# Functional Myeloid-Derived Suppressor Cell Subsets Recover Rapidly after Allogeneic Hematopoietic Stem/Progenitor Cell Transplantation

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A B S T R A C T

Myeloid-derived suppressor cells (MDSCs) are regulatory cell populations that have the ability to suppress effector T cell responses and promote the development of regulatory T cells (Tregs). They are a heterogeneous population of immature myeloid progenitors that include monocytic and granulocytic subsets. We postulated that given the rapid expansion of myeloid cells post-transplant, these members of the innate immune system may be important contributors to the early immune environment post-transplant. To evaluate the kinetics of recovery and function of MDSCs after allogeneic hematopoietic stem cell transplant (HSCT), 26 patients undergoing allogeneic HSCT were studied at 6 time points in the first 3 months after HSCT. Both MDSC subsets recovered between 2 and 4 weeks, well before the recovery of T and B lymphocytes. MDSC subset recovery positively correlated with T, B, and/or double-negative T cell numbers after HSCT. MDSCs isolated from patients post-transplant were functional in that they suppressed third-party CD4$^+$ T cell proliferation and Th1 differentiation and promoted Treg development. In conclusion, functional MDSC are present early after HSCT and likely contribute to the regulatory cell population post-transplant.

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) involves rapid expansion of the hematopoietic compartment after infusion of hematopoietic stem cell containing products that have been either harvested from marrow/cord blood or collected by growth factor mobilization. All populations contain large numbers of immature myeloid cells that contain a potent immune regulatory population known as myeloid-derived suppressor cells (MDSCs).

MDSCs are a heterogeneous population of activated immature myeloid progenitor cells; precursors to macrophages, granulocytes, and dendritic cells that have been prevented from fully differentiating into mature cells [1,2]. MDSCs are potent suppressors of T cells through direct cell contact, increased production of arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO) and reactive oxygen species, and induction of regulatory T cells (Tregs) [1,2].

The expansion and functional importance of MDSCs in cancer and noncancer pathogenic conditions has been recognized. MDSC numbers are increased, and they act as immune regulatory cells in autoimmune diseases [3], solid organ transplantation [4,5], and allergic asthma [6]. We have reported that the numbers of MDSCs are positively correlated with the severity of murine colitis and that adoptive transfer of MDSCs isolated from murine colitis models or generated in vitro ameliorate murine intestinal inflammation [7].

Allogeneic HSCT transplantation is associated with a massive expansion of myeloid cell compartment, both in the donor with granulocyte colony-stimulating factor (G-CSF) mobilized hematopoietic stem cell collection and in the recipient early post-transplant. Both G-CSF and granulocyte-macrophage (GM)-CSF have been shown to expand MDSCs [8]. Cytokines prominent in the early post-transplant period,

such as tumor necrosis factor (TNF)-α, IFN-γ, and IL-6, are known to activate or license MDSCs to become potent suppressors of multiple aspects of the immune system [9]. Small series have suggested that MDSCs are present in the grafts and recipients early after HSCT [10-12].

MDSC are divided into 2 main subsets, monocytic (M-MDSC) and granulocytic (G-MDSC), based on expression of monocytic or granulocytic cell surface markers [1,9]. Each subset may have different functions under varying inflammatory conditions. In a murine asthma model, M-MDSC inhibited whereas G-MDSC exacerbated airway inflammation [6]. Furthermore, timing appears to be important. In a sepsis model, MDSCs exaggerated inflammation in the early stage but suppressed inflammation in the later stage of sepsis [13].

In murine GVHD transplant models, MDSC accumulation in recipient mice post-transplantation was positively correlated with the severity of graft-versus-host disease (GVHD) [14]. The infusion of embryonic stem cell– or bone marrow–derived MDSCs before transplantation provided protection from lethal acute GVHD, leading to long-term survival in 37% [15] or 82% [16] of recipient mice. Adoptive transfer of MDSCs preserved graft-versus-leukemic (GVL) effects of allogeneic host T cells in a GVL model [15]. Mougiakakos et al. [10] found that CD14+HLA-DR−/lowIDO+ monocytic MDSCs in peripheral blood mononuclear cells (PBMCs) are significantly increased in patients with acute GVHD and that these MDSCs could suppress the proliferation of lymphocytes through IDO in vitro. More recently, another study showed extracorporeal photopheresis treatment in patients with GVHD rapidly increased the percentage of circulating G-MDSCs in PBMCs, which efficiently suppressed effector CD4 T cell responses [17]. G-MDSCs may be underappreciated in previous studies of leukocytes post-transplant because they sediment with red blood cells in conventional density gradient cell preparations (eg, Ficol) and do not survive freeze–thaw well.

To date, reconstitution of MDSC subsets after allogeneic HSCT and the relation of MDSC subsets to acute GVHD have not been well defined. We were interested in understanding the kinetics of MDSC recovery post-HSCT, their functionality, and whether they were associated with acute GVHD.

## METHODS

### Patients

Patients undergoing allogeneic HSCT were enrolled on a trial evaluating early immune recovery after allogeneic HSCT. This study was approved by the ethics committee of the University of Manitoba. All patients and/or their parents signed informed consent. Patient demographics and transplant outcomes are presented in Table 1.

There was an equal representation of myeloablative (busulfan/fludarabine or total body irradiation based) and nonmyeloablative conditioning regimens. Aplastic anemia patients received fludarabine/cyclophosphamide with adjusted cyclophosphamide dosing for the patient with Fanconi anemia. GVHD prophylaxis was tacrolimus and mini-methotrexate (5 mg/m² on days +1, +3, and +6, with an additional dose on day +11 for unrelated donors). Antithymocyte globulin was used in the aplastic anemia patients and if there was less than 10/10 HLA match. Most patients received G-CSF (filgrastim)–mobilized grafts from family or unrelated donors with a target cell dose of 5 × 10⁶ CD34 cells/kg. Filgrastim (5 µg/kg/day) was administered daily starting on day +1 in the cord blood recipient until neutrophil recovery above 1.5 × 10⁹/L but was otherwise not used in the other subjects unless there was delayed count recovery.

All recipients had documentation of donor engraftment. Neutrophil engraftment day was defined as the first day of 3 consecutive days with absolute neutrophil count greater than .5 × 10⁹/L. Platelet engraftment day was defined as the first of 7 consecutive days with platelet count greater than 20 × 10⁹/L without transfusion support for at least 7 days. Acute GVHD was graded according to the modified Glucksberg criteria [18]. In the first

### Table 1
Patient Demographics and Transplant Outcomes (N = 26)

| Characteristic | Value |
| --- | --- |
| Sex (female/male) | 8/18 |
| Disease | |
| Acute myelogenous leukemia | 11 |
| Non-Hodgkin lymphoma | 4 |
| Myelodysplastic/fibrotic syndrome | 4 |
| Acute lymphoblastic leukemia | 3 |
| Chronic lymphocytic leukemia | 1 |
| Severe aplastic anemia/Fanconi anemia | 2 |
| Common variable immunodeficiency | 1 |
| Median age at transplant, yr (range) | 43 (13-62) |
| Donor source | |
| G-CSF mobilized PBSCs (16 unrelated, 6 related) | 22 |
| Bone marrow (2 unrelated, 1 related) | 3 |
| Cord blood (1 unrelated) | 1 |
| Conditioning regimen | |
| Myeloablative | 13 |
| Nonmyeloablative | 13 |
| Acute GVHD | |
| None or GVHD grade I | 13 |
| GVHD grades II-IV | 13 |
| Median days to achieve ANC 500/µL (range) | 18 (12-28) |
| Median days of platelet recovery to 20 × 10⁹/L* (range) | 21 (13-47) |
| Median CD34+ stem cells transplanted, ×10⁶/kg (range) | |
| PBSCs | 5.4 (2.0-8.0) |
| Bone marrow | 2.7 (1.5-3.8) |
| Cord blood | .21 |

PBSC indicates peripheral blood stem cell; ANC, absolute neutrophil count.
* Six patients did not have a nadir of platelets below 20 × 10⁹/L.

100 days post-transplant, 2 patients died (primary disease) and 6 patients had progression or relapse of malignancy.

### Isolation of WBCs

Twenty-four milliliters of peripheral blood was drawn on the day before the start of the conditioning regimen, with 1 additional sample drawn each between days +4 and +5, +7 to +9, +14 to +16, +21 to +23, +27 to +29, and +80 to +100 after HSCT, for a total of 7 samples per subject. WBCs were isolated from peripheral blood using HetaSep (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer protocol. All blood samples were processed and analyzed 24 hours after drawing.

### Antibodies and Flow Cytometry

WBCs were stained with fluorescence-labeled antibodies against surface molecules for MDSC subsets (HLA-DR, CD45, CD33, CD15, CD66b, and CD14), B cells (CD19), and T cells (CD3, CD4, and CD8) (eBioscience, San Diego, CA; Biolegend, San Diego, CA) according to manufacturer recommendations. After staining, cells were acquired and analyzed using flow cytometry (FACS Canto II; BD Biosciences, San Jose, CA) and FlowJo software (TreeStar, San Carlos, CA) [10,17]. For intracellular staining of Arg-1 and iNOS, cells were first stained with fluorescence-labeled antibodies against HLA-DR, CD33, CD15, and CD14 and then fixed and permeabilized using eBioscience intracellular fixation/permeabilization buffer. Cells were then stained with Percp-Cy5.5–labeled anti-iNOS (Santa Cruz Biotechnology, Dallas, TX) and PE-labeled anti–Arg-1 (R&D systems, Minneapolis, MN), and analyzed by flow cytometry (FACS Canto II). The FACS Canto II was maintained by daily running BD 7-color microbeads to ensure the accuracy and consistency of the subpopulation analysis. Single staining tubes and isotype control staining tubes were used to do the compensation for all runs.

### T Cell Proliferation and Differentiation Assay

To evaluate the function of the MDSC subsets, M-MDSCs and G-MDSCs were enriched by positive selection from the peripheral blood using CD14+ selection kit and CD15+ selection kit (Stem Cell Technologies) according to the manufacturer protocol. The purity of M-MDSC and G-MDSC cells was confirmed by flow cytometry. The control population was CD14−CD15− cells that contained all lymphocytes. The suppressive effect of MDSC subsets on lymphocyte proliferation and T cell differentiation was evaluated as described previously [10]. Briefly, third-party PBMCs were isolated from healthy donors and labeled with carboxyfluorescein succinimidyl ester (CFSE). The 1 × 10⁵ CFSE-labeled PBMCs were then stimulated with anti-CD3/CD28 microbeads

(1.8 µL/well, Life Technologies, Grand Island, NY) in the presence of M-MDSCs, G-MDSCs, or control cells at the ratios of PBMC/MDSC/control cells (1:1 and 1:.5) in 96-well plates for 5 days. Cells were then stained with fluorescence-labeled antibodies against CD4 and IFN-γ for detecting CD4^{+} T cell proliferation and Th1 differentiation. To evaluate the effects of MDSC subsets on the differentiation of Tregs, $1 \times 10^5$ CFSE-labeled PBMCs were stimulated with anti-CD3/CD28 microbeads (1.8 µL/well) in the presence of recombinant human transforming growth factor-β (5 ng/mL; Peprotech, Rocky Hill, NJ) for 5 days. Stimulator cells were added into the culture at the beginning. After 5 days, cells were stained with fluorescence-labeled antibodies against CD4, CD25, and Foxp3 to detect Tregs. Cells were analyzed by flow cytometry.

### Statistical Analysis

Values were expressed as means ± standard deviation when normally distributed and with boxplots when not normally distributed. Mann-Whitney tests were used to compare MDSC subset values between GVHD groups. ANOVA followed by the Newman-Keuls multiple comparison test was used to compare production of mediators in MDSC subsets. Spearman correlations were used to assess the relationship between cell values. All statistical analyses were performed using GraphPad Prism, version 5 (GraphPad Prism, La Jolla, CA). $P < .05$ was considered to be statistically significant.

## RESULTS

### MDSC Subsets Recover Early in Patients after Allo-HSCT

Several markers have been used to define human MDSCs and subsets. Commonly used for human MDSC characterizations are CD33, CD11b, CD14, HLA-DR, CD15, or CD66b, and, in
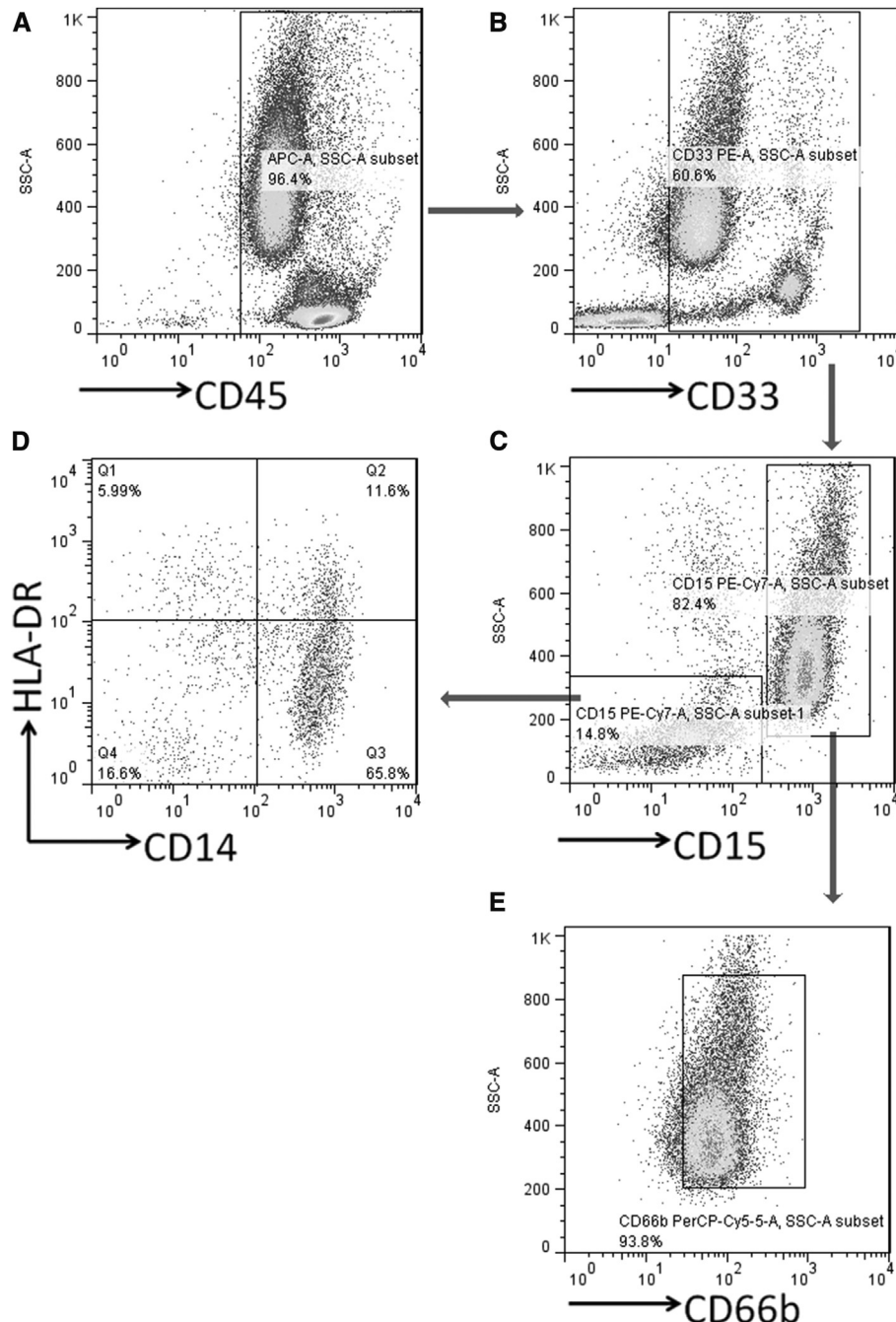


**Figure 1.** Gating strategy of MDSC subsets. (A) CD45^{+} cells; (B) CD33^{+} cells; (C) CD15^{−} cells and CD15^{+} cells; (D) CD14^{+}HLA-DR^{−/low} cells, M-MDSCs; and (E) CD66^{+} cells, G-MDSCs.

some cases, lineage negative [19-21]. We used $CD33^+CD14^+HLA\text{-}DR^{-/low}$ to define M-MDSCs and $CD33^+CD15^+CD66b^+$ to define G-MDSCs (Figure 1). As expected, the total WBC count fell immediately post-HSCT due to the conditioning regimen and recovered over the next 3 weeks (Figure 2A). The median number of days to neutrophil and platelet recovery was 18 and 21 days, respectively (Table 1). The median percentage of M-MDSCs fell from 1.44% (interquartile range [IQR], .45% to 3.19%) at the pre-conditioning samples to .06% (IQR, .01% to .09%) at days 4 and 5 post-transplant and then recovered to 3.93% (IQR, 1.53% to 10.02%) of total WBCs at days 14 to 16 post-transplant (Figure 2B). This translated into the median number of

M-MDSCs dropping from $5.2 \times 10^4$/mL (IQR, 2.14 to $9.9 \times 10^4$/mL) at the preconditioning samples to $.02 \times 10^4$/mL (IQR, .01 to $.16 \times 10^4$/mL) at days 4 to -5 post-transplant and then recovered to $5.9 \times 10^4$/mL (IQR, .46 to $15.21 \times 10^4$/mL) at days 14 to 16 post-transplant (Figure 2C). Recovery of G-MDSCs similarly occurred early post-transplant but appeared slightly more delayed compared with M-MDSCs (Figure 2D,E). Median G-MDSC numbers remained below normal ranges until days +21 to +23, with recovery to normal ranges occurring by days +27 to +29 post-transplant. Despite some patients still being leukopenic early post-transplant, over 60% of patients had MDSC subset levels in the normal range at these early post-transplant time points.
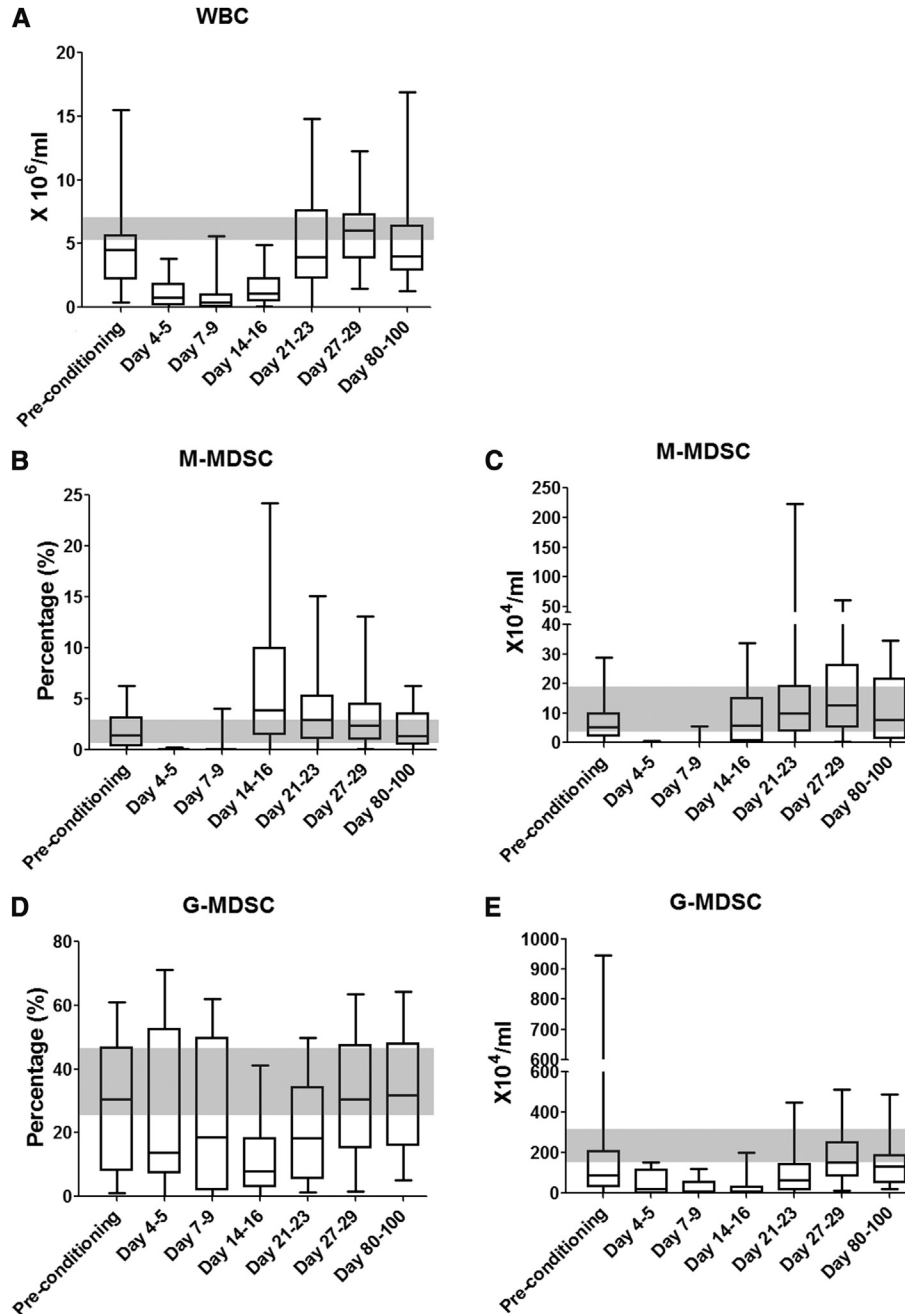


**Figure 2.** Time course of recovery of MDSC subsets in patients after allo-HSCT. WBCs were isolated, and the phenotype of MDSC subsets were analyzed through flow cytometry. The recovery of WBCs (A), percentage (B) and number (C) of M-MDSCs, and percentage (D) and number (E) of G-MDSCs. Shaded area is the median with IQRs of 12 healthy donors.
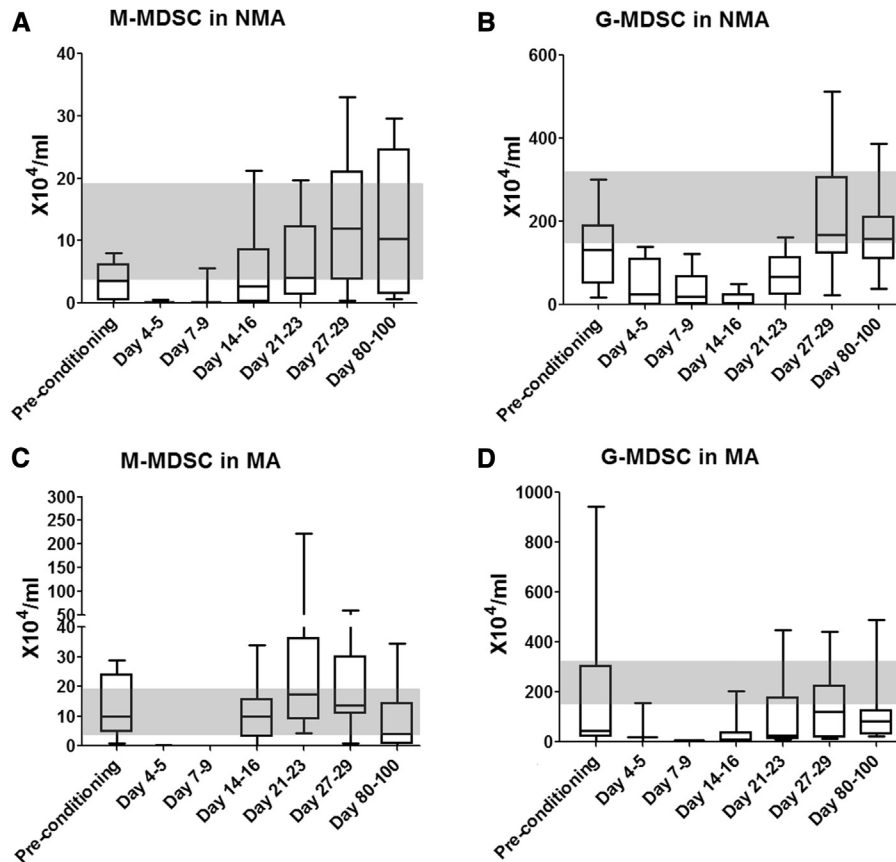
**Figure 3.** Recovery of MDSC subsets in patients accepting myeloablative (MA) or nonmyeloablative (NMA) chemotherapy regimens. (A) M-MDSC recovery in patients receiving NMA, (B) G-MDSC recovery in patients receiving NMA, (C) M-MDSC recovery in patients receiving MA, and (D) G-MDSC recovery in patients receiving MA. Shaded area is the median with IQRs of 12 healthy donors.

No apparent difference in the recovery of M- and G-MDSCs was observed with type of conditioning regimen. In both myeloablative and nonmyeloablative conditioning regimens, median M-MDSC numbers were in the normal range by days +14 to +16 (Figure 3A,C) and G-MDSC numbers by days +27 to +29 (Figure 3B,D).

The recovery of adaptive immune CD19+ B lymphocytes and CD3+ T lymphocytes were also evaluated. Neither B lymphocyte nor T lymphocyte numbers recovered in the first 100 days after allo-HSCT (Figure 4A,B), consistent with previous reports [22,23]. Double-negative (DN) T cells, defined as CD3+CD4−CD8−, exist as a small population of T lymphocytes in peripheral blood and lymphoid organs [24]. DN T cells have shown to be capable of controlling GVHD [24]. As shown in Figure 4C, the median number of DN T cell decreased from $3.19 \times 10^4$/mL (IQR, .83 to $5.63 \times 10^4$/mL) at the precondition to $.26 \times 10^4$/mL (IQR, .10 to $.84 \times 10^4$/mL) at days 7 to 9 post-transplant and then recovered to $2.20 \times 10^4$/mL (IQR, .48 to $5.68 \times 10^4$/mL) at days 27 to 29 post-transplant. Taken together, our results indicated that immune recovery of both M- and G-MDSC subsets was much more rapid than recovery of CD19+ B and CD3+ T cells.

### Relationship of MDSC Subsets with Other Immune Cells

Because MDSC subsets can modulate the functions of other immune cells, such as T cells and Tregs, we wanted to evaluate the relationships between the immune reconstitution of MDSC subsets with other immune cells, in particular whether the speed of MDSC recovery in individual patients was associated with T cell or B cell recovery. First, the number of MDSC subsets at different time points post-transplantation was evaluated for correlation with other immune cells using the Spearman test. As shown in Table 2, M-MDSC recovery positively correlated with G-MDSC recovery at days 14 to 16 and days 80 to 100 post-HSCT and positively correlated with CD19+ B cell recovery at days 21 to 23. G-MDSC recovery positively correlated with CD19+ B cell recovery at days 14 to 16, days 21 to 23, and days 80 to 100 post-HSCT; positively correlated with CD3+ T cells at days 14 to 16, days 21 to 23, and days 27 to 29 post-HSCT; and positively correlated with DN T cells at days 14 to 16 and days 21 to 23 post-HSCT. We then analyzed whether MDSC recovery in individual patients can speed up the recovery of T and B cell recovery. There was no trend of MDSC recovery with the speed of recovery of T and B cells in our study, but we did not evaluate later time points more relevant to T and B cell recovery post-transplant.

### Relationship of MDSC Subsets with Acute GVHD

Of 26 subjects in this study, 13 developed higher grade acute GVHD (grades II to IV) and 13 had no or lower grade acute GVHD (grades 0 to I). MDSC subsets were compared at various time points between these 2 patient groups. As shown in Figure 5A, patients who later developed higher grade acute GVHD had higher numbers of G-MDSCs before the start of conditioning compared with those without or lower grade acute GVHD. At other time points, M-MDSC recovery or G-MDSC recovery was not associated with GVHD.
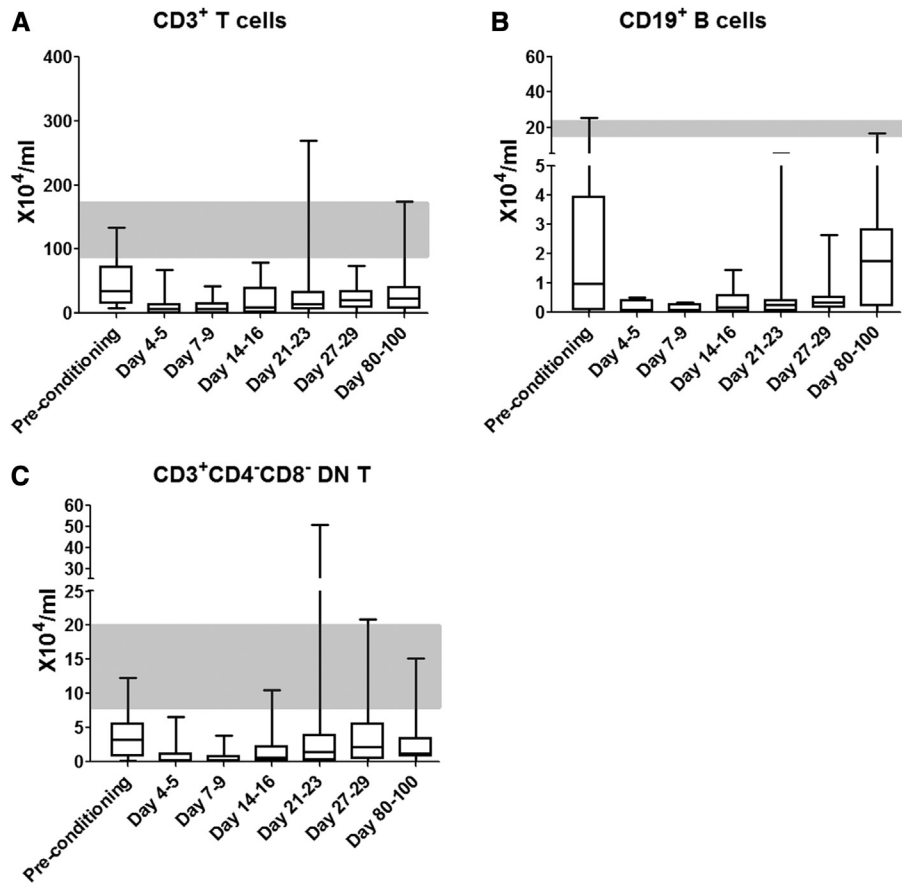
**Figure 4.** Time course of recovery of T lymphocytes, B lymphocytes, and DN T cells in patients after allo-HSCT. WBCs were stained with fluorescence-labeled antibodies against CD3 and CD19 for T and B lymphocytes and for CD3, CD4, and CD8 for DN T cells and then analyzed by flow cytometry. (A) Recovery of T lymphocytes, (B) recovery of B lymphocytes, and (C) recovery of DN T cells. Shaded area is the median with IQRs of 12 healthy donors.

This differs from a very recent report in which acute GVHD patients had lower percentages of M-MDSCs at day +15 but higher percentages of M-MDSCs at day +30 compared with

**Table 2**
Relationship of MDSC Subsets with Other Immune Cells

| | M-MDSCs | | G-MDSCs | |
|---|---|---|---|---|
| | R | P | R | P |
| Days 14-16 | | | | |
| M-MDSCs | | | .667 | <.001 |
| G-MDSCs | .667 | <.001 | | |
| CD19$^+$ B cells | .212 | .340 | .676 | <.001 |
| CD3$^+$ T cells | .321 | .146 | .715 | <.001 |
| CD3$^+$CD4$^-$CD8$^-$ DN Tregs | .464 | .026 | .795 | <.001 |
| Days 21-23 | | | | |
| M-MDSCs | | | .210 | .304 |
| G-MDSCs | .210 | .304 | | |
| CD19$^+$ B cells | .604 | .001 | .551 | .004 |
| CD3$^+$ T cells | .134 | .514 | .447 | .022 |
| CD3$^+$CD4$^-$CD8$^-$ DN Tregs | .354 | .076 | .422 | .032 |
| Days 27-29 | | | | |
| M-MDSCs | | | .119 | .564 |
| G-MDSCs | .119 | .564 | | |
| CD19$^+$ B cells | .273 | .187 | .246 | .236 |
| CD3$^+$ T cells | −.101 | .632 | .468 | .018 |
| CD3$^+$CD4$^-$CD8$^-$ DN Tregs | .005 | .980 | .389 | .050 |
| Days 80-100 | | | | |
| M-MDSCs | | | .464 | .022 |
| G-MDSCs | .464 | .022 | | |
| CD19$^+$ B cells | .108 | .625 | .421 | .045 |
| CD3$^+$ T cells | −.297 | .158 | −.265 | .210 |
| CD3$^+$CD4$^-$CD8$^-$ DN Tregs | −.237 | .264 | −.185 | .386 |

R indicates correlation coefficient.

nonacute GVHD patients [25]. Interestingly, in the present study, where G-MDSC number recovered to normal range at days 27 to 29, the ratio of G-MDSC at days 27 to 29-to-G-MDSC at preconditioning baseline (G-MDSCR) might correlate with the incidence of GVHD (Figure 5B). In patients who developed acute GVHD (grades II to IV), the median G-MDSCR was .498 (IQR, .263 to 1.791), whereas those with no or lower grade acute GVHD, the G-MDSCR was 2.335 (IQR, 1.409 to 6.050) (Figure 5B). Our results suggest G-MDSC subsets before the start of conditioning or G-MDSCR may be associated with the later development of acute GVHD, but this needs to be confirmed in a larger independent cohort.

### Effects of MDSC Subsets on T Cell Responses

Next, we asked whether the MDSC in patients early post-HSCT were functional; specifically, whether they could modulate the function of third-party T cells. MDSC subsets were isolated from patients at days 80 to 100 post-transplant. Our results showed that both M-MDSCs and G-MDSCs suppressed proliferation of third-party CD4$^+$ T cells in a dose-dependent manner, with G-MDSCs being more potent inhibitors of T cell proliferation (Figure 6A,B). To evaluate the effect of MDSC subsets on the development of Th1 and Tregs, we measured expression of CD4$^+$IFN-$\gamma^+$Th1 and expansion of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the coculture system of anti-CD3/CD28 stimulated third-party lymphocytes. Both M-MDSCs and G-MDSCs suppressed the differentiation of Th1 (Figure 6C,D) and promoted the
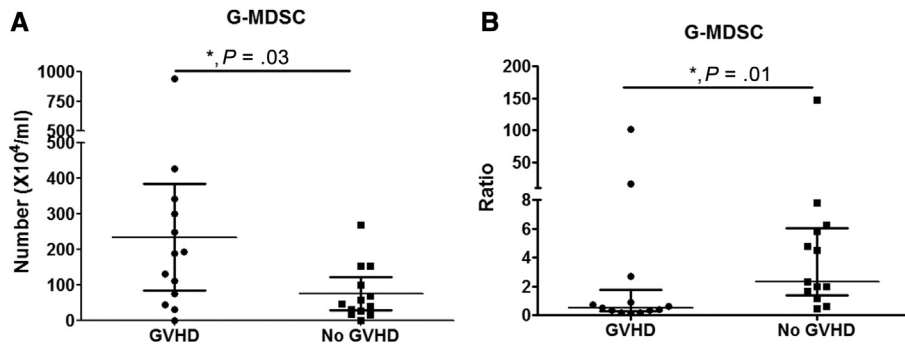
**Figure 5.** Relationship of G-MDSCs with grades II to IV acute GVHD. (A) G-MDSC numbers at preconditioning between patients who developed high grade acute GVHD later and patients who developed low grade GVHD or without GVHD. (B) Ratio of G-MDSC number at days 27 to 29 over the baseline at the preconditioning. *P < .05.

development of Tregs (Figure 6E,F) when compared with the control cell group.

### Production of Soluble Mediators of Arg-1 and iNOS in MDSC Subsets

MDSCs suppress effector T cell responses by multiple mechanisms [1], 2 of which occur through Arg-1 and iNOS. We evaluated the Arg-1 and iNOS activity in MDSC subsets in patients pre- and post-transplant. G-MDSCs, but not M-MDSCs, expressed significantly higher levels of Arg-1 and iNOS compared with healthy donors, both at preconditioning and multiple time points post-transplant (Figure 7A,B). Levels of Arg-1 or iNOS expressed in G-MDSCs in the pre-conditioning samples were around 5 and 2 times higher than those in healthy donors, respectively, suggesting these MDSC subsets were activated/licensed before transplant. G-MDSCs showed temporal down-regulation of the expression of Arg-1 and iNOS at days +14 to +16 after HSCT, but both recovered by days +27 to +29 to high levels, which remained so until days +80 to +100 post-HSCT. Interestingly, there was no difference in the expression levels of Arg-1 and iNOS in M-MDSCs among healthy donors and patients (Figure 7A,B).

### DISCUSSION

The aim of this study was to analyze the kinetics of immune reconstitution (both numerical and functional) of MDSC subsets in patients undergoing allogeneic HSCT. In the present study, we reported the early recovery of both M-MDSC and G-MDSC subsets post-HSCT, well before the recovery of adaptive immune CD3+ T and CD19+ B cells. We also demonstrated both MDSC subsets were functional, which can regulate T cell responses. The present study especially highlighted the possible important roles of G-MDSCs in allogeneic HSCT: G-MDSCs had stronger suppressive function on lymphocyte proliferation than M-MDSCs, the percentage and number of G-MDSCs were significantly higher than those of M-MDSCs, G-MDSCs showed stronger positive correlation with other immune cells, and G-MDSCs may predict the development of acute GVHD. Most studies of immune recovery post-transplant used frozen cells isolated using density gradients such as Ficoll. Different from M-MDSCs, G-MDSCs do not survive freeze–thaw well and will sediment with RBCs in most density gradient separations. This needs to be considered in future studies evaluating early regulatory networks in transplant and other settings.

The early post-transplant period is characterized by the rapid expansion of myeloid cells [22,23]. After successful allogeneic HSCT, monocytes are the first cells to recover, followed soon thereafter by granulocytes, platelets, and natural killer cells. Expansion of lymphocytes, correlating to cognate immune reconstitution, does not occur to any great extent until after 100 days post-HSCT [22,23]. In the present study, we demonstrate that both M-MDSCs and G-MDSCs recover in the first month post-HSCT, with M-MDSCs recovering 1 to 2 weeks earlier than G-MDSCs (Figure 1). This is consistent with the knowledge that monocytes recover earlier than granulocytes in patients post-HSCT. Recovery of both MDSC subsets appears to be similar regardless of whether a myeloablative or nonmyeloablative conditioning regimen is used (Figure 3). Both MDSC subsets recover with the same tempo post-HSCT (Table 2). Our results showed that both MDSC subsets isolated from patients post-transplant can suppress T lymphocyte proliferation, inhibit CD4+ Th1 differentiation, but promote the development of Tregs. All this suggests that functional MDSCs appear early post-HSCT, and these cells could play a role in later modulating T cell responses that lead to both GVHD and GVL.

Although Mougiakakos et al. [10] reported that M-MDSCs are significantly increased in PBMCs of patients with acute GVHD, we could not confirm this using our patient population. Both studies may be limited by small patient numbers. Interestingly, our results show that the ratio of G-MDSCs at days 27 to 29 over baseline before the start of conditioning is significantly higher in patients who develop no or grade I acute GVHD compared with patients who developed higher grade acute GVHD. This suggests that rapid recovery of G-MDSCs or even higher G-MDSCs at days 27 to 29 in patients after allogeneic HSCT may result in a lower risk to develop acute GVHD. We found that patients who later went on to develop higher grades (II to IV) of acute GVHD had more pretransplant circulating G-MDSCs (Figure 5). This is difficult to explain, given the assumption that most MDSCs would be destroyed by the conditioning regimen. One explanation might be that MDSCs present before the start of conditioning are licensed and secrete large amounts of soluble inhibitors, such as Arg-1, iNOS, and IDO, during the cell death process. This could impact the function of alloreactive T cells in the graft that are important in the initiation of GVHD. Our results show that G-MDSCs expressed higher levels of Arg-1 and iNOS in patients at preconditioning compared with those in healthy donors. We also found that the M-MDSC subset in both patients and healthy donors expressed dramatically lower levels of Arg-1 and iNOS than G-MDSCs. This is different from murine MDSCs because Arg-1 and iNOS are thought to be the markers for M-MDSCs in mice [1]. Recent
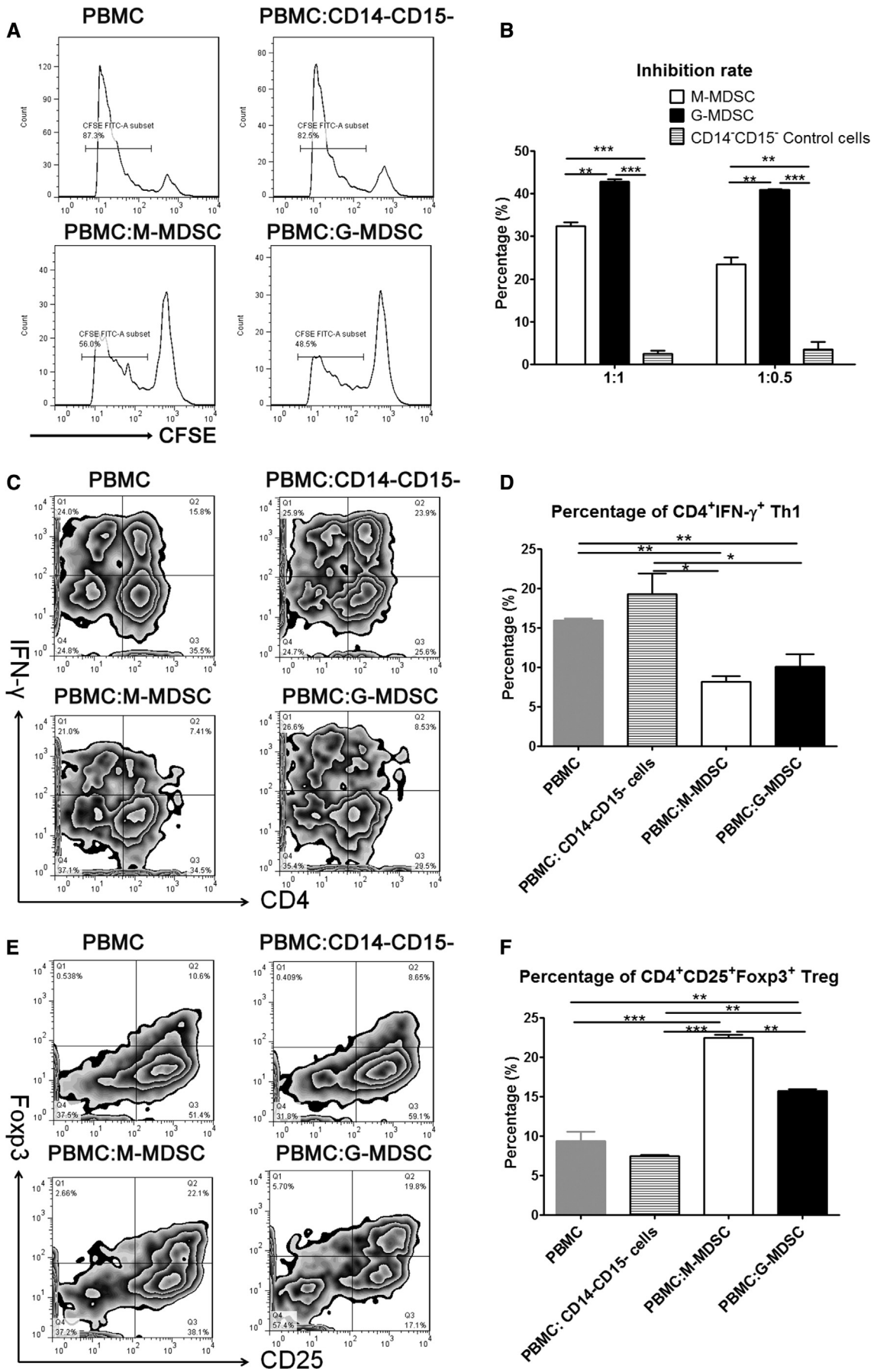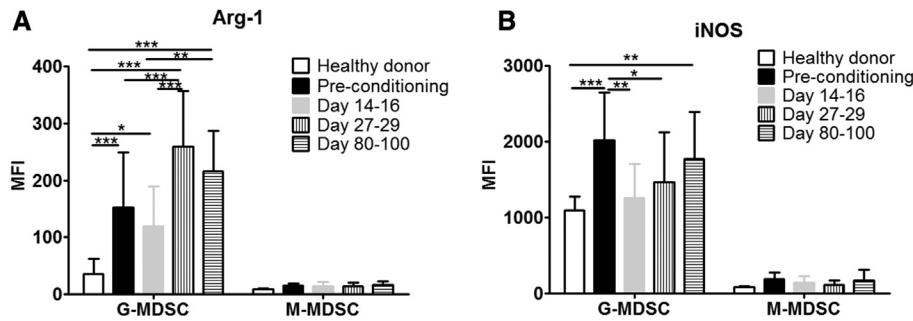
**Figure 7.** Expression of Arg-1 and iNOS in MDSC subsets of patients after allo-HSCT. The expression of Arg-1 and iNOS in MDSC subsets were detected by intracellular staining of Arg-1 and iNOS and analyzed by flow cytometry. (A) Expression levels of Arg-1 in MDSC subsets. (B) Expression levels of iNOS in MDSC subsets. $*P < .05$, $**P < .01$, $***P < .001$.

studies showed that the suppressive function of G-MDSCs in patients with GVHD is partially dependent on the activity of Arg-1 [17], whereas the suppressive function of M-MDSCs in patients post-HSCT is partially dependent on IDO [10]. A large clinical trial is ongoing to confirm this observation in which we will study both the function of MDSCs in patients before conditioning and evaluate the soluble mediator expression pattern and possible roles by these MDSCs at later time points post-transplant.

The expansion and activation of MDSCs usually requires 2 signals. The first signal provided by growth factors such as G-CSF and GM-CSF promotes the differentiation and expansion of MDSCs from myeloid progenitors [2,9]. The second signal, provided by proinflammatory cytokines such as IFN-$\gamma$, IL-6, and TNF-$\alpha$, leads to activation or licensing of their immune regulatory function, including up-regulation of soluble mediators, such as Arg-1 and iNOS [2,9]. In the presence of GM-CSF with TNF-$\alpha$, IL-1$\beta$, or IL-6, PBMCs from healthy donors can be converted into CD33$^+$ MDSC-like cells [26]. The combination of GM-CSF and IL-6 or GM-CSF and G-CSF can also induce the differentiation and activation of MDSC from bone marrow cells in human and mice [8].

Interestingly, these growth factors and proinflammatory cytokines, which can induce the differentiation and activate MDSCs, have also been found to be important in the pathogenesis of GVHD [27,28]. Serum levels of IFN-$\gamma$, TNF-$\alpha$, and IL-6 are increased in the first 2 to 3 weeks after allo-HSCT [28,29], and G-CSF was also increased in patients post-transplantation [10]. Our data show that both MDSC subsets recovered within 1 month post-transplantation. We hypothesize that the early post-transplant immune environment has the required elements to expand and activate MDSCs and that MDSC recovery and function in patients post-transplant is dependent on the growth factor and proinflammatory cytokines milieu post-transplant.

One study has indicated that the percentage of M-MDSCs is correlated with the serum levels of G-CSF, IL-6, and IL-10 in patients after allo-HSCT [10]. Clinically, growth factors are variably used post-transplant to speed neutrophil recovery. MDSCs induced by different growth factors and/or cytokines may have different suppressive functions [8]. Therefore, differences in clinical practice with respect to

post-transplant growth factor administration may be affecting both the number and function of MDSC in the post-transplant milieu and may be an explanation for differences in GVHD with hematopoietic growth factor usage. Increasing MDSC activity selectively post-transplant—through cytokine administration or cellular infusion—may be a tactic for preventing/treating GVHD while preserving the GVL effect.

Taken together, we demonstrate that both M-MDSC and G-MDSC subsets recover early after HSCT, well before adaptive immune CD3$^+$ T and CD19$^+$ B cells; G-MDSCs/M-MDSCs recovery positively correlated with other immune cells at different times after HSCT; and G-MDSCs in patients at the preconditioning time point and the ratio of G-MDSCs at days 27 to 29 over baseline may predict the development of acute GVHD. These early post-HSCT MDSC subsets are functional; they suppress T cell proliferation and Th1 differentiation while promoting the development Tregs. This suggests that MDSCs may play an important role in the pathophysiology of GVHD and GVL and potentially are a target for therapeutic manipulation.

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**Figure 6.** Function assay of MDSC subsets in patients after allo-HSCT. (A) Representative figures of CD4$^+$ T lymphocyte proliferation (gated on CD4$^+$ T cells). (B) Inhibition rate of MDSC subsets on lymphocyte proliferation. (C) Representative figure of percentages of CD4$^+$IFN-$\gamma^+$ Th1 cells. (D) Percentage of CD4$^+$IFN-$\gamma^+$ Th1 cells. (E) Representative figure of percentages of CD4$^+$CD25$^+$Foxp3$^+$ Tregs (gated on CD4$^+$ T cells). (F) Percentages of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the coculture. The results represent 1 of 2 patient experiments. $*P < .05$, $**P < .01$, $***P < .001$.

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