Published in final edited form as:

J Immunol Methods. 2012 July 31; 381(1-2): 14–22. doi:10.1016/j.jim.2012.04.004.

Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples

Athanasios Kotsakis^{1,2,*}, Malgorzata Harasymczuk^{1,*}, Bastian Schilling¹, Vasilis Georgoulis², Athanassios Argiris¹, and Theresa L. Whiteside¹

¹University of Pittsburgh Cancer Institute and University of Pittsburgh School of Medicine, Departments of Pathology, Medical Oncology, Immunology and Otolaryngology, Pittsburgh, PA, 15213 USA

²Department of Medical Oncology, University Hospital of Heraklion, 71100, Crete, Greece

Abstract

Myeloid-derived suppressor cells (MDSC) present in the human peripheral blood, represent a heterogeneous population of cells with monocytic and granulocytic features. To provide guidelines for reliable assessments of the frequency and function of MDSC, we compared fresh vs. cryopreserved peripheral blood mononuclear cell (PBMC) samples obtained from normal controls and patients with cancer. PBMC were obtained from 4 healthy donors and 21 patients with cancer. They were stained with labeled antibodies, and the frequency of DR⁻/LIN⁻/CD11b⁺, DR⁻/LIN⁻/CD15⁺, DR⁻/LIN⁻/CD33⁺ and DR^{-low}/CD14⁺ cells was determined by flow cytometry before and after cryopreservation. CFSE-based suppressor assays were used to test inhibitory functions of MDSC. Arginase I expression and reactive oxygen species (ROS) upregulation in MDSC subsets were evaluated by flow cytometry. The DR^{-low}/CD14⁺ and DR⁻/LIN⁻/CD11b⁺ subsets of MDSC were found to be more resistant to the cryopreservation/thawing procedure compared to the DR⁻/LIN⁻/CD15⁺ and DR⁻/LIN⁻/CD33⁺ subsets. The frequency of the latter two MDSC subsets was significantly reduced after cryopreservation. All but DR⁻/LIN⁻/CD15⁺ cells inhibited proliferation of autologous CFSE-labeled CD4⁺ cells but lost suppressor activity after cryopreservation. Only DR⁻/LIN⁻/CD15⁺ cells were positive for Arginase I, but lost its expression after cryopreservation. Only fresh DR⁻/LIN⁻/CD11b⁺ and DR⁻/LIN⁻/CD15⁺ cells produced ROS after *in vitro* stimulation.

Studies of human MDSC should be performed in fresh blood samples. If samples have to be cryopreserved, monitoring of CD11b⁺ and CD14⁺ MDSC subsets provides the most reliable results. Arginase I expression or stimulated ROS production assessed by flow cytometry are useful markers for MDSC subsets only in fresh samples.

Keywords

myeloid-derived suppressor cells (MDSC); MDSC subsets; cell cryopreservation; flow cytometry

© 2012 Elsevier B.V. All rights reserved.

Corresponding Author: Theresa L. Whiteside, Ph.D., Professor of Pathology, Otolaryngology and Immunology, University of Pittsburgh Cancer Institute, 5117 Centre Ave., Pittsburgh, PA 15213, Tel. 412-624-0096, Fax: 412-624-0264, Whitesidetl@upmc.edu.
*Drs. Kotsakis and Harasymczuk have equally contributed to the work presented in this manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The immune system in patients with cancer is negatively regulated by many different mechanisms. Among them, regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) play a prevailing role. MDSC represent a heterogeneous population of variably-matured myeloid cells, which mediate suppression of anti-tumor immune responses (Nagaraj and Gabrilovich, 2010). Their presence at the tumor site or in the peripheral circulation has been correlated with poor prognosis, and MDSC may serve as a predictive marker for the clinical outcome following oncologic treatment (Diaz-Montero, 2009; Nefedova, 2004; Johansson, 2010). For these reasons, MDSC are currently under extensive investigation, and numerous clinical or laboratory studies are in progress to determine the role MDSC play in cancer progression.

In humans, MDSC are defined based on their phenotype, which incorporates several well known surface markers such as CD11b or HLA-DR. Historically, MDSC have been also defined by the expression of a common myeloid marker, CD33, and by the lack of expression of the markers characteristic of mature lymphoid cells such as CD3, CD19, and CD56 (Nagaraj, 2010). In contrast, in mice, MDSCs share common phenotypic markers Gr-1 and CD11b and lack of expression of markers typical of mature macrophages and dendritic cells. In particular, granulocytic subpopulation of MDSC is defined as CD11b⁺/LY6G⁺/LY6C^{low}, while the monocytic subset has been defined as CD11b⁺/LY6G⁻/LY6C^{hi} (Youn, 2008). Initially, Almand and colleagues have classified human MDSC in advanced non-small cell lung cancer (NSCLC), squamous cell carcinoma of the head and neck (SCCHN) and breast cancer patients as HLA-DR⁻/LIN⁻ cells (Almand, 2001). More recently, other groups have shown that the frequency of MDSC, defined as DR⁻/LIN⁻/CD33⁺ cells, is increased in renal cancer and melanoma (Mirza, 2006; Daud, 2008; Kusmartsev, 2008). Diaz-Montero et al considered MDSC to be DR⁻/LIN⁻/CD33⁺/CD11b⁺ in breast cancer patients (Diaz-Montero, 2009). MDSC have been also defined as CD14⁻/CD15⁺/CD33⁺/CD11b⁺ cells in patients with advanced NSCLC (Liu, 2010) and as DR⁻/CD14⁺ cells in hepatocellular carcinoma, melanoma, prostate and renal cancer (Hoechst, 2008; Filipazzi, 2007; Poschke, 2010; Vuk-Pavlovic, 2010; van Crujisen, 2008). Thus, the phenotypic profile of MDSC in patients with cancer is not well established, and it still remains to be determined which of the above listed subsets are clinically relevant in human cancer.

Functionally, MDSC are also heterogeneous, and they produce a broad range of factors able to suppress T-cell functions including Arginase I, reactive oxygen species (ROS) or nitric oxide (NO) (Nagaraj, 2010; Poschke, 2010; Corzo, 2009). However, it has not been entirely clear whether these various functions are mediated by all MDSC subsets or are confined to certain of these subsets only. One reason for this uncertainty is that measurements of the MDSC frequency and function in patients' peripheral blood have been variously performed using freshly-collected, shipped or cryopreserved specimens (Diaz-Montero, 2009; Gabitass, 2011). Both shipping, which often involves considerable delays in sample processing, and cryopreservation might have negative impact on the viability of various peripheral blood cell subsets, including MDSC. For measurements of changes in MDSC frequency using samples collected in the course of clinical trials, mononuclear cells are often cryopreserved for batch testing in one assay to avoid assay-to-assay variability. Yet, considerable losses in various cell subsets that might occur during this process are seldom considered as a significant source of variability in the results, and comparisons of fresh with cryopreserved samples are rarely performed. Effects of cryopreservation on MDSC subsets are not known, but given the phenotypic and functional heterogeneity of these cells, it might be expected that some subsets survive cryopreservation and thawing better than others.

As reliable serial monitoring of changes in MDSC frequency during disease progression and therapy is required for evaluating their potential role in cancer, we have compared MDSC frequency and functions in fresh and cryopreserved specimens obtained from normal donors and patients with cancer. Based on our results, a recommendation is made that a reliable assessment of the various MDSC subsets in human peripheral blood requires freshly-harvested specimens.

Materials and Methods

Patients and healthy donors

Patients' peripheral blood and access to medical records were obtained after getting the approval of the Institutional Review Board at the University of Pittsburgh (IRB#0403105). All subjects signed an informed consent before providing specimens for this study. Peripheral blood samples were collected from 4 normal controls (NC), 17 patients with squamous cell head and neck cancer (HNSCC) and 4 with melanoma who were seen in the Oncology Outpatient Clinic between 2010 and 2011 and were randomly selected for blood donation.

Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was drawn (30–40 mL) into heparinized tubes from NC and patients with cancer. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradients (GE Healthcare Bioscience) within 2 h of the blood draw. Cells were resuspended in AIM-V medium (Invitrogen), washed in medium and then were counted using a cell counter (Beckman-Coulter). Cells were divided into two equal aliquots, one to be immediately used for experiments, the other to be cryopreserved stored at -80°C and thawed for testing at a later date.

Cryopreservation and thawing procedure

Aliquots of cells ($10\text{--}15 \times 10^6/\text{mL}$) to be cryopreserved were centrifuged, washed in DMEM medium (20% FBS, 1% L-Glutamine, 1% Pen-Strep), resuspended in 20% (v/v) DMSO-freeze medium and transferred into cryovials (1 mL/vial). Cells were cryopreserved by placing the cryovials in a liquid N_2 vapors. Cells were stored at -80°C for various time periods. For analysis, cryovials were removed from the -80°C nitrogen freezer, and transferred to the 37°C water bath. Cells were resuspended in 9 mL of complete medium (RPMI-1640 plus 10% human AB serum). After two washes, the cells were counted in a trypan blue dye, resuspended in the complete medium and stored in 4°C refrigerator for not more than 4 h prior to staining and flow-cytometry analysis or functional studies.

Antibodies for surface markers

The following labeled anti-human monoclonal antibodies (mAbs) were used for staining: anti-lineage-FITC, including anti-CD3, -CD14, -CD16, -CD19, -CD20 and -CD56 (BD Pharmingen, San Diego, CA), anti-CD33-PE, anti-HLA-DR-ECD, anti-CD11b-PE-Cy5, anti-CD14-PE, anti-CD15-PE-Cy5 (all from Beckman Coulter, Brea, CA), anti-CD33-PE-Cy7 (eBioscience, San Diego, CA). Sheep polyclonal anti-human Arginase I-FITC Ab was purchased from R&D Systems (Minneapolis, MN). The appropriate isotypes were used as negative controls and were purchased from the above listed vendors. Prior to staining, each antibody was titrated to determine its optimal dilution using freshly isolated PBMC obtained from healthy donors.

Staining of PBMC

Freshly-isolated PBMCs were stained for flow cytometry analysis. After adding the specific mAbs for each surface marker, cells were incubated for 30 min at room temperature (RT), washed twice by adding 1–2 mL of phosphate buffered saline (PBS) to each tube. Cells then fixed with 4% (w/v) paraformaldehyde in PBS for 20 min in the dark. For intracellular Arginase I staining, the eBioscience permeabilization procedure was used, and Raji cells served as a positive control, while CD4⁺ T cells served as a negative control. The isotype control Ab was used in all cases.

Flow cytometry

Samples were acquired and the frequency of the MDSC subsets analyzed by flow cytometry in an EPICS XL-MCL flow cytometer equipped with EXPO32 software (Beckman Coulter). At least 1×10^5 events were acquired for analysis. Briefly, following the initial FS/SC discrimination, the gate was set on DR⁻/LIN⁻ cells, as shown in Figure 1A. Next, we gated on the subpopulations defined as MDSC, including CD33⁺, CD11b⁺ and CD15⁺ cells and their combinations. In order to identify CD14⁺ cells, the gate was first set on DR⁻/low cells and then on the CD14⁺ population (Figure 1B). To evaluate Arginase I expression, we first gated on DR⁻/LIN⁻ cells and then determined the percent of positive cells in the CD33⁺CD11b⁺, CD14⁺ and CD15⁺ cell subsets.

ROS detection

Oxidation-sensitive dye, dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, CA), was used to measure ROS production by MDSC. Cells were incubated at 37°C in medium in the presence of 10 μ M DCFDA for 15 min. To induce activation, cells were incubated with 50 ng/ml phorbol 12-myristate-13-acetate (PMA) and 2 μ M ionomycin in the presence of DCFDA (Sigma, St. Louis, MO). Unstimulated and stimulated cells were then stained with the cocktails of labeled Abs as described above. The Abs added covered all of the four phenotypic subsets of MDSC. After 30 min incubation on ice, cells were washed twice with cold PBS and analyzed by four-color flow cytometry. Gating was the same as used for Arginase I assays. Aliquots of Ab-stained cells which were not incubated with DCFDA served as controls.

Isolation of MDSC-enriched subsets for suppression assays

PBMC were incubated with anti-HLA-DR Ab-coated magnetic beads (Miltenyi) for 10 min and separated on AutoMACS (Miltenyi, Auburn, CA). Negatively-selected cells (DR⁻) were then incubated with anti-CD11b or anti-CD14 or anti-CD15 Ab-coated magnetic beads for 10 min to positively identify DR⁻/CD11b⁺ or DR⁻/CD14⁺ or DR⁻/CD15⁺ cells. The protocol was optimized for the use of 50×10^6 PBMC, and the separation programs “depletes” were used for the first step and “possls” for the second step, respectively.

A third and final separation was performed by using anti-CD4⁺ magnetic beads to isolate CD4⁺ T cells from the negatively selected cells (DR⁻/CD11b⁻). The CD4⁺ cells were used in suppression assays, as described below.

Suppression assays

Isolated DR⁺ (control) and DR⁻/LIN⁻/CD11b⁺, DR⁻/LIN⁻/14⁺ or DR⁻/LIN⁻/15⁺ populations enriched in MDSC were all obtained from the same healthy donor's PBMC and were co-cultured with autologous DR⁻/CD11b⁻ or CD14⁻ or CD15⁻/CD4⁺ (responder cells) in CFSE-based proliferation assays. CD4⁺ cells were labeled with CFSE, as previously described (Mandapathil, 2009), at 1×10^5 /well and were co-incubated with the separated MDSC aliquoted into wells of 96-well plates at CD4⁺/MDSC or control cell ratios of 1:1

and 2:1 for 4 days at 37°C in an atmosphere of 5% CO₂ in air. To induce proliferation, CD4⁺ cells were stimulated with beads coated with anti-CD3/anti-CD28 Abs (ratio 2:1, Miltenyi) in the presence of 500 IU/mL of IL-2 (Peprotech, Rocky Hills, NJ).

Statistical analysis

Comparisons of the frequency of the following cell subsets in fresh and frozen samples were performed: DR⁻, LIN⁺, DR⁻/LIN⁻, DR⁻/LIN⁻/CD33⁺, DR⁻/LIN⁻/CD11b⁺, DR⁻/LIN⁻/CD15⁺, DR^{-low}/CD14⁺, DR⁻/LIN⁻/CD11b⁺ co-expressing CD33⁺. The results were analyzed by Wilcoxon signed rank tests. The data, expressed as percentages of total PBMC are presented as medians ± upper and lower quartiles in box plots. Differences with p values <0.05 were considered significant.

Results

Human MDSC have been variously phenotyped as DR⁻/LIN⁻/CD33⁺, DR⁻/LIN⁻/CD11b⁺, DR⁻/LIN⁻/CD15⁺, and DR⁻/CD14⁺ cell subsets (Nagaraj, 2010; Greten, 2011). Therefore, we tested the samples of peripheral blood for the frequency of these four cell subsets. To determine the effect of cryopreservation on the frequency of the MDSC subsets, blood samples from 25 individuals (4 normal controls and 21 cancer patients) were obtained. PBMC were isolated and analyzed prior to and after freezing and thawing. In addition, when sufficient numbers of PBMC were available, the frequency of DR⁻/LIN⁻/CD33⁺ cells co-expressing CD11b⁺ and of DR⁻/LIN⁻/CD33⁺ cells co-expressing CD15⁺ was determined. Arginase I expression was measured in 8 paired fresh and cryopreserved samples. ROS production by MDSC subsets was evaluated in paired samples in 8 patients and 4 normal donors. Routinely, the PBMC viability after cryopreservation and thawing, as determined by trypan-blue exclusion, was 80% and the cell recovery was 70 to 80%. Morphology of monocytic (DR⁻/CD14⁺) and granulocytic (DR⁻/LIN⁻/CD15⁺) subsets of MDSC is shown in Figure 1C.

Frequency of the MDSC subsets in fresh and frozen samples

The median frequency of total DR⁻ cells was found to be decreased from 67% to 61% (p=0.001) in frozen/thawed samples. While the frequency of LIN⁺ cells increased from 91% to 96% (p=0.001) after cryopreservation and thawing of cells, that of total DR⁻/LIN⁻ cells was significantly decreased (p<0.0001) as shown in Figure 2. The percentage of DR⁻/LIN⁻/CD33⁺ cells was also significantly reduced in the frozen/thawed samples (p=0.001), the median proportion of these cells decreasing by 47% after thawing (Figure 2). Similarly, a significant decrease was observed in the percentage of DR⁻/LIN⁻/CD15⁺ cells (p=0.035), which were reduced by 69% after a freeze/thaw procedure. In contrast, the percentages of monocytic subpopulations (DR⁻/LIN⁻/CD11b⁺ and DR^{-low}/CD14⁺ cells) were not significantly different (p=0.768 and p=0.981, respectively) in fresh vs. cryopreserved/thawed samples.

While the DR⁻/LIN⁻/CD11b⁺ cell frequency was not influenced by the cryopreservation procedure (p=0.1), this cell subset consisted of two subpopulations of MDSC: one population comprised DR⁻/LIN⁻/CD11b⁺ cells co-expressing CD33⁺ and the other DR⁻/LIN⁻/CD11b⁺ cells that were CD33⁻ (Figure 1). In agreement with the data reported above, only the frequency of DR⁻/LIN⁻/CD11b⁺ cells co-expressing CD33⁺ was significantly lower (p=0.041) in frozen/thawed compared to fresh samples (Figure 2), while DR⁻/LIN⁻/CD11b⁺ cells were not affected by freezing/thawing.

MDSC frequency in cryopreserved samples obtained from cancer patients vs. healthy donors

Because of the possibility that cells in patients' blood samples have increased sensitivity to cryopreservation relative to normal cells, we compared the frequency of the four MDSC subsets ($DR^{-}/LIN^{-}/CD33^{+}$, $DR^{-}/LIN^{-}/CD11b^{+}$, $DR^{-}/LIN^{-}/CD15^{+}$ and $DR^{-}/CD14^{+}$) in fresh and cryopreserved/thawed samples obtained from healthy donors versus those obtained from patients with HNSCC or melanoma. No significant differences were observed (data not shown). The $DR^{-}/LIN^{-}/CD33^{+}$ and $DR^{-}/LIN^{-}/CD15^{+}$ MDSC subsets were equally sensitive to cryopreservation whether studied in PBMC of healthy donors or patients with cancer, and the other two subsets were relatively resistant, as described above.

Time period of sample cryopreservation

To determine whether the duration of cryopreservation influences the frequency of the different MDSC subsets, we compared PBMC which were cryopreserved for 1–15 days, or longer than 2 weeks (16 days up to 4 months). No significant differences were observed (data not shown).

Suppressor functions of $DR^{-}/11b^{+}$, $DR^{-}/14^{+}$ and $DR^{-}/15^{+}$ cells

Suppressor assays were performed with cells enriched in MDSC, i.e., $DR^{-}/11b^{+}$, $DR^{-}/14^{+}$ and $DR^{-}/CD15^{+}$ cells isolated from the peripheral blood of healthy donors using AutoMACS. These cell subsets were tested for their ability to inhibit proliferation of stimulated autologous $CD4^{+}$ T cells. Figure 3 shows that freshly isolated $DR^{-}/11b^{+}$ and $DR^{-}/14^{+}$ MDSC subsets mediated suppression of $CD4^{+}$ cells at the 1:1 R:S cell ratio. In contrast, the $DR^{-}/15^{+}$ cells, isolated on AutoMACs did not inhibit proliferation of autologous $CD4^{+}$ cells. When autologous DR^{+} cells obtained from the same donor were used as a control for DR^{-} cells (Figure 3D), $CD4^{+}$ T cell proliferation was not inhibited. Following cryopreservation/thawing, we were only able to recover sufficient numbers of cells to perform the CFSE-based assay for the $DR^{-}/11b^{+}$ subset of MDSC. As shown in Figure 3, after cryopreservation/thawing $DR^{-}/11b^{+}$ cells no longer suppressed proliferation of autologous $CD4^{+}$ T cells.

Arginase I expression in MDSC subsets

Flow cytometry-based assay for Arginase I expression in the cells was used as an alternative to surface markers for the detection of MDSC. Arginase I expression was measured prior to and after cryopreservation in all four MDSC subsets. We first gated on $Lin^{-}DR^{-}$ cells (Figure 4, center). Next, we determined its expression in the $CD33^{+}/CD11b^{+}$, $CD14^{+}$ and $CD15^{+}$ cell subsets (see top and bottom panels). Arginase I was found to be expressed only in $DR^{-}/Lin^{-}/CD15^{+}$ MDSC (Figure 4). Following cryopreservation, these cells were no longer positive for the enzyme.

ROS detection

Using the gating strategy described above, we also determined the ability of cells within each of the monocytic and granulocytic subsets to up-regulate ROS production (Figure 5). ROS production upon PMA/ionomycin stimulation increased in two MDSC subsets, $DR^{-}/LIN^{-}/CD11b^{+}$ and $DR^{-}/LIN^{-}/CD15^{+}$ based on the changes observed in the MFI (the stimulated/unstimulated cell ratio) using PBMC obtained from cancer patients. However, upon cryopreservation and thawing of PBMC, ROS production was decreased or absent in these two MDSC subsets. No stimulated ROS activity was detected in $DR^{-}/CD14^{+}$ and $DR^{-}/LIN^{-}/CD33^{+}$ cells tested as fresh PBMC obtained from patient with cancer.

Discussion

MDSC represent a heterogeneous population of immature cells that have been reported to play a significant role in the development of immune tolerance in cancer (Serafini, 2006). Recent data have shown that tumor progression may be associated with the accumulation of immature cells in the primary tumor site and in the periphery, as well (Bronte, 1998, Bronte, 2000; Kusmartsev, 2004). Although MDSC have gained a great deal of attention recently, their precise phenotypic and functional definition is a significant problem (Greten, 2011). Several markers have been proposed and used to define human MDSC and, as a result, at least four distinct MDSC subsets have been identified in human peripheral blood. These subsets have been arbitrarily singled out and monitored for their frequency in the peripheral blood of patients with cancer by various investigators (Nagaraj, 2010; Greten, 2011). Further, in many of these studies PBMC have been cryopreserved and thawed for phenotypic and occasionally for functional analyses. Currently, there is no consensus as to which of these subsets should be monitored in order to establish the frequency of MDSC in the peripheral circulation, and it is unclear to what extent MDSC phenotype and functions are affected by cryopreservation procedures.

Although cryopreservation of PBMC is a standard and widely used procedure, especially in serial monitoring aimed at avoiding day-to-day assay variability, data indicate that it may negatively influence the viability and function of hematopoietic cells. For example, Costantini et al showed that cryopreservation induces a consistent set of changes in PBMCs which have a significant impact in the further analysis of the lymphocyte phenotype and functions (Costantini, 2003). In a recent study, Treg frequency was reported to be reduced after cryopreservation (Elkord, 2009). It is unknown to what extent cryopreservation affects the viability or modifies expression of MDSC markers. In various reported studies which have evaluated the frequency of MDSC, there is often no mention of whether the protocol employed fresh or cryopreserved samples (e.g., Kusmartsev, 2004). Thus, an accurate analysis of MDSC in human blood specimens is currently problematic for two reasons: (a) it is unclear which subset of the four that are commonly studied by flow cytometry in various laboratories relates best to immune suppression seen in cancer patients and (b) effects of cryopreservation on the phenotype, frequency and functions of MDSC subsets are not known. To address the second problem, we investigated the impact of cryopreservation on phenotypic markers in MDSC subtypes, as well as the expression of Arginase I and ROS production upon stimulation in these cell subsets.

Our data show that PBMC cryopreservation/thaw leads to a significant reduction in the frequency of not all but only two of the four subtypes of human MDSC. The frequency of DR⁻/LIN⁻/CD11b⁺ cells and DR⁻/CD14⁺ cells was unaltered in fresh and frozen/thawed samples. Importantly, almost all CD14⁺ (more than 95%) were also CD11b⁺ (Figure 1), suggesting that DR⁻/CD14⁺/CD11b⁺ monocytic subset of MDSC is resistant to damages induced by freezing/thawing. On the other hand, the granulocytic subpopulations, including CD15⁺ cells are more sensitive to freezing/thawing. Our results showed that cryopreservation had a significant and selective impact on the MDSC viability and recovery, which significantly altered the content of the tested MDSC population. Therefore, all flow cytometry results obtained with cryopreserved PBMC samples should be interpreted cautiously and perhaps restricted the analysis of the monocytic subsets.

While the cryopreservation has a negative impact on survival of selected MDSC subsets, its impact on functions of recovered MDSC is much greater. The surviving monocytic MDSC subsets, i.e., DR⁻/LIN⁻/CD11b⁺ which mediated suppression of CD4⁺ T-cell proliferation when tested fresh, completely lost suppressor functions after cryopreservation. Our failure to isolate adequate numbers of DR⁻/LIN⁻/CD33⁺, DR⁻/CD14⁺ or DR⁻/LIN⁻/CD15⁺ cells

from frozen/thawed samples for functional analyses again emphasizes the necessity for working with fresh PBMC when functional studies are considered. It is possible that rate-control freezing of PBMC might be more advantageous than rapid freezing in preserving their functions.

Arginase I expression can now be measured by flow cytometry in various MDSC subsets, and the assay provides a potential measure of this enzyme activity in MDSC (assuming its expression correlates with enzymatic activity) without the need to isolate the cells for CFSE-based co-culture suppressor assays. Arginase I is an enzyme which hydrolyzes the amino acid L-arginine to ornithine and urea. In cancer patients, it is produced at high levels by tumor cells and is implicated in promoting tumor growth by the inhibition of immune cells (Chang, 2001; Suer, 1999; Singh, 2000). In the absence of L-arginine, the T cells are arrested in G0–G1 of the cell-cycle which leads to the inhibition of their proliferation and functions (Tadmor, 2011). Recently, it has been suggested that MDSC are another source of Arginase I and contribute to its high levels observed in sera of cancer patients. Although Arginase I was reported as detectable in monocytic and granulocytic MDSC (Ostrand-Rosenberg, 2010), in our hands, only granulocytic DR[−]/LIN[−]/CD15⁺ MDSC expressed Arginase I. This is in agreement with the report of elevated expression of the enzyme in granulocytic CD15⁺ cells in renal cancer patients from Ochoa's group (Zea, 2005). However, in contrast to their data, the DR[−]CD15⁺ cells we obtained, nearly all of which expressed Arginase I, failed to inhibit proliferation of autologous CD4⁺ responder cells. This implies that MDSC subsets can mediate suppressor functions by diverse mechanisms which operate as defined by the tumor milieu. As DR[−]/LIN[−]/CD15⁺ granulocytic MDSC do not survive cryopreservation, it was not surprising to find that Arginase I expression was completely lost after freezing and thawing.

The ability to produce ROS upon stimulation can be used as another measure of MDSC function, and it has been reported to be one of the major mechanisms of MDSC-induced immune suppression in cancer patients (Corzo, 2009). We, therefore, used this assay as a surrogate functional marker for MDSC. We found that stimulated ROS production was detectable in DR[−]/LIN[−]/CD11b⁺ and DR[−]/LIN[−]/CD15⁺ subsets in PBMC of cancer patients and that upon cryopreservation/thawing of PBMC, this activity was significantly reduced or lost.

In summary, the present study shows that cryopreservation/thawing of PBMC has a significant impact on the frequency of MDSC as measured in *ex vivo* assays. Studies of MDSC subsets in human blood samples should be performed using fresh, not frozen/thawed cells, especially when functional analyses are performed. The monocytic CD11b⁺ and CD14⁺ MDSC are least sensitive to cryopreservation/thawing of PBMC. Cell recovery, viability and phenotypic profiles of these monocytic MDSC subsets appeared to be relatively undamaged by this process. Thus, monitoring the frequency of these two MDSC subsets in cryopreserved serial samples, e.g., in the context of clinical trials, might perhaps be rationalized. However, functional assays, including Arginase I expression or stimulated ROS production, were profoundly affected by cryopreservation/thawing even in the monocytic MDSC subsets.

Acknowledgments

Supported in part by the NIH grant PO-1 CA109688 to TLW. A. Kotsakis is support by a scholarship from the Hellenic Society of Medical Oncology.

References

1. Almand B, Clark JI, Nikitina E, van Baynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J. Immunol.* 2001; 166:678–689. [PubMed: 11123353]
2. Bronte V, Apolloni E, Cabrelle A, Ronca R, Serafini P, Zamboni P, Restifo NP, Zanovello P. Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. *Blood.* 2000; 96:3838–3846. [PubMed: 11090068]
3. Bronte V, Wang M, Overwijk WW, Surman DR, Pericle F, Rosenberg SA, Restifo NP. Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. *J. Immunol.* 1998; 161:5313–5320. [PubMed: 9820504]
4. Chang CI, Liao JC, Kuo L. Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. *Cancer Res.* 2001; 61:1100–1106. [PubMed: 11221839]
5. Corzo CA, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Sotomayor E, Padhya T, et al. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J. Immunol.* 2009; 182:5693–5701. [PubMed: 19380816]
6. Costantini A, Mancini S, Giuliodoro S, Butini L, Regnery CM, Silvestri G, Montroni M. Effects of cryopreservation on lymphocyte immunophenotype and function. *J. Immunol. Meth.* 2003; 278:145–155.
7. Daud AI, Mirza N, Lenox B, Andrews S, Urbas P, Gao GX, Lee JH, Sondak VK, et al. Phenotypic and functional analysis of dendritic cells and clinical outcome in patients with high-risk melanoma treated with adjuvant granulocyte macrophage colony-stimulating factor. *J. Clin. Oncol.* 2008; 26:3235–3241. [PubMed: 18591558]
8. 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. [PubMed: 18446337]
9. Elkord E. Frequency of human T regulatory cells in peripheral blood is significantly reduced by cryopreservation. *J. Immunol. Meth.* 2009; 347:87–90.
10. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, Castelli C, Mariani L, Parmiani G, Rivoltini L. 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; 25:2546–2553. [PubMed: 17577033]
11. 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; 60:1419–1430. [PubMed: 21644036]
12. Greden TF, Manns MP, Korangy F. Myeloid derived suppressor cells in human diseases. *Int. Immunopharmacol.* 2011; 11:802–807. [PubMed: 21237299]
13. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP, Greden TF, Korangy F. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)/CD25(+)/Foxp3(+) T cells. *Gastroenterol.* 2008; 135:234–243.
14. Johansson CC, Mouggiakakos D, Trocme E, All-Ericsson C, Economou MA, Larsson O, Seregard S, Kiessling R. Expression and prognostic significance of iNOS in uveal melanoma. *Int. J. Cancer.* 2010; 126:2682–2689. [PubMed: 19847812]
15. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J. Immunol.* 2004; 172:989–999. [PubMed: 14707072]
16. Kusmartsev S, Su Z, Heiser A, Dannull J, Eruslanov E, Kubler H, Yancey D, Dahm P, Vieweg J. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin. Cancer Res.* 2008; 14:8270–8278. [PubMed: 19088044]
17. Liu CY, Wang YM, Wang CL, Feng PH, Ko HW, Liu YH, Wu YC, et al. Population alterations of L-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14/CD15+/CD33+

- myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. *J. Cancer Res. Clin. Oncol.* 2010; 136:35–45. [PubMed: 19572148]
18. Mandapathil M, Lang S, Gorelik E, Whiteside TL. Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression. *J. Immunol. Meth.* 2009; 346:55–63.
 19. Mirza N, Fishman M, Fricke I, Dunn M, Neugh AM, Frost TJ, Lush RM, Gabrilovich DI. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res.* 2006; 66:9299–9307. [PubMed: 16982775]
 20. Nagaraj S, Gabrilovich DI. Myeloid-derived suppressor cells in human cancer. *Cancer.* 2010; 16:348–353.
 21. Nefedova Y, Huang M, Kusmartsev S, Bhattacharya R, Cheng P, Salup R, Jove R, Gabrilovich D. Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. *J. Immunol.* 2004; 172:464–474. [PubMed: 14688356]
 22. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer Immunol. Immunother.* 2010; 59:1593–1600. [PubMed: 20414655]
 23. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. *Cancer Res.* 2010; 70:4335–4345. [PubMed: 20484028]
 24. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Sem. Cancer Biol.* 2006; 16:53–65.
 25. Singh R, Pervin S, Karimi A, Cederbaum S, Chaudhuri G. Arginase activity in human breast cancer cell lines: N(omega)-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. *Cancer Res.* 2000; 60:3305–3312. [PubMed: 10866325]
 26. Suer Gokmen S, Yoruk Y, Cakir E, Yorulmaz F, Gulen S. Arginase and ornithine, as markers in human non-small cell lung carcinoma. *Cancer Biochem. Biophys.* 1999; 17:125–131. [PubMed: 10738908]
 27. Tadmor T, Attias D, Polliack A. Myeloid-derived suppressor cells - their role in haemato-oncological malignancies and other cancers and possible implications for therapy. *Br. J. Haematol.* 2011; 153:557–567. [PubMed: 21477210]
 28. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol.* 2008; 181:5791–5802. [PubMed: 18832739]
 29. van Crujlsen H, van der Veldt AA, Vroiling L, Oosterhoff D, Boxterman HS, Scheper RJ, Giaccone G, Haanen JB, et al. Sunitinib-induced myeloid lineage redistribution in renal cell cancer patients: CD1c+ dendritic cell frequency predicts progression-free survival. *Clin. Cancer Res.* 2008; 14:5884–5892. [PubMed: 18794101]
 30. Vuk-Pavlovic S, Bulur PA, Lin Y, Qin R, Szumlanski CL, Zhao X, Dietz AB. Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer. *The Prostate.* 2010; 70:443–455. [PubMed: 19902470]
 31. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta S, McDermott D, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* 2005; 65:3044–3048. [PubMed: 15833831]

Figure 1. Gating strategy for the identification of the different subtypes of human MDSC in PBMC and morphology of monocytic vs. granulocytic MDSC subsets

(**A**) After determining FS/SS, the gate is set on DR⁻/LIN⁻ cells and then on the CD33⁺, CD11b⁺ and CD15⁺ MDSC subpopulations. The majority (nearly 90%) of DR⁻/LIN⁻/CD33⁺ are also positive for CD11b, as opposed to DR⁻/LIN⁻/CD11b⁺ cells, where only half of the cells are positive for CD33⁺. Almost all DR⁻/LIN⁻/CD15⁺ cells are positive for CD33. (**B**) Gating strategy for the subtyping of DR⁻/CD14⁺ MDSC. Almost all of the cells are also CD11b⁺. (**C**) Giemsa stained monocytic (DR⁻/CD14⁺) and granulocytic (DR⁻/LIN⁻/CD15⁺) subsets of MDSC.

Figure 2. The frequency of different MDSC subsets in freshly-isolated vs. cryopreserved/thawed PBMC

The frequency of all MDSC subtypes was significantly decreased, except for DR⁻/LIN⁻/CD11b⁺ and DR⁻/CD14⁺ cells. The p-values have been calculated using the Wilcoxon's signed rank test. Each box plot represents 21 independent determinations.

Figure 3. Suppression of proliferation of autologous CD4⁺ responder cells by freshly isolated vs. cryopreserved MDSC

CSFE-labeled CD4⁺ responder cells incubated alone or co-incubated with different MDSC subsets for 4 days were tested for proliferation inhibition. The percent inhibition values are indicated in relevant plots. (A) Unstimulated responder cells, no proliferation (B) Anti-CD3/anti-CD28 Ab-stimulated CD4⁺ responder cells, (C) Responder cells co-cultured with DR⁻/CD11b⁺ or CD14⁺ or CD15⁺ at the 1:1 ratio (D) Responder cells co-cultured with autologous DR⁺ cells at the 1:1 ratio. The inhibition of proliferation shown in C is attributed to the fresh DR⁻/CD11b⁺, since fresh autologous HLA-DR⁺ cells used as controls did not cause inhibition of CD4⁺ proliferation.

Figure 4. Arginase I expression in different subsets of MDSC

The enzyme expression was measured by flow cytometry as described in Materials and Methods. The lower center panel shows positive staining with Raji cells and the isotype control for Arginase I. With freshly harvested PBMC, the gate was first set on LIN⁻DR⁻ cells (center panel). Next, the CD14⁺ and CD15⁺ subsets were discriminated (right) and after staining for Arginase I, the % of positive cells within each subset was determined. Only the CD15⁺ subset was positive for Arginase I. In parallel, the CD11b⁺ and CD33⁺ subsets were identified (left) and shown to be negative for Arginase I.

Figure 5. Up-regulation of ROS expression in PMA/ionomycin-activated MDSC

Increased ROS expression in stimulated vs. unstimulated PBMC was measured by flow cytometry as described in Materials and Methods. The gating strategy on DR⁻/LIN⁻ cells and the discrimination of the MDSC were the same as shown in Figure 4. Empty control contains cells without DCFDA. Unstimulated cells were incubated with DCFDA, while stimulated cells were incubated with PMA/ionomycin and DCFDA. Stimulated ROS upregulation was observed in DR⁻/LIN⁻/CD11b⁺ and DR⁻/LIN⁻/CD15⁺ MDSC.