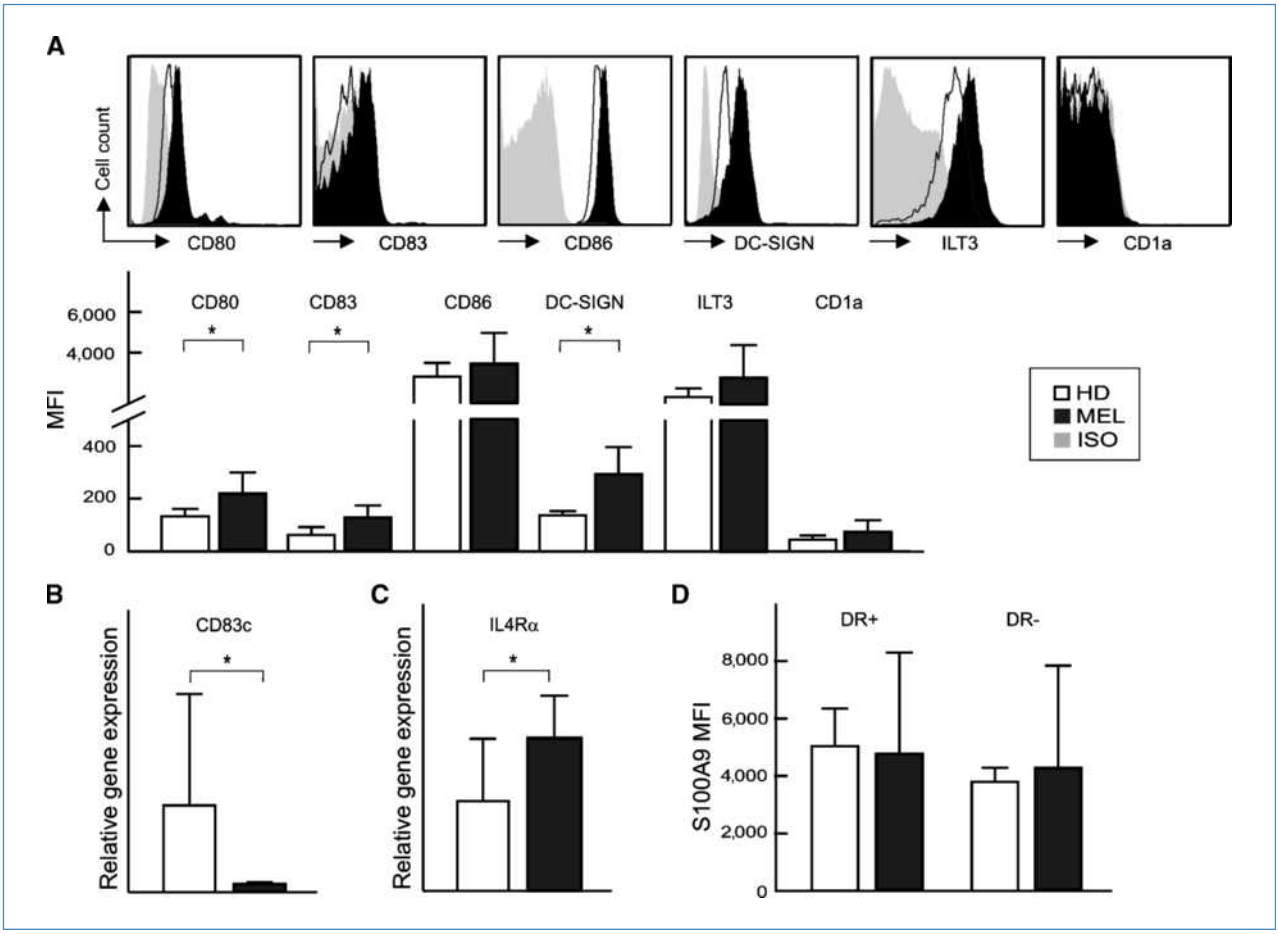

Figure 2. MM-MDSC show increased expression of cell surface markers associated with myeloid differentiation and maturation. A, representative histograms (top) and MFI values (bottom) depicting the phenotypic profile of healthy donor ($n \geq 4$) and MM ($n \geq 7$) CD14$^+$HLA-DR$^{-/low}$ cells. Levels of (B) *CD83c* and (C) *IL-4Rα* mRNA ($n = 6$), and (D) S100A9 protein ($n \geq 5$) in MM and healthy donor CD14$^+$HLA-DR$^{-/low}$ cells. Columns, mean; bars, SD; *, $P < 0.05$; black, MM; white, healthy donors; gray, isotype.

MM-MDSC show signs of oxidative stress

The protective effects of nor-NOHA and catalase indicate a role for oxidative stress in MDSC-mediated T-cell suppression. We therefore assessed levels of oxidative stress in freshly isolated PBMCs. MM CD14$^+$HLA-DR$^{-/low}$ cells retained higher levels of oxidized DCF than equivalent healthy donor cells, providing evidence for a more pro-oxidative intracellular milieu (Fig. 5A).

NADPH-oxidase is an enzyme with a central role in the production of cellular ROS. Quantitative PCR analyses showed that the most important NADPH-oxidase subunits, p47^{phox} and gp91^{phox}, were not differentially expressed between healthy donor and MM CD14$^+$HLA-DR$^{-/low}$ cells (Fig. 5A, right), suggesting an alternative source of oxidative stress.

MM-MDSC express high Stat3, which is required for T-cell suppression

Several immunosuppressive pathways, including ROS production, can be regulated by Stat3, which is also known to be involved in the differentiation arrest and expansion of MDSC (34). We found increased expression of Stat3 protein, but not mRNA, in MM compared with healthy donor CD14$^+$HLA-DR$^{-/low}$ cells (Fig. 5B). Importantly, we also observed significantly higher amounts of phosphorylated Stat3 (pStat3) in HLA-DR$^{-/low}$ than in HLA-DR$^+$CD14$^+$ cells, indicating increased Stat3 activation in the HLA-DR$^{-/low}$ subpopulation (shown for melanoma HLA-DR$^+$ and HLA-DR$^{-/low}$ cells in Fig. 5C). Patient CD14$^+$HLA-DR$^{-/low}$ cells responded more strongly to IFNα stimulation by increasing pStat3 levels, whereas H_2O_2 treatment did not affect pStat3 (Fig. 5C, middle). A correlation analysis revealed an association between levels of pStat3 and cellular oxidative stress (Fig. 5C, right). Importantly, addition of the Stat3 inhibitor AG490 almost completely abolished the ability of MDSC to suppress T-cell proliferation and IFNγ production. In most experiments, AG490-pretreated MM-MDSC could even improve T-cell responses above the levels observed for T cells stimulated alone. This is the first report indicating that active Stat3 signaling is instrumental for MDSC-mediated T-cell suppression in human cancer.

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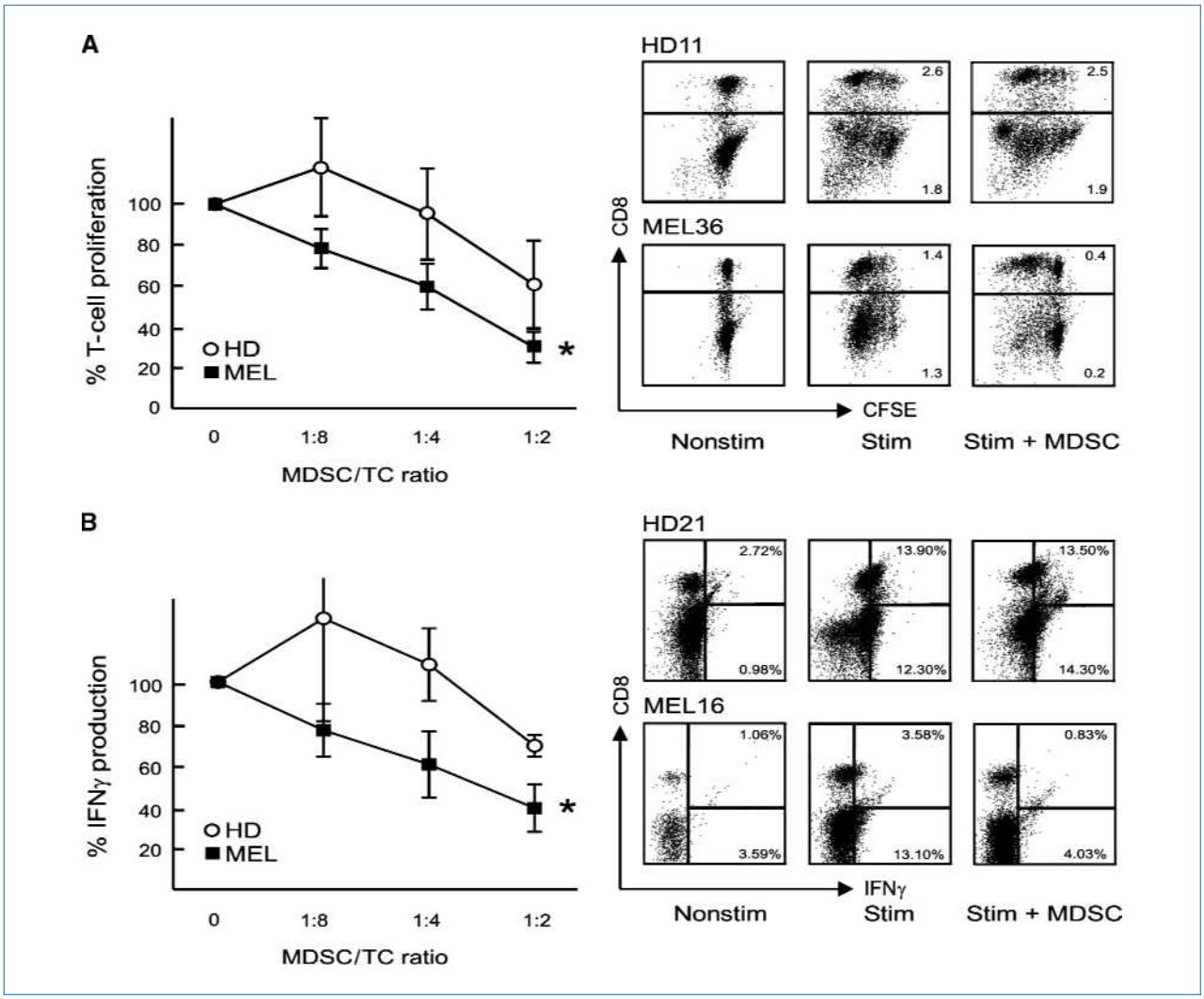

Figure 3. Suppression of T-cell proliferation and IFNγ production by MM-MDSC. A, proliferation and (B) IFNγ production of purified T cells stimulated 5 d in the presence or absence (set as 100%) of 25,000, 12,500, or 6,250 autologous MDSC (n = 6; columns, mean; bars, SEM; *, $P \leq 0.05$ in the ANOVA test for the means within a group) with representative dot plots showing (A) carboxyfluorescein succinimidyl ester (CFSE) dilution and division indices, and (B) %IFNγ+ proportion of CD8+ and CD8– T cells in MM patients and healthy donors (MDSC/T cell, ratio 1:4). Stim, stimulated; nonstim; nonstimulated.

Discussion

In recent years, MDSC were recognized as important suppressors of antitumor T-cell responses.

In keeping with two previous reports, we find significantly increased numbers of $CD14^+HLA\text{-}DR^{-/low}$ cells in the blood of patients with advanced melanoma compared with healthy individuals (20, 21). These $CD14^+HLA\text{-}DR^{-/low}$ cells are monocyte like, but were termed MM-MDSC based on their function because "suppressive activity is the ultimate defining characteristic (of MDSC)" (11). Patients with no active disease or in regression returned to physiologic frequencies of $CD14^+HLA\text{-}DR^{-/low}$ cells, indicating a causal relationship between melanoma disease status and the presence of MDSC.

The percentage of $Lin^-HLA\text{-}DR^-CD33^+$ cells, a phenotype frequently described for human cancer–associated MDSC, was similar in MM patients and healthy donors. The frequencies of these cells observed by us and by Daud and colleagues (14) in patients with resected melanomas were slightly lower than those reported by others in several types of malignancies (2, 13, 15).

Our results revealed Arg1 as one of the main suppressive mechanisms used in melanoma-MDSC–mediated suppression of T-cell proliferation. However, several suppressive mechanisms seem to be involved because no treatment could completely restore proliferation, nor rescue IFNγ production.

We noticed a positive correlation between reduction of suppression by the arginase inhibitor nor-NOHA and the antioxidative catalase. This agrees with the downregulated expression of iNOS we found in blood-derived MM-MDSC because L-arginine depletion by Arg1 overexpression has been shown to (*a*) cause decreased iNOS expression (35)

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and (*b*) shift L-arginine metabolism by iNOS toward superoxide generation (36). Suppression of T-cell proliferation therefore results from a mix of nutrient starvation and oxidative stress, which has been described to affect lymphocyte effector functions and T-cell receptor–mediated signaling (23, 37).

In contrast to Filipazzi and colleagues (20), we could not detect a substantial role for TGFβ in the suppression of T-cell proliferation by MM-MDSC. However, CD14$^+$HLA-DR$^{-/low}$ TGFβ$^+$ cells in their study were most prominent after a vaccination combined with GM-CSF. Because GM-CSF is associated with MDSC expansion (38), it is possible that the repertoire of MDSC described by Filipazzi and colleagues (20) was shaped by extrinsic GM-CSF administration. In this context, it is important to note that MDSC in the periphery might be significantly different from cells that become directly exposed to high cytokine concentrations within the tumor microenvironment, a situation potentially mimicked by cytokine administration.

On the other hand, the cells studied by us are likely to resemble more a peripheral MDSC population induced mainly by tumor-secreted factors. Phenotypic changes induced by

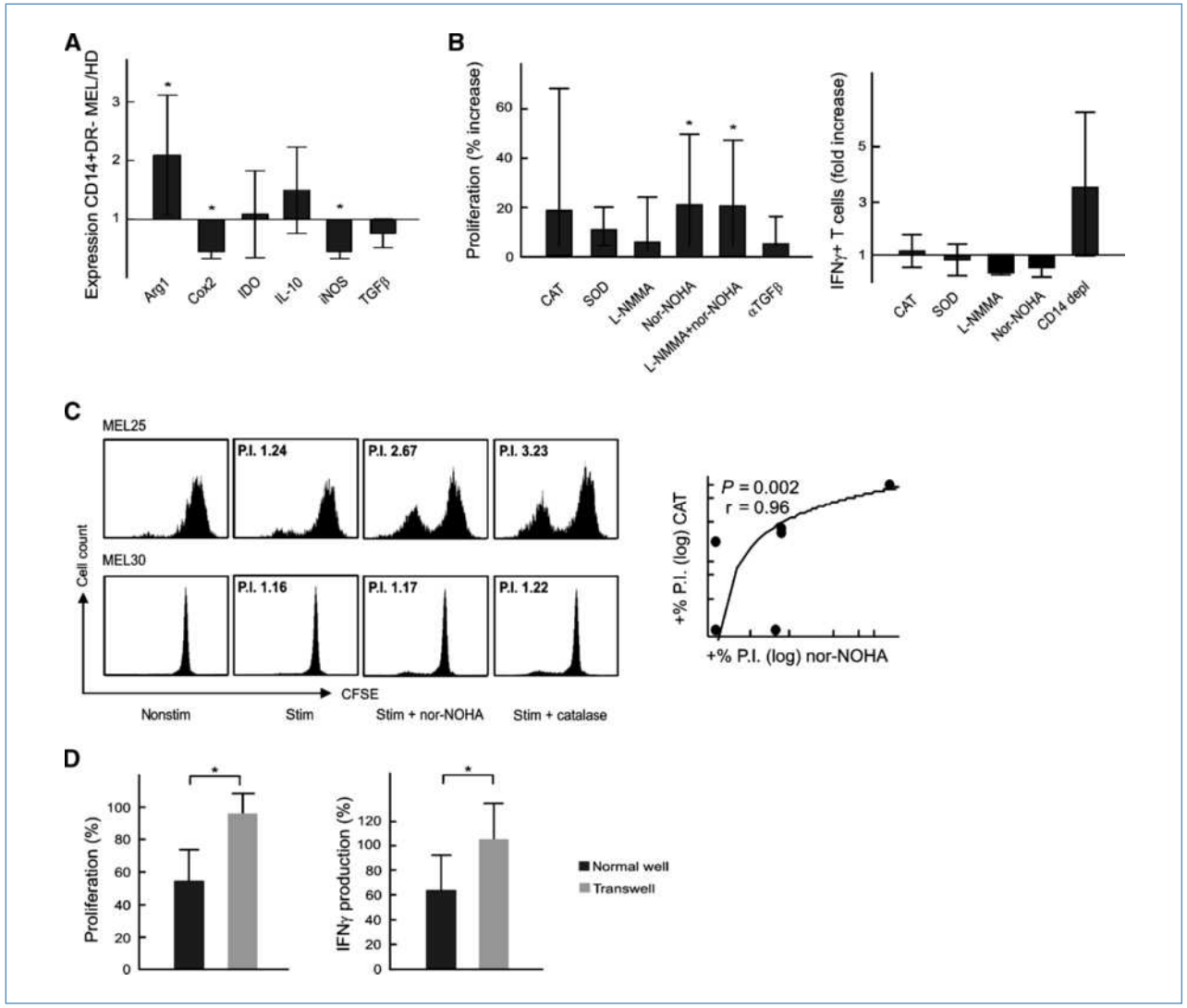

Figure 4. Mechanism of MDSC-mediated T-cell suppression. A, comparison of gene expression levels of candidate suppressive molecules in healthy donor and MM CD14$^+$HLA-DR$^{-/low}$ cells (n = 6); Cox2, cyclooxygenase 2; IDO, indoleamine 2,3-dioxygenase. B, effect of "salvage agents" to candidate suppressive mechanisms on T-cell proliferation (n = 6; columns, mean increase in percentage of divided cells; bars, SD) and IFNγ production (n = 6; columns, mean fold increase in percentage of IFNγ+ cells; bars, SD; *, $P < 0.05$); CAT, catalase; SOD, superoxide-dismutase; L-NMMA, NG-monomethyl-L-arginine-acetate. C, correlation analysis (right) between improvement in proliferation index (P.I.) after nor-NOHA and catalase treatment relative to nontreated controls in MM PBMCs (n = 6) and representative proliferation assays (left) for MEL25 (top) being responsive to both nor-NOHA and catalase treatment, or MEL30 (bottom) responsive to neither (numbers in histograms, proliferation index). D, proliferation and IFNγ production in T cells cocultured at a 1:1 ratio with MM-CD14$^+$HLA-DR$^{-/low}$ cells (n = 8) in normal 96-well or in Transwell plates. Columns, mean percentage of divided or IFNγ+ cells compared with T cells stimulated alone in the respective plate; bars, SD; *, $P < 0.05$.

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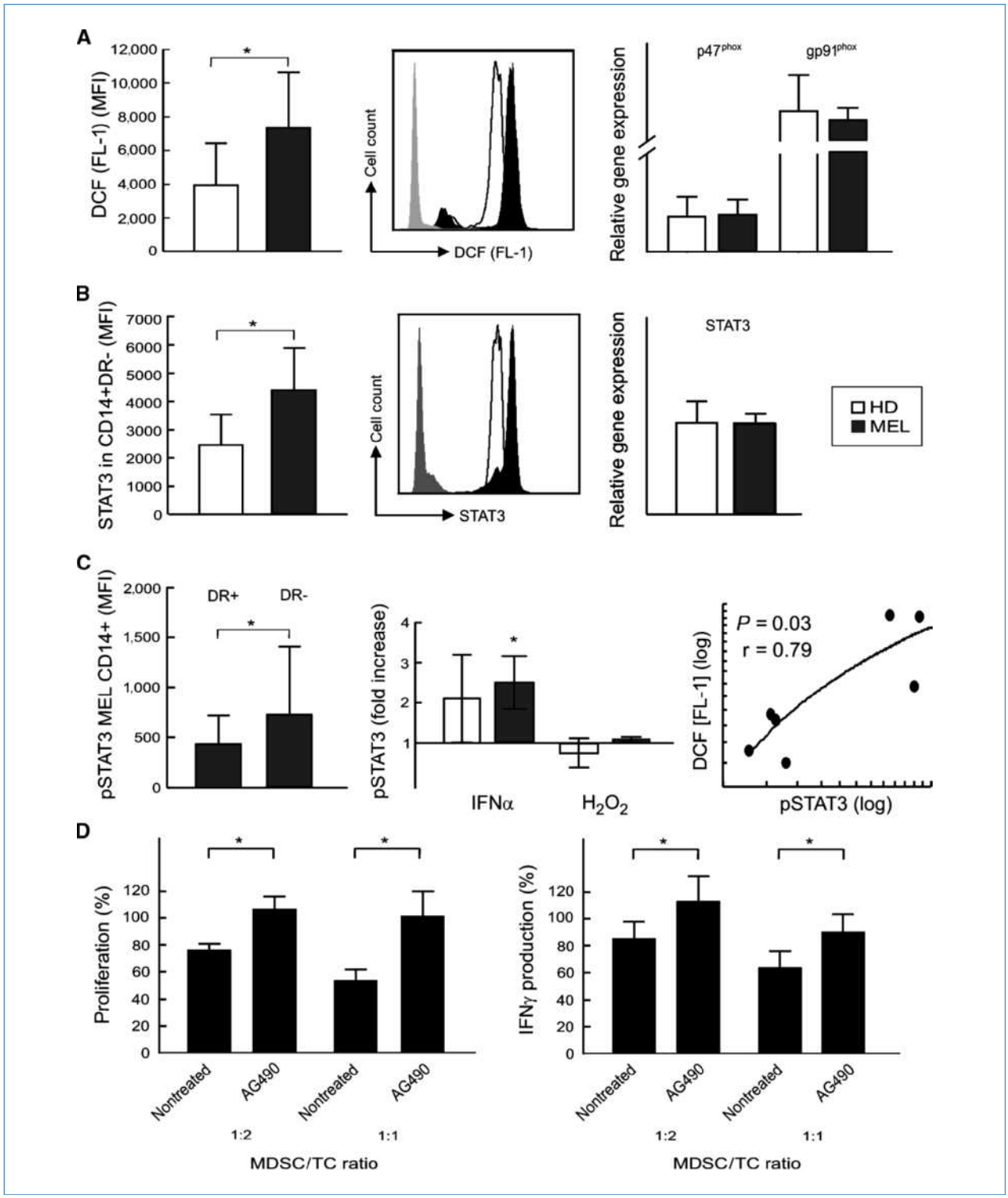

Figure 5. Oxidative stress and its regulation in MDSC. A, MFI (left) and representative histogram (middle) of intracellular oxidation levels (DCF) in MM ($n = 5$) and healthy donor ($n = 9$) $CD14^+HLA\text{-}DR^{-/low}$ cells. Expression levels of important subunits of the major ROS-producing enzyme NADPH-oxidase in MM and healthy donor $CD14^+HLA\text{-}DR^{-/low}$ cells ($n = 6$; right). B, Stat3 MFI (left), representative histogram (middle), and mRNA levels (right) in MM and healthy donor $CD14^+HLA\text{-}DR^{-/low}$ cells ($n \geq 6$). C, pStat3 MFI in $HLA\text{-}DR^{+/-}$ $CD14^+$ cells *ex vivo* (left) and upon IFNα or H_2O_2 treatment (right; $n = 5$) and correlation of DCF- and pStat3-MFI in $CD14^+HLA\text{-}DR^{-/low}$ cells ($n = 7$). Columns, mean; bars, SD; *, $P < 0.05$. D, proliferation and IFNγ production in T cells cocultured with MDSC ($n = 8$) pretreated with 0 or 10 μmol/L of the Stat3 inhibitor AG-490. Columns, mean percentage of divided or IFNγ+ cells compared with T cells stimulated alone; bars, SD; *, $P < 0.05$.

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cytokine-free cultures indicate that the observed MDSC phenotype might result from environmental stimuli whose effects are reversible, as MDSC are known to decrease shortly after tumor resection (2, 39). The increase of MDSC in cancer patient blood, which is often the only accessible population in humans, indicates either a systemic immune-suppressive environment or provides a snapshot of MDSC on their way to the tumor and other peripheral sites.

Hoechst and colleagues (18) showed that CD14$^+$HLA-DR$^{-/low}$ MDSC from hepatocellular carcinoma patients induced T_{reg} *in vitro*. We could neither observe a correlation between blood MM-MDSC and T_{reg} frequencies nor a MDSC-mediated increase of cells with the T_{reg} phenotype after culturing MDSC with autologous T cells.

Strikingly, CD14$^+$ monocyte–like MM-MDSC expressed markers normally found on dendritic cells or macrophages, such as CD83 and DC-Sign, whereas most reports describe MDSC as a mixed population of cells bearing mainly characteristics of immature cells (40). To our knowledge, no increased expression of CD80, CD83, or DC-Sign has thus far been reported for human MDSC.

CD80 ligates either CD28 in a costimulatory fashion, or CTLA-4, which transduces inhibitory signals in T cells and is associated with T_{reg}-mediated suppression. In a murine ovarian cancer model, CD80 was upregulated on MDSC and its ligation of CTLA-4 on T_{reg} was crucial for T-cell suppression (41). In addition, suggesting a functional relevance for CD80 expression on MM-MDSC, CD80-deficient mouse MDSC showed reduced Arg1 expression and activity (42).

The function of CD83, often used as a marker for mature dendritic cell, is presently unknown. However, increasing evidence suggests involvement of surface and soluble CD83 in immune suppression (26–28). After stimulation or exposure to high dosages of tumor necrosis factor α, IL-4, or GM-CSF, many cells of myeloid lineage upregulate CD83 (43), whereas the soluble CD83 splice variant CD83c is downregulated (26). CD83c can inhibit T-cell proliferation (26) and is associated with shorter treatment-free survival in chronic lymphocytic leukemia (44). In contrast to surface CD83, soluble CD83c was less expressed in MM-MDSC and most likely does not contribute to T-cell suppression.

DC-Sign, a C-type-lectin receptor present on macrophages and dendritic cells, allows nonactivated T cells to cluster with antigen-presenting cells while scanning their surface for potential T-cell receptor ligands.

It could be speculated that the expression of molecules such as CD80, CD83, and DC-Sign on MM-MDSC could enable them to establish antigen-independent contact with T cells, allowing suppressive factors to act for a longer time and over shorter distances. However, blocking these molecules on MM-MDSC did not significantly affect their suppressive capacity, suggesting that their overexpression either has no functional relevance or alternatively mediates effects independent of T-cell contact. These interesting findings still warrant further investigation, including the definition of additional markers explaining our observation that cell-to-cell contact or at least close cellular proximity is necessary for MDSC-mediated T-cell suppression, as T-cell responses remained intact when both populations were physically separated.

IL-4Rα (CD124) is a subunit of the IL-4 and IL-13 receptor, both involved in MDSC activation, and has been postulated as a MDSC marker (29, 30). IL-4Rα/Stat6 engagement triggers the expression of TGFβ, Arg1, and iNOS (3). However, the functional relevance of CD124 for MDSC-mediated suppression is not completely clear; in some mouse models, CD124$^-$ and CD124$^+$ cells of MDSC phenotype are equally suppressive (7) and tumor-bearing CD124-knockout mice still accumulate MDSC (45). A recent study in melanoma and colon cancer patients showed upregulation of CD124 on polymorphonuclear and/or CD14$^+$ cells in some individuals and a concomitant correlation between CD124 expression and suppression of mixed lymphocyte reactions (30). We found significantly higher levels of the IL-4Rα gene product in MM than in healthy donor CD14$^+$HLA-DR$^{-/low}$ cells. CD124 surface expression is often low and does not seem to be present in all patients. Therefore, it remains unclear whether IL-4Rα is a suitable marker for MDSC identification, but its role in MDSC activation makes it an interesting target of further studies.

Two recent publications showed that S100A9, a calcium-binding protein with leukocyte chemotactic function, is produced by mouse MDSC and can in an autocrine fashion cause MDSC differentiation arrest and tumor homing (31, 32). We detected no differential expression of S100A9 in MM-MDSC, but cannot exclude a functional role for S100A9 in human MDSC, e.g., through differential regulation of its receptor.

A large number of signals important for MDSC expansion and suppressiveness, including M-CSF, GM-CSF, IL-6, VEGF, IL-10, S100A9, and TGFβ, are dependent on Stat3 signaling (31, 46). In murine MDSC, pStat3 is increased and Stat3 activation prevents myeloid cell differentiation, thereby promoting MDSC expansion (34, 47), whereas Stat3 knockdown or inhibition leads to reduced numbers of CD11b$^+$Gr1$^+$ cells (34, 48). To our knowledge, this is the first report confirming the increase of total and pStat3 in human MDSC. In agreement with the preclinical observations described above, we could show that inhibition of Stat3 in MM-MDSC not only prevented them from exerting suppression on T cells but even turned them into cells with stimulatory capacity.

We could further show a correlation between intracellular oxidative stress and pStat3 levels. In agreement with our T-cell proliferation data, we find that MDSC have high levels of intracellular oxidants. Because H_2O_2 treatment did not influence pStat3 levels, this indicates that Stat3 activation may lead to the production of ROS but not vice versa.

Cheng and colleagues (31) have shown in mouse models that S100 signaling through Stat3 induces NADPH-oxidase transcription, leading to ROS production by MDSC. We did not detect the upregulation of important NADPH-oxidase subunits, but ROS could be generated from several other sources, such as iNOS in presence of low L-arginine levels (36). This would be in agreement with our finding that a positive correlation exists between melanoma MDSC–mediated T-cell suppression by Arg1 and ROS.

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In conclusion, we find that $CD14^{+}HLA\text{-}DR^{-/low}$ cells circulating in patients with advanced MM represent a new type of MDSC: these cells seem monocytic and their suppression partially relies on Arg1, in agreement with a subpopulation of MDSC described in mouse models (3). Conventionally, MDSC are described as a heterogeneous mixture of immature myeloid immunosuppressive cells (3, 40). In contrast, our results provide evidence that MM-MDSC concurrently express markers of immature and mature myeloid cells. The increased expression and activity of Stat3 along with its importance for MDSC-mediated suppressive activity indicates for the first time a central role of this signal-transducing molecule in human MDSC.

It has become clear that human MDSC differ strongly between patient populations, probably shaped by the mix of factors secreted by individual tumor types. An improved understanding of the interactions between cancer cells and different types of hematopoietic cells seems of crucial importance for the development of novel therapies. Our findings encourage the use of MDSC-targeting strategies such as Stat3 and arginase inhibitors, antioxidant treatment, and CTLA-4 blocking agents as adjuvants to conventional chemoimmunotherapy of melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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