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Graft Monocytic Myeloid-Derived Suppressor Cell Content Predicts the Risk of Acute Graft-versus-Host Disease after Allogeneic Transplantation of Granulocyte Colony-Stimulating Factor–Mobilized Peripheral Blood Stem Cells

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A B S T R A C T

Myeloid-derived suppressor cells (MDSCs) are powerful immunomodulatory cells that in mice play a role in infectious and inflammatory disorders, including acute graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation. Their relevance in clinical acute GVHD is poorly known. We analyzed whether granulocyte colony-stimulating factor (G-CSF) administration, used to mobilize hematopoietic stem cells, affected the frequency of MDSCs in the peripheral blood stem cell grafts of 60 unrelated donors. In addition, we evaluated whether the MDSC content in the peripheral blood stem cell grafts affected the occurrence of acute GVHD in patients undergoing unrelated donor allogeneic stem cell transplantation. Systemic treatment with G-CSF induces an expansion of myeloid cells displaying the phenotype of monocytic MDSCs ($Lin^{low/neg}HLA\text{-}DR^{-}CD11b^{+}CD33^{+}CD14^{+}$) with the ability to suppress alloreactive T cells in vitro, therefore meeting the definition of MDSCs. Monocytic MDSC dose was the only graft parameter to predict acute GVHD. The cumulative incidence of acute GVHD at 180 days after transplantation for recipients receiving monocytic MDSC doses below and above the median was 63% and 22%, respectively ($P = .02$). The number of monocytic MDSCs infused did not impact the relapse rate or the transplant-related mortality rate ($P > .05$). Although further prospective studies involving larger sample size are needed to validate the exact monocytic MDSC graft dose that protects from acute GVHD, our results strongly suggest the modulation of G-CSF might be used to affect monocytic MDSCs graft cell doses for prevention of acute GVHD.

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) consist of a group of morphologically and functionally heterogeneous population of myeloid progenitor cells, dendritic cells, and immature myeloid cells that suppress immune responses in vivo and in vitro [1]. In mice, the expression of the Gr-1 antigen (Ly6C/Ly6G) and cellular morphology have been used to characterize 2 major populations of MDSCs: granulocytic MDSCs expressing $CD11b^{+}Ly6G^{+}Ly6C^{int}CD115^{low}$ and monocytic MDSCs (M-MDSCs) expressing $CD11b^{+}Ly6G^{-}Ly6C^{+}CD115^{+}$ [2,3].

Human MDSC phenotype is less defined. Both granulocytic and monocytic human MDSCs express CD33, CD11b, and low/negative levels of HLA-DR with variable expression of CD15 (granulocyte MDSC) and CD14 (M-MDSC). These populations are able to inhibit alloreactive responses mediated by T lymphocytes and natural killer (NK) cells through a variety of mechanisms that include L-arginine depletion by arginase 1 and the inducible nitric oxidase, generation of reactive oxygen species, release of transforming growth factor-β and IL-10, cysteine sequestration, and regulatory T cell (Treg) induction [1,4]. In physiological situations, immature myeloid cells mature into dendritic cells, macrophages, and neutrophils/granulocytes upon entering the peripheral blood. In pathological conditions, growth factors boost immature myeloid cell expansion and interfere with their normal differentiation, inducing the MDSC phenotype [1].

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Table 1
Patient, Graft, and Transplant Characteristics

Characteristic	Value
Number of patients	60
Number of donors	60
Median patient age at transplant, yr (range)	44 (18-67)
Median donor age, yr (range)	34 (20-43)
Median follow-up, mo (range)	17 (13 days to 53 mo)
Diagnosis	
Acute myeloid leukemia	6 (10%)
Non-Hodgkin lymphoma	21 (35%)
Multiple myeloma	12 (20%)
Hodgkin disease	17 (28%)
Chronic lymphocytic leukemia	4 (6%)
Male/female patients	39/21 (65%/35%)
Male/female donors	46/14 (77%/23%)
HLA disparity	
10/10 alleles	30 (50%)
9/10 alleles	17 (28%)
8/10 alleles	13 (22%)
Conditioning regimen	
Cyclophosphamide (100 mg/kg) + thiotepa (10 mg/kg)	19 (32%)
Cyclophosphamide (60 mg/kg) thiotepa (10 mg/kg) + fludarabine (60 mg/m^2)	17 (28%)
Cyclophosphamide (60 mg/kg) + thiotepa (10 mg/kg) + fludarabine (120 mg/m^2) + total body irradiation (2 Gy)	2 (3%)
Fludarabine (90 mg/m^2) + melphalan (100-140 mg/m^2)	15 (25%)
Fludarabine (90 mg/m^2) + total body irradiation (2 Gy)	4 (7%)
Fludarabine (150 mg/m^2) + busulfan (.8 mg/kg)	3 (5%)
GVHD prophylaxis	
Methotrexate with cyclosporine	52 (87%)
Mycophenolate mofetil with cyclosporine	8 (13%)

Although MDSCs have been primarily studied in cancer patients in which they are responsible of immune escape phenomena [5,6], there has been growing interest in understanding their role in infectious and inflammatory disorders including acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). GVHD represents 1 of the most frequent complications of allo-HSCT and remains a major cause of morbidity and mortality related to this therapy. Advances in aGVHD prevention and treatment, new conditioning regimens, and better donor selection have improved the outcomes of patients undergoing allo-HSCT [7].

In preclinical mouse models, cotransplantation of functional MDSCs in a fully MHC-mismatched mouse model of HSCT led to a decrease in aGVHD severity and mortality without abrogating the graft-versus-tumor (GVT) effect [8]. These cells were obtained in vitro through exposure of bone marrow cells to granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IL-13. Additional evidence to support the involvement of MDSCs in aGVHD was shown in patients in which a specific subset of MDSC expressing the monocytic CD14 marker and low levels or lack of the antigen presenting HLA-DR molecules (CD14$^+$HLA-DR$^{low/neg}$ cells) accumulate after allo-HSCT [9]. These cells suppressed the proliferation of autologous T cells and their frequency correlated significantly with the serum levels of G-CSF, which in fact has been demonstrated to induce T cell–suppressive CD14$^+$ myeloid cells [10].

Based on these findings, the aim of the present study was first to investigate the effect of G-CSF administration, used to mobilize hematopoietic stem cells, on the frequency of CD14$^+$ M-MDSCs (Lin$^{low/neg}$HLA-DR$^-$CD11b$^+$CD33$^+$CD14$^+$) in the peripheral blood stem cell (PBSC) grafts of 60 unrelated donors and second to search for a correlation between the number of M-MDSCs infused with the graft and the incidence of aGVHD.

METHODS

Patients and Donors

All patients were transplanted from their respective unrelated donors at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan. Written informed consent was obtained, and the institutional review board approved the study (Comitato Etico Indipendente, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, protocol number INT45/12; 4/24/2012). Patients, donors, and transplant characteristics are described in Table 1. Stem cell mobilization, collection, treatment, and storage were done according to the standard operating procedures approved by the Joint Accreditation Committee of International Society for Cellular Therapy Europe and the European Group for Blood and Marrow Transplantation.

Donors were mainly young men (46 men versus 14 women; median age 34 [range, 20 to 43]) treated with G-CSF [filgrastim] 10 μg/kg/day for 5 days before leukoapheresis. All patients received allo-HSCT for hematologic malignancies (Table 1). Three patients (5%) received fludarabine (150 mg/m^2) and busulfan (.8 mg/kg), 4 patients (7%) received fludarabine (90 mg/m^2) combined with low-dose total body irradiation (2 Gy), and all other patients received a conditioning regimen based either on fludarabine (90 mg/m^2) and melphalan (100 to 140 mg/m^2; 15 patients [25%]) or thiotepa (10 mg/kg) and cyclophosphamide (60 to 100 mg/m^2; 38 patients [63%]) with or without fludarabine (60 to 120 mg/m^2) (Table 1). GVHD prophylaxis consisted of cyclosporine and short-course methotrexate (n = 52 [87%]) or mycophenolate mofetil (n = 8 [13%]). Diagnosis and clinical grading of aGVHD were performed using the international standard criteria [7]. Sixty age-matched healthy control subjects were also included in the study.

Chimerism Analysis

Chimerism analysis was carried out as previously described [11] on peripheral blood collected monthly after transplantation.

Flow Cytometry and Graft Content Analysis

Sixty PBSC grafts collected by apheresis procedure were analyzed by flow cytometry at the moment of transplantation, and the number of CD34$^+$ hematopoietic stem cells, total CD3$^+$ lymphocytes, CD4$^+$ helper and CD8$^+$ cytotoxic T cells, CD19$^+$ B lymphocytes, and CD16$^+$-CD56$^+$ NK cells was determined using Trucount tubes containing fluorescent beads as an internal standard (BD Biosciences, San Jose, CA) [12] and the appropriate monoclonal antibodies (Supplemental Table 1). M-MDSCs (defined as Lin$^{low/neg}$HLA-DR$^-$CD11b$^+$CD33$^+$CD14$^+$), Tregs (CD4$^+$CD25$^+$CD127$^-$FoxP3$^+$), and invariant NK T cells (iNKT; CD3$^+$TCRVα24-Jα18$^+$TCRVβ11$^+$CD4$^+$ and CD3$^+$TCRVα24-Jα18$^+$TCRVβ11$^+$CD4$^-$) were analyzed by flow cytometry retrospectively on frozen samples [6,13] using the listed fluorochrome-coupled monoclonal antibodies (Supplementary Table 1). Staining of cells was performed at 4°C for 20 minutes in the dark in fluorescent activated cell sorter staining buffer (1× PBS supplemented with 2% FBS). For intracellular FoxP3 staining, after extracellular staining, cells were permeabilized and stained according to manufacturer instructions (FoxP3 Staining Kit; Miltenyi Biotec, Bergisch-Gladbach, Germany). Cell acquisition and analysis were performed on a FacsCalibur cytometer using CellQuest software (BD Biosciences) or on a MACS Quant cytometer using MACS Quantify Software (Miltenyi Biotec). A minimum of 5 × 10^5 events were collected for accurate data acquisition.

Immunomagnetic Cell Separation

Immunomagnetic separations were performed using Automacs Pro Separator (Miltenyi Biotec). For CD3$^+$ lymphocyte isolation, peripheral blood mononuclear cells isolated by Ficoll density gradient (Lymphoprep; Axis-Shield, Oslo, Norway) were suspended in separation buffer (PBS supplemented with .5% BSA) and incubated with anti-CD3 microbeads (Miltenyi Biotec) for 15 minutes at 4°C. After washing, cells were separated through a double-column positive selection. For M-MDSC enrichment, PBSCs were first incubated with anti-HLA-DR microbeads (Miltenyi Biotec) to deplete HLA-DR positive cells. Thereafter, CD33$^+$ cells were separated using anti-CD33 microbeads (Miltenyi Biotec). Separations were performed according to manufacturer instructions. Purity of the selected populations was evaluated by flow cytometry, demonstrating an efficiency of separation above 90% in all experiments.

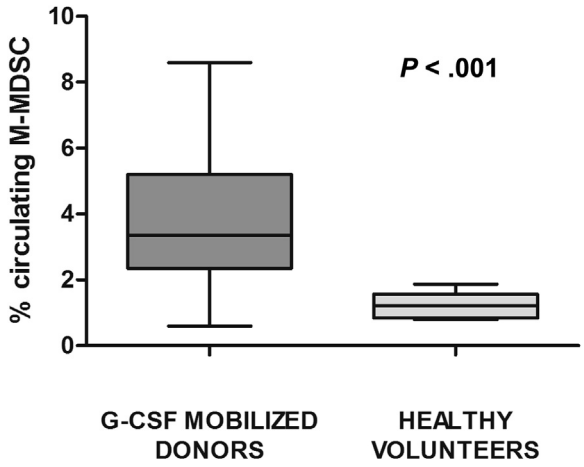

Figure 1. G-CSF treatment of HSCT donors induces an increase in the frequencies of circulating M-MDSCs in respect to healthy nontreated volunteers as assessed by flow cytometric evaluation of $Lin^{low/neg}$ HLA-DR^- $CD11b^+$ $CD33^+$ $CD14^+$ ($P = .0002$). Bars represent median values, whereas whiskers represent minimum and maximum.

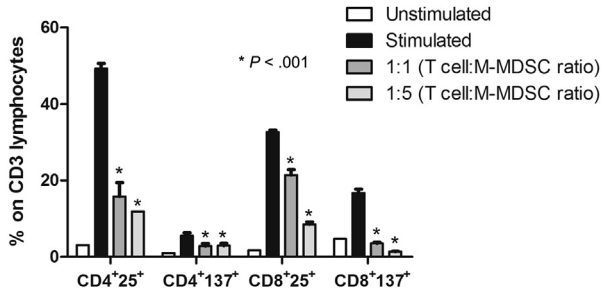

Figure 2. In vitro co-culture of immunomagnetic selected CD3 T lymphocytes isolated from peripheral blood of HSCT donors and M-MDSCs selected through a double-step immunomagnetic separation. PBSC graft cells were first depleted of HLA-DR^+ cells, and thereafter myeloid cells expressing the CD33 antigen were enriched through positive selection. Purity of all populations was assessed by flow cytometry, demonstrating an efficiency of separation of more than 90% in all experiments. $CD3^+$ lymphocytes were stimulated with CD2/CD3/CD28 microbeads with or without different ratios of M-MDSCs (1:1 or 1:5). Cells were also plated without microbeads as negative control. Immunosuppressive properties of M-MDSCs were assessed by flow cytometric analysis of the surface activation markers CD25 and CD137. Co-culture experiments demonstrate the ability M-MDSCs to suppress T cell activation in a dose-dependent manner on both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Experiments were carried out in triplicate.

$CD3^+$/MDSC Co-Culture

Selected $CD3^+$ lymphocytes and MDSCs were co-cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine at a ratio of 1:1 and 1:5 in the presence of anti-CD2/CD3/CD28 biotin beads according to manufacturer instructions (Miltenyi Biotec). Three to 5×10^5 T lymphocytes were plated in triplicate in 24-well flat bottom plates. As positive control, lymphocytes were stimulated in the absence of MDSCs; as negative control, $CD3^+$ cells and MDSCs were incubated in the absence of stimuli. After 4 days of culture, cells were harvested and expression of the surface activation markers CD25 and CD137 [11,14] was evaluated through flow cytometry.

Statistical Analysis

Class comparisons for clinical variables and graft cell subset doses infused were made with the Mann-Whitney test or Fisher's exact test for categorical variables. Probability of aGVHD was calculated with the cumulative incidence procedure, with relapse and death without aGVHD within 180 days as competing events. Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis. All analysis were carried out using GraphPad Prism 5.0 (GraphPad, San Diego, CA) and R (The R Project [http://www.r-project.org/], packages "ROCR" and "cmprsk"). Multivariate analysis was performed by fitting a logistic regression model with a backward selection procedure using SPSS version 20. $P < .05$ was deemed statistically significant.

RESULTS

G-CSF Induces an Increase of the M-MDSCs Population in Peripheral Blood of Mobilized PBSC Donor

We first tested the hypothesis that G-CSF administration, routinely used for the mobilization of $CD34^+$ stem cells of healthy PBSC donors, may induce an in vivo expansion of MDSCs. Specifically, we analyzed the phenotype and the frequency of MDSCs in the peripheral blood of PBSC donors (n = 60) and in age-matched healthy control subjects (n = 60). In line with previous reports from patients treated with G-CSF or GM-CSF for autologous PBSC transplantation [10,15], we detected an expansion of a monocytic $CD14^+$ myeloid population displaying the phenotype of M-MDSCs ($Lin^{low/neg}$HLA-$DR^-$$CD11b^+$$CD33^+$$CD14^+$; Supplemental Figure 1). There was a significant increase in the number of circulating M-MDSCs in the peripheral blood of the G-CSF–treated donors compared with the age-matched healthy untreated subjects (3.36% and 1.21% of total $CD45^+$ cells, respectively, $P < .001$, Mann-Whitney t-test) (Figure 1). Further analysis demonstrated that G-CSF treatment resulted in a variable expansion of M-MDSC within the donor population analyzed (.8% to 7.8% of total $CD45^+$ cells). There was no significant correlation between the number of circulating M-MDSCs cells and the age or sex of the PBSC donors ($P > .05$; Fisher's exact t-test; median age 34 years [range, 20 to 43]).

Immune-Suppressive Activity of G-CSF–Induced M-MDSCs

We analyzed whether the $Lin^{low/neg}$HLA-$DR^-$$CD11b^+$-$CD33^+$$CD14^+$ M-MDSC population identified in the peripheral blood of PBSC donors exerted an immune-suppressive activity in vitro. M-MDSCs and autologous $CD3^+$ T cells were isolated from the peripheral blood of G-CSF–treated donors (n = 10) using immunomagnetic selection strategies (see Methods). Highly purified $Lin^{low/neg}$HLA-$DR^-$$CD11b^+$-$CD33^+$$CD14^+$ cells were recovered (>90% as assessed by flow cytometry) and thereafter co-cultured for 4 days with autologous T cells in the presence of T cell stimulators (IL-2 and anti-CD2/CD3/CD28 Abs).

Flow cytometric analysis demonstrated that G-CSF–induced M-MDSCs, significantly suppressed T cell responses as demonstrated by the analysis of the expression of CD25 and CD137 activation markers on $CD4^+$ and $CD8^+$ T cells. A significant decrease in CD25 and CD137 expression levels was detected both on $CD3^+CD4^+$ helper and $CD3^+CD8^+$ cytotoxic lymphocytes ($P < .05$, Mann-Whitney t-test) (Figure 2).

Patients and aGVHD Incidence

Sixty patients received G-CSF–mobilized PBSC donor grafts for hematological malignancies. Most patients had lymphoma (n = 42; 70%) and myeloma (n = 12; 20%), whereas 6 patients had acute myeloid leukemia (n = 6; 10%) (Table 1). All patients reached a full donor chimerism before day 30 after transplantation as assessed by analysis of variable number of tandem repeats on peripheral blood. Platelet recovery (count >20,000/μL) was achieved at a median of day 9 post-transplantation (range, 3 to 20), whereas neutrophils reached values great than 500/μL on day 11 (range, 3

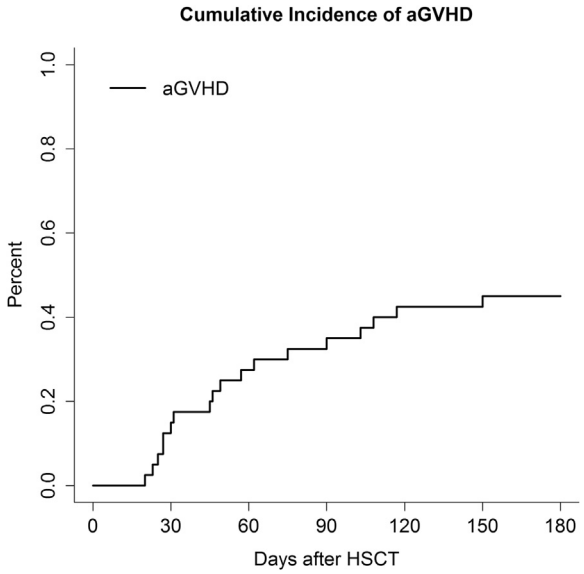

Figure 3. Probabilities of aGVHD were calculated with the cumulative incidence procedure, with relapse and death without aGVHD within 180 days as competing events (48%).

to 17), indicating successful engraftment in all patients (data not shown).

The cumulative incidence of aGVHD at 180 days after transplantation for the 60 patients receiving a G-CSF–mobilized PBSC allograft from unrelated donors was 48% (Figure 3). Overall, 2 patients (5%) developed grade IV aGVHD, 3 patients (10%) developed grade III aGVHD, 10 patients (38%) had grade II aGVHD, and 13 patients (47%) developed grade I aGVHD. All patients developed cutaneous aGVHD, whereas 4 patients (12.5%) developed concomitant hepatic, 3 (10%) intestinal, 2 (5%) gastric, and 2 (5%) multiorgan aGVHD. Nine patients developed chronic GVHD 115 days after transplantation (n = 9; 15%).

Several clinical variables have been shown to correlate with aGVHD onset, particularly, donor–recipient sex mismatch, donor and patient age, the degree of HLA incompatibility, and the type of conditioning regimen [16-18]. In our cohort of patients, none of these parameters was significantly associated with the development of aGVHD ($P > .05$, Fisher's exact t-test, Supplementary Table 2). Median follow-up was 17 month (range, 13 days to 53 months). Incidence of relapse was 20% (12/60), whereas transplant-related mortality was 12% (7/60).

PBSC Graft Content and aGVHD

The PBSC graft content of different cell population previously shown to impact on aGVHD [13,19-23] was analyzed. Enumeration of total T cells, B cells, NK cells, CD4 and CD8 T cells, Treg lymphocytes ($CD4^+CD25^+CD127^-FoxP3^+$), and iNKT ($CD3^+TCRV\alpha24\text{-}J\alpha18^+TCRV\beta11^+CD4^+$ and $CD3^+TCRV\alpha24\text{-}J\alpha18^+TCRV\beta11^+CD4^-$) was performed using flow cytometry. The absolute doses of the graft cell populations infused to patients at the time of PBSC transplantation are shown in Table 2. No significant correlation was found between the number of cells infused and aGVHD development ($P > .05$, Mann-Whitney t-test). In contrast with recent reports [13], in our study analysis failed to reveal a correlation between either the total iNKT population or the 2 subsets $CD4^+$ and $CD4^-$ ($P > .05$, Mann-Whitney t-test) and aGVHD. Cumulative incidence of aGVHD at 180 days after transplantation for recipients receiving cell doses below and above the median were 45% and 60%, respectively ($P = .73$, Gray's test), for $CD4^+$ iNKT and 50% and 55%, respectively ($P = .65$, Gray's test), for $CD4^-$ iNKT. Similarly, there was no association between the number of Tregs infused and aGVHD. At 180 days after transplantation, patients receiving Treg doses below and above the median had a cumulative incidence of 55% and 44%, respectively ($P = .58$ Gray's test).

Table 2
PBSC Graft Content of 60 G-CSF–Treated Donors Analyzed by Flow Cytometry

	Mean (10^6/kg body weight)	SD	Median (10^6/kg body weight)	Range	Mann-Whitney Test P
CD34	7.26	3.33	6.7	3.3-14.9	.52
CD45	1041.18	416.51	978.4	423.8-2029.5	.83
CD3	284.56	111.67	261	130-546	.47
CD4	179.73	84.59	153.7	59.7-427.7	.43
CD8	96.04	39.56	86.1	46-178.6	.94
CD19	67.05	43.25	57.7	6.4-232.4	.58
CD16-56	39.59	18.57	38	6.35-81.7	.22
Tregs	3.27	1.16	3.3	1.48-6.15	.90
iNKT $CD4^+$	.42	.35	.3	.0001-1.29	.30
iNKT $CD4^-$	.32	.30	.2	.0001-1.09	.09
M-MDSCs	39.13	29.98	33.6	3.35-159.99	.02

SD indicates standard deviation.
Mann-Whitney t-test was performed to assess possible associations between quantity of cells infused and aGVHD onset. The only population significantly associated with aGVHD was $Lin^{low/neg}$ $HLA\text{-}DR^-$ $CD11b^+$ $CD33^+$ $CD14^+$, M-MDSCs. P values are referred to Mann-Whitney test performed comparing patients developing aGVHD versus those not experiencing the complication.

Because the main feature of M-MDSCs that distinguishes them from the other cellular components is their ability to suppress T cells responses, we speculated that the M-MDSC graft content could have an impact on the incidence of aGVHD. The hypothesis that the M-MDSC graft content may be related to aGVHD development comes from mouse experiments indicating that adding functional MDSCs to donor grafts alleviates GVHD while preserving the GVT effect [8]. Indeed, class comparison analysis demonstrated that higher doses of M-MDSCs in the graft are associated with a minor risk of developing aGVHD ($P = .02$, Mann-Whitney t-test, Figure 4). Patients developing aGVHD received a median dose of 28.5×10^6 cells/kg body weight (range, 3.35 to 94.2), whereas patients not developing the complication received a

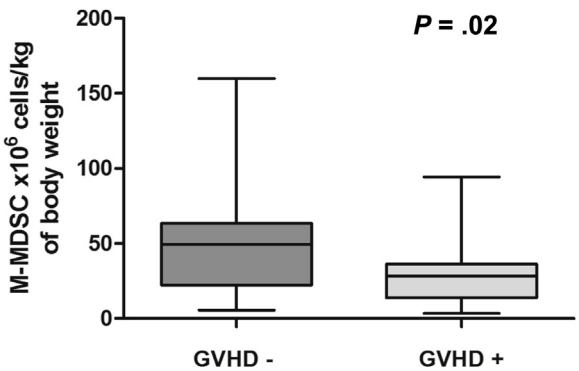

Figure 4. Box plots showing the absolute number of infused M-MDSCs with respect to aGVHD. Significantly lower quantity of M-MDSC/kg body weight were infused in patients who developed aGVHD with respect to those who did not (median 28.5×10^6/kg versus 49.4×10^6/kg total $CD45^+$ cells, Mann-Whitney t-test $P = .02$). Bars represent median values, whereas whiskers represent minimum and maximum.

median dose of 49.4 × 10^6 cells/kg body weight (range, 5.6 to 159.9). In addition, a significant correlation between the number of M-MDSCs infused at the time of transplantation and the severity of aGVHD was found (median 22.64 M-MDSC/kg body weight in grades II to IV aGVHD versus 39.93 M-MDSC/kg body weight in grades 0 to I aGVHD, Mann-Whitney *t*-test $P = .017$).

The cumulative incidence of aGVHD at 180 days after HSCT for recipients receiving cell doses below and above the median for total M-MDSCs was 63% and 22%, respectively ($P = .02$, Gray's test, Figure 5). No correlation was found between number of M-MDSCs infused and incidence of chronic GVHD ($P > .05$, Mann-Whitney *t*-test). Our results suggest a role of donor M-MDSCs in the early post-transplant immune suppression that might be involved in regulating alloreactive T cells responsible of aGVHD. Notably, the number of M-MDSCs infused did not impact the relapse rate or the transplant-related mortality rate ($P > .05$, Mann-Whitney *t*-test). Patients receiving doses above and below the median quantity of M-MDSCs displayed the same 1-year overall survival and event free survival rates ($P > .05$; log-rank test), suggesting this population does not alter hematopoietic reconstitution and immune surveillance after transplantation.

The Graft Cell Dose of $Lin^{low/neg}HLA\text{-}DR^{-}CD11b^{+}CD33^{+}CD14^{+}$ Predicts for the Occurrence of aGVHD

To identify a dose of infused M-MDSCs able to exert a protective effect on aGvHD, data were analyzed by a receiver operating characteristic (ROC) curve model (Figure 6). Sensitivity and specificity were plotted in a ROC curve, a plot of the false-positive rate and true-positive rate for every possible dose of M-MDSCs infused. The graft dose of 37,13 × 10^6 M-MDSCs cells/kg of body weight is able to discriminate patients developing aGVHD after MUD allo-HSCT with a specificity of 84,2% and a sensitivity of 76,6% (Figure 6, AUC 0.71). Furthermore, a multivariate regression analysis adjusted for variables with *P*-values <0.5 in class comparison analysis was performed. Taking into account MDSC cell doses below or above the cut-off value identified by the ROC analysis, multivariate analysis confirmed that the number of M-MDSC/kg of body weight is the only independent factor associated with the occurrence of aGVHD (Table 3).

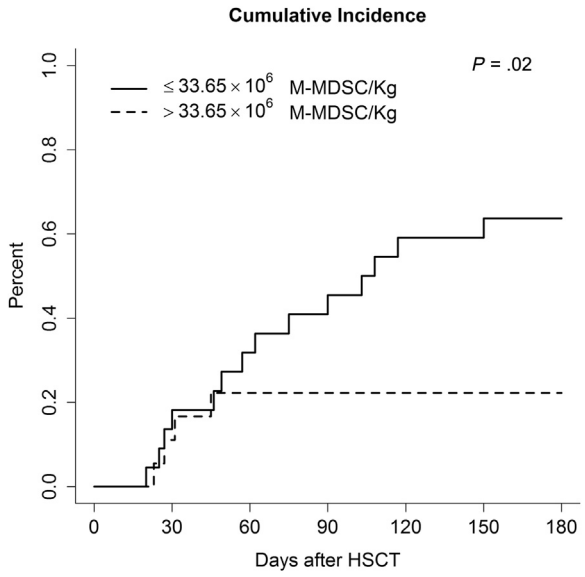

Figure 5. Cumulative incidence of aGVHD at 180 days after unrelated HSCT in patients receiving below (solid line) versus above (dashed line) the median dose of M-MDSCs (64% and 22%, respectively, Gray's test $P = .02$).

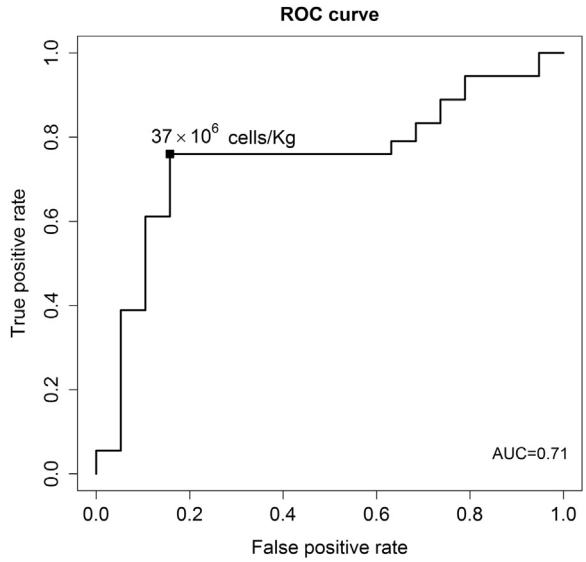

Figure 6. The dose of 37 × 10^6 M-MDSC/kg body weight is associated with the best combination of sensitivity and specificity. Receiver-operating characteristic curve displaying the false-positive rate versus the true-positive rate for every possible dose of M-MDSCs infused (area under the curve [AUC] .71).

DISCUSSION

Recent experimental evidence support the view that MDSCs have an immunoregulatory role in allo-HSCT, but no clear data are available in humans [24]. It has been shown that treatment of mice with *exogenous* G-CSF induces MDSCs that upon adoptive transfer into secondary recipients can suppress GVHD [25]. In humans, a single study indicated that the frequency of monocitic MDSC was significantly increased after allo-HSCT, especially in patients with aGVHD [9]. A significant correlation between the levels of MDSCs and the concentration of G-CSF, IL-6, and IL-10 in the sera of patients was also found [9], in line with the idea that the common denominator of the accumulation of MDSCs is a driving inflammatory milieu [24,26]. These cytokines, as well as MDSCs, declined steadily over time after a peak that occurred early after allo-HSCT [9]. In view of the developing opinion that MDSCs regulate alloreactivity after transplantation and that G-CSF stimulates the accumulation of MDSCs in vitro [26] and in vivo [4], we explored the effect of G-CSF–based mobilization regimens on MDSC phenotype, function, and

Table 3
Variables from the Multivariate Analysis Describing the Probability of Developing aGVHD

Variables	Odds Ratio	95% Confidence Interval	*P*
HLA mismatches	1.52	.15-15.06	.72
Sex mismatch	0.93	.08-10.55	.95
Conditioning regimen	1.29	.15-11.05	.81
$CD34^{+}$ cells	3.19	.47-21.55	.234
$CD3^{+}$ cells	2.71	.44-16.56	.28
$CD4^{+}$ cells	2.45	.24-25.04	.44
$CD16/CD56^{+}$ cells	.34	.07-1.62	.17
M-MDSC cells	.10	.02-.486	.04

Results from the multivariate analysis with a backward-stepping procedure that included all the variables with a $P \leq .5$ in class comparison analyses. M-MDSCs were the only factor significantly associated with aGVHD occurrence ($P = .04$).

frequency and the impact of MDSCs infused within the G-CSF–mobilized PBSC grafts on aGVHD.

By studying a larger cohort than in the previous report [4] and in analogy with data in mice indicating that mouse bone marrow cells cultured with G-CSF and GM-CSF resulted in the generation of $CD11b^+Gr1^+$ MDSCs able to suppress both $CD4^+$ and $CD8^+$ alloresponse in vitro [8,25], we confirmed that systemic treatment with G-CSF induces an expansion of myeloid cells displaying the phenotype of M-MDSCs ($Lin^{low/neg}HLA\text{-}DR^-CD11b^+CD33^+CD14^+$). Because describing an aberrant myeloid phenotype is not sufficient for defining MDSCs, we demonstrated that the purified M-MDSCs have the ability to suppress alloreactive T cell responses in vitro and therefore meets the definition of MDSC. In allogeneic HSCT, exogenous MDSCs alleviate GVHD in mice [8]; therefore, we reasoned that the M-MDSC graft dose might impact on aGVHD and manipulating MDSCs may be a promising therapeutic strategy. Indeed, we demonstrated that the incidence of aGVHD was reduced when higher levels of M-MDSCs were present among the infused donor cells. Even when the age of the donor or recipient, gender, and HLA mismatches were taken into account, the level of infused M-MDSCs was the only significant predictor of aGVHD ($P = .02$, Mann-Whitney t-test and $P = .04$ multivariate analysis).

In clinical studies, the use of G-CSF–mobilized peripheral blood as a stem cell source is associated with enhanced GVT effects without amplification of significant aGVHD [27], although there are still some concerns about the fact that it could increase the risk of leukemogenesis acting on immature myeloid precursors [28]. The mechanism by which G-CSF ameliorates GVHD still remains controversial, although it is known that G-CSF can induce a T helper 2 (TH2) polarization of donor T cells while opposing T helper 1 differentiation [29]. This TH2 polarization of the donor lymphocytes is thought to down-regulate aGVHD, whereas on the other hand it could be durable and could increase late infectious complications [28]. In addition to direct effects of G-CSF on donor T cell populations, additional data suggest that G-CSF may reduce GVHD through effects on dendritic cells, monocytes, NK cells, and NKT cells [30]. In in vitro studies and experimental model systems, G-CSF was shown to promote the emergence of tolerogenic immature dendritic cell subsets and granulocyte-monocyte precursors that dampen allogeneic and mitogenic responses by recruiting regulatory T cell populations [31]. Our results provide evidence of a new mechanism by which G-CSF–mobilized peripheral blood can modulate aGVHD, supporting the hypothesis that G-CSF directly affects regulatory M-MDSC frequencies in grafts favoring the control of the alloreactivity of donor T cells in the post-transplant period.

The mechanism of MDSC-induced GVHD protection remains incompletely defined. MDSCs suppressive functions have been attributed to arginase-1, inducible nitric oxide synthase, reactive oxygen species, and/or peroxynitrite [8,32-34], indicating a fair level of discordance. The recently published human study [9], analyzing MDSCs of the phenotype equivalent to the one we observed, revealed indoleamine 2,3-dioxygenase as the key mediator of the immunosuppressive activity of MDSCs in allo-HSCT. We believe the presence of high level of MDSCs at the time of transplantation is required to suppress T cell activation and thus reduce GVHD development. In support of this hypothesis, it has been shown that a rapid and significant expansion of MDSCs in peripheral lymphoid tissues of recipient mice before receiving a T cell–replete bone marrow graft led to the abrogation of aGVHD. Several studies reported that MDSCs isolated in vivo from G-CSF–treated mice or MDSCs generated in vitro with various cytokines significantly prevented GVHD. On the other hand, MDSCs did not control ongoing GVHD when given later than the activation and proliferation of allogeneic T cells in vivo [25].

Because it is known that GVT responses depend on T cell reactivity, it is reasonable to question whether increased levels of M-MDSC also down-regulate GVT responses. Murine models have generated promising data in this sense, demonstrating the adoptive transfer of MDSCs can result in the successful control of GVHD without compromising GVT effects [8,25]. Highfill et al. [8] demonstrated that in vitro generated MDSCs migrate to the site of allo-priming, mainly lymph nodes and spleen, shortly after transplantation and suppress normal antigen presentation to alloreactive T cells. Other reports focused on their ability to produce IL-10 and induce IL-10 producing cells such as Tregs and different types of antigen-presenting cells [31] or to suppress alloreactive T cells through soluble secretion factor and direct cell–cell contact [1]. In our study no significant correlation was found between the number of G-CSF–induced M-MDSCs and tumor relapse or with transplant-related mortality. Likewise, the 1-year overall survival and event-free survival rates were not influenced by quantity of M-MDSCs infused at the moment of transplantation. This is in line with MacDonald et al. [31], who reported that G-CSF–induced regulatory myeloid cells, upon adoptive transfer, prevent GVHD without compromising GVT in mice.

In patients undergoing allo-HSCT, other regulatory populations present in PBSCs grafts such as Tregs and iNKT have been demonstrated to correlate with aGVHD onset and grade [13,22,23]. Nevertheless, in our study on a quite homogenous population of patients receiving the same type of transplantation (HLA compatibility, number of $CD34^+$ and $CD3^+$ cells infused, conditioning regimen, and GVHD profilaxis), only M-MDSC graft levels are predictive of aGVHD. Stem cell grafts with higher Tregs or iNKT content do not correlate with less aGVHD. The discrepancy with previously published reports might be due to several critical differences among the studies, mainly type of transplantation, conditioning regimen used, and study cohort. In addition, given the relatively small sample size, our study might be underpowered to detect the role of the analyzed parameters. The fact that in such a cohort the M-MDSC graft content is the only variable significantly associated with aGVHD highlights the potential key role of this cell population as an intrinsic antagonist of GVHD. Whether other mobilizing agents affect M-MDSC graft content is still not known but would be interesting to assess. Plerixafor, for instance, might influence M-MDSCs by binding to and blocking the chemokine receptor type 4 expressed on the cell surface as it does on stem cells, favoring their migration in the bloodstream [35,36].

Although our data are determined in a relatively small cohort and that further analyses in prospective studies are required to validate the M-MDSC graft dose discriminating patients at higher risk of developing aGVHD, our results provide the proof of concept that MDSC-based approaches might constitute a therapeutic option for aGVHD. Moreover, given that MDSC levels can be modulated in vivo through cytokines routinely used during stem cell mobilization process, MDSCs therapies might be more feasible and less expensive than expanding and manipulating other cellular subsets such as Tregs and iNKT in vitro. In conclusion, our report points to an important role of G-CSF–induced

M-MDSCs in suppressing aGVHD and will likely enhance interest in developing clinical protocols aimed at modulating G-CSF to affect M-MDSC graft cell doses to prevent a GVHD.

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SUPPLEMENTARY DATA

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