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Plasmacytoid Dendritic Cell Count on Day 28 in HLA-Matched Related Allogeneic Peripheral Blood Stem Cell Transplant Predicts the Incidence of Acute and Chronic GVHD

Reena Rajasekar,[1] *Vikram Mathews,*[1] *Kavitha M. Lakshmi,*[1] *Shanmugaapriya Sellathamby,*[1] *Biju George,*[1] *Auro Viswabandya,*[1] *Dolly Daniel,*[2] *Mammen Chandy,*[1] *Alok Srivastava*[1]

[1] Department of Hematology, Christian Medical College, Vellore, India; and [2] Department of Clinical Pathology and Blood Bank

Correspondence and reprint requests: Vikram Mathews, MD, Department of Haematology, Christian Medical College and Hospital, Vellore 632004, India (e-mail: vikram@cmcvellore.ac.in).

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ABSTRACT

Dendritic cells (DC) are antigen-presenting cells involved in induction and regulation of immune responses. We investigated the impact of the number of infused and day 28 dendritic cells on the development of acute and chronic GVHD (aGVHD, cGVHD). Monocytoid (MC) and plasmacytoid (PC) dendritic cells were characterized as lin$^-$HLA-DR$^+$CD11c$^+$ and lin$^-$HLA-DR$^+$CD123$^+$, respectively. Sixty-eight consecutive patients who underwent HLA matched related granuloyte-colony stimulating factor (G-CSF) mobilized allogeneic PBSCT, from February 2005 to May 2006, were included in the analysis. Twenty-three patients developed aGVHD (grade II-IV) and 21 patients had cGVHD. On a univariate analysis the day 28 total DC and the day 28 MC and PC dendritic cells as continuous variables were significantly associated with development of aGVHD and cGVHD. Using an ROC plot analysis a cutoff value for total DC = 10.7/μL, MC = 9.7/μL, and PC = 4.5/μL on day 28 gave the highest likelihood ratios for aGVHD (2.7, 2.14, and 3.29, respectively). On a multivariate analysis, a low day 28 PC ($\leq$4.5/μL) together with patient age retained their risk for aGVHD (hazard ratio [HR] = 65.1 and 1.0, *P*-values .000 and .036, respectively), whereas for cGVHD only a low day 28 PC remained significant (HR = 11.8, *P* = .008). These results suggest that the PC dendritic cell count in the peripheral blood on day 28 is a strong predictor for development of GVHD in recipients of an allogeneic matched related PBSCT.

KEY WORDS

Acute GVHD • Chronic GVHD • Plasmacytoid dendritic cell count • GVHD prediction • PBSCT

INTRODUCTION

Allogeneic stem cell transplantation (SCT) remains the best therapeutic option for a number of malignant and nonmalignant hematologic diseases. Significant obstacles limiting the efficacy of allogeneic SCT are the occurrence of regimen-related toxicity (RRT), graft-versus-host disease (GVHD), tumor relapse, engraftment failure, and susceptibility of patients to opportunistic infections after transplantation [1-4]. Development of both acute and chronic GVHD (aGVHD, cGVHD) is a major limitation of peripheral blood SCT (PBSCT), contributing significantly to morbidity and mortality [5,6]. There are limited robust predictors of GVHD after a matched related allogeneic PBSCT.

After SCT, immune response is determined early by both mature immunocompetent cells transferred within the allograft and by immune cells that develop de novo from transplanted stem cells. Different cell lineages reconstitute at different rates, and this could have an impact on clinical outcomes post transplantation [7]. Dendritic cells (DCs) are antigen-presenting cells involved in induction and regulation of immune responses. DC precursors in blood constitute <1%

of mononuclear cells [8]. According to their potential ability to induce naïve T cell differentiation to Th1 and Th2 effector cells, 2 distinct lineages of DCs have been identified, namely, monocytoid (MC) and plasmacytoid (PC) DCs, which do not express lineage markers (CD3, CD14, CD16, CD19, CD20, and CD56) but are positive for HLA-DR and either CD11c or CD123 (IL-3 receptor α-chain), respectively [9,10].

Despite a 10-fold higher dose of transplanted T cells in PBSC recipients compared to marrow recipients, aGVHD does not develop in a significantly higher proportion among PBSC recipients as one would have anticipated based on the T cell dose. This is attributed to G-CSF selectively mobilizing PC DCs and the ability of activated PC DCs to prime T lymphocytes to produce Th2 cytokines that has been hypothesized to favor immunotolerance [11,12].

We undertook a prospective analysis to study the impact of the number of infused and day 28 total DC and subsets, namely MC (monocytoid, lin-HLA-DR$^+$CD11c$^+$) and PC (plasmacytoid, lin$^-$HLA$^-$DR$^+$CD123$^+$) DCs, on the development of aGVHD and cGVHD.

PATIENTS, MATERIALS, AND METHODS

All patients who underwent an allogeneic HLA matched related PBSCT at our center, between February 2005 and May 2006, were included in this study. A written informed consent was obtained from all patients, and in the case of minors, from their parents or legal guardians. Patients were nursed in rooms with a positive-pressure HEPA filtered air.

Conditioning

Thirty-four (50%) patients were conditioned using a conventional myeloablative regimen consisting of busulphan (16 mg/kg) and cyclophosphamide (120 mg/kg). The rest received a reduced-intensity conditioning (RIC) regimen; in the majority this was fludarabine based (Table 1).

Graft Source and Engraftment

Granulocyte-colony stimulating factor (G-CSF) stimulated PBSCs were collected from the donor with a target cell dose of 5×10^8 MNC/kg body weight of the recipient. Neutrophil engraftment was defined as the first of 3 consecutive days with ANC $>0.5 \times 10^9$/Lt, whereas platelet engraftment was defined as the first of 3 consecutive untransfused platelet counts $>20 \times 10^9$/Lt. Samples of PBSC harvests and post-transplant day 28 peripheral blood were obtained in sodium heparin vacutainer tubes for flowcytometry analysis.

Table 1. *Baseline Characteristics*

Characteristics	Value n (%)/median (range)
Patients	—
Males	43 (63.2)
Age (years)	27 (3-55)
Donors	—
Males	36 (52.9)
Age (years)	29 (9-63)
Diagnosis	—
Acute lymphoblastic leukemia	6 (8.8)
Acute myeloid leukemia	31 (45.6)
Acute undifferentiated leukemia	1 (1.5)
Aplastic anemia	12 (17.6)
Biphenotypic acute leukemia	2 (2.9)
Chronic myeloid leukemia	5 (7.4)
Dyskeratosis congenita	1 (1.5)
Myelodysplastic syndrome	6 (8.8)
Myelofibrosis	2 (2.9)
Paroxysmal nocturnal hemoglobinuria	1 (1.5)
β-Thalassemia major	1 (1.5)
Conditioning regimen	—
Myeloablative	34 (50)
Bu/Cy	20 (29.4)
Cy/TBI	14 (20.6)
Reduced intensity	34 (50)
Flu/Mel	12 (17.6)
Flu/Cy	15 (21.1)
Flu/Cy/ATG	3 (4.4)
Flu/Bu/Cy	1 (1.5)
Flu	1 (1.5)
Ida/Flu/Cytosine	2 (2.9)

ATG indicates antithymocyte globulin; Bu, Busulfan; Cy, Cyclophosphamide; Flu, Fludarabine; Mel, Melphalan; TBI, total-body irradiation.

GVHD Prophylaxis

Cyclosporine and short methotrexate was used as GVHD prophylaxis. Cyclosporine was administered at a dose of 2.5 mg/kg intravenously over 4 hours twice daily starting on day −4, and changed to oral administration at 5 mg/kg twice daily when mucositis had resolved. Cyclosporine levels were monitored and the dose adjusted to achieve a target level of 100-300 ng/mL. The methotrexate dose was 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3, 6, and 11 (in 5 cases the day 1 dose was 15 mg/m^2 and the day 3, 6, and 11 doses were 10 mg/m^2). If mucositis was severe (grade IV) or bilirubin >20 mg/L, the day 6 and day 11 doses were omitted. Acute GVHD was treated with dexamethasone or methyl prednisone. Steroid refractory GVHD was treated as per the discretion of the treating physician. Patients who died before day 28 were excluded from DC analysis.

Definition of Outcomes

Incidence and severity of GVHD was defined as per established criteria [13]. Overall survival (OS)

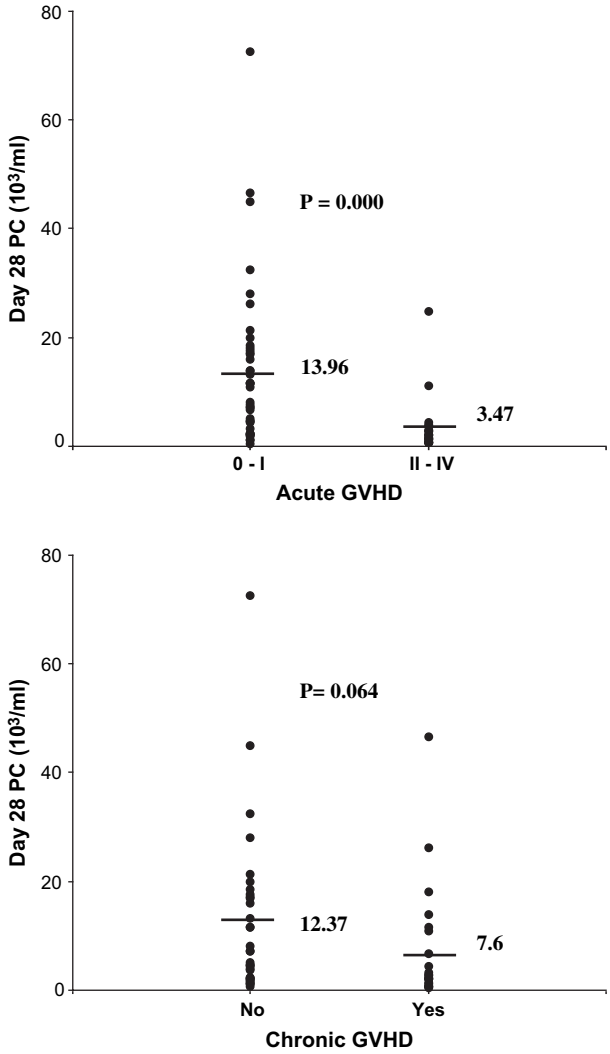
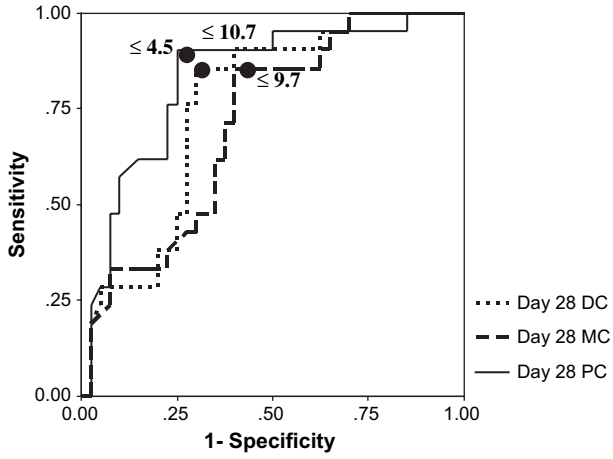


Figure 1. Mean day-28 PC counts and GVHD.



ROC Cutoff	Sensitivity	Specificity	AUC
Day 28-DC ≤ 10.7 / ul	81	70	0.759
Day 28-MC ≤ 9.7 / ul	86	60	0.712
Day 28-PC ≤ 4.5 / ul	91	73	0.830

Figure 2. Predictive sensitivity and specificity testing of day 28 DC, day 28 MC, and day 28 PC cell count for aGVHD using an ROC plot.

was defined as time from initiating treatment to last follow-up or death. Event-free survival was calculated from time of initiating therapy to last follow-up or an event (relapse or death).

DCs Enumeration

DC were enumerated using FITC-conjugated monoclonal antibodies directed against leukocyte lineage markers (Lin: anti-CD3, CD14, CD16, CD19, CD20, CD56, BD Biosciences, San Jose, CA), PE-conjugated anti-CD11c (BD Biosciences), PE-conjugated anti-CD123 (BD Biosciences), and PerCP-conjugated anti-HLA-DR (BD Biosciences). The whole blood was incubated with appropriate antibody combinations (MC = Lin, HLA-DR, CD11c, PC = Lin, HLA-DR, CD123) for 15 minutes at room temperature in the dark followed by red cell lysis with ammonium chloride. The cells were then washed twice in phosphate-buffered saline. Events were then acquired on a FACSCalibur flowcytometer (Becton Dickenson, Mansfield, MA. Data analysis was performed using CellQuestPro software (Becton Dickenson). Dead cells were gated out before the final analysis using side-scatter/forward-scatter dot-plots. A minimum of 75,000 events were acquired per analysis. Previously described method for identification and enumeration of DC subsets was used [14]. Cells negative for lineage markers (Lin^-) were gated and then analyzed for HLA-DR and CD11c for MC and CD123 expression for PC. An absolute DC value (μL) was determined by multiplying the percentage of MC and PC DCs with the total number of white blood cells.

Apart from DCs, graft and posttransplant day 28 peripheral blood samples were analyzed for CD34, CD133, CD3, CD4, CD4CD45RA, CD4CD45RO, CD8, CD8CD45RA, CD8CD45RO, CD19, and NK cell ($CD16^+$, $CD56^+$, $CD3^-$) counts.

Statistical Analysis

Statistical analyses were performed with SPSS for Windows 11.01 version (SPSS Inc., Chicago, IL). χ^2 and Mann-Whitney *U*-tests was used for parametric and nonparametric data, respectively. A receiver operating characteristic (ROC) curve was used to designate the cutoff level for total DC (sum of MC and PC), MC and PC DC count. The effects of high versus low day 28 DC, MC, and PC count on outcomes were tested using Kaplan-Meier methods. Log-rank tests were used to

Table 2. *Comparison of Low and High Day 28 PC# Patient Groups*

Characteristics n (%)/median (range)	Low PC (≤4.5/μL) (n = 30)	High PC (>4.5/μL) (n = 31)	*P*-Value
Patient	—	—	—
Males	20 (66.7)	18 (58.1)	.600
Age (years)	29 (5- 53)	25 (3-55)	.201
Female donor to male recipient	10 (33.3)	11 (35.5)	1.000
Conditioning regimen	—	—	—
Myeloablative	10 (33.3)	22 (71)	.005
Graft (×10⁶/kg)	—	—	—
CD34	12.5 (3.2-74.2)	12.2 (2.3-44.4)	.604
CD133	10.1 (3.9-53)	10.1 (2-42.5)	.660
CD3	189 (61.1-403)	166.4 (47.1-515.7)	.267
CD19	11.5 (3.4-293.1)	21.8 (1.6-248.5)	.141
CD4	116.6 (35.9-256.6)	92.2 (17.9-272.1)	.162
CD4CD45RA	43 (10.7-139.3)	31.1 (4.7-132.1)	.248
CD4CD45RO	46.3 (18.2-120)	46.4 (12.2-196.3)	.624
CD8	79.8 (22.5-212.9)	80.2 (15.6-354.5)	.697
CD8CD45RA	45 (11.3-130)	47.2 (12.7-175.3)	.593
CD8CD45RO	26.2 (8.4-85.1)	24 (5.2-189.5)	.498
$CD16^+CD56^+CD3^-$	17.7 (2.6-90)	17.1 (4.7-74)	.778
MC	0.8 (0.1-2.6)	0.9 (0.2-3.3)	.341
PC	2 (0.1-9.6)	2.3 (0.4-14.8)	.530
Engraftment (days)	–	—	—
ANC >500/mm³	11.5 (9-18)	14 (10-18)	.013
Platelet >20,000/mm³	15 (9-160)	11 (8-39)	.004
Day-28 subsets (×10⁶/Lt)	—	—	—
CD3	610.4 (11.9-15116.3)	750.4 (297.2-2789.8)	.248
CD19	36.3 (1-449.3)	19.8 (1-302)	.109
CD4	314.5 (6.7-1670.9)	371.6 (161.6-1926.6)	.175
CD4CD45RA	110.1 (3-904.2)	120.3 (35.8-509.7)	.267
CD4CD45RO	162.8 (5.7-708.9)	216.6 (82-872.1)	.106
CD8	270.7 (7-13226.8)	340.7 (91.1-1663.1)	.111
CD8CD45RA	132 (3-1774)	231.9 (40.2-876.4)	.017
CD8CD45RO	111.3 (2.1-6308.9)	131.2 (32.2-923.3)	.273
$CD16^+CD56^+CD3^-$	173.5 (24.1-704.8)	230.3 (90.4-542.1)	.028
MC	2.4 (0.5-16.9)	16.8 (2.8-151.8)	.000
PC	2.1 (0.5-4.4)	14 (4.6-72.6)	.000
Acute GVHD (grade (II-IV))	19 (63.3)	2 (6.5)	.000
Chronic GVHD	14 (51.9)	7 (25.9)	.093
Survival	—	—	—
Relapse	1 (3.3)	7 (22.6)	.053
Death	6 (20)	6 (19)	1.000

MC indicates monocytoid dendritic cell; PC, plasmacytoid dendritic cell.

measure significant differences between strata. Cox models were used to assess the proportional hazards of various subsets both in the graft and those engrafted after transplant. To confirm outcomes and to adjust for potential confounding factors, multivariate Cox proportional hazards models were also done. A probability type 1 error <.05, was considered the threshold of statistical significance.

Table 3. *Multivariate Relative Risk (RR) Analysis for GVHD*

	Acute GVHD		Chronic GVHD	
Variable	**RR (95% CI)**	***P*-value**	**RR (95% CI)**	***P*-value**
Patient age	1.0 (1.0-1.09)	.036	1.0 (0.98-1.06)	.457
Female-to-male transplant	0.4 (0.14-1.45)	.179	1.7 (0.68-4.52)	.249
Myeloablative Conditioning	2.3 (0.85-6.03)	.101	1.9 (0.70-5.36)	.205
Day 28 NK cell count	1.0 (1.0-1.0)	.415	1.0 (1.0-1.0)	.627
Day 28-DC low <10.7/μL	0.1 (0.01-1.52)	.105	0.1 (0.02-0.69)	.020
Day 28-MC low <9.7/μL	2.1 (0.15-31.27)	.570	3.1 (0.61-16.04)	.172
Day 28-PC low <4.5/μL	65.1 (7.34-577.06)	.000	11.8 (1.89-73.41)	.008

DC, Total dendritic cell; MC, monocytoid dendritic cell; PC, plasmacytoid dendritic cell; CI, confidence interval.

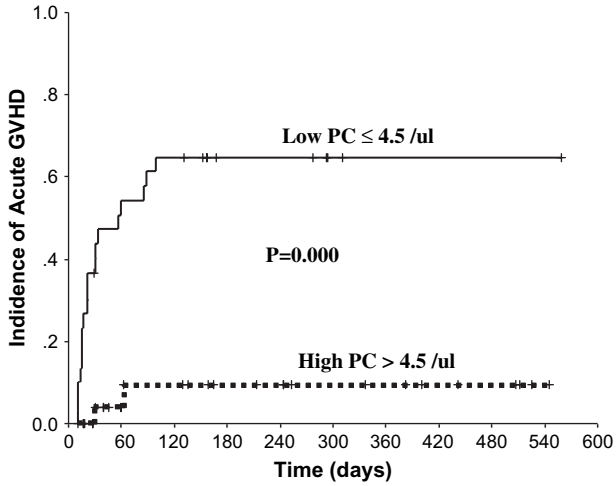


Figure 3. Cumulative incidence of aGVHD by day 28 PC.

RESULTS

Patient and Graft Characteristics

There were 68 patients (43 men and 25 women) with a median age 27 years (range: 3-55). The mean follow-up was 405 days. Patient characteristics are summarized in Table 1.

DC Count and GVHD

Twenty-three patients developed aGVHD (grade II-IV) and 21 patients had cGVHD. Seven patients died before day 28 and were excluded from the analysis; 2 of these patients had aGVHD. The mean absolute day 28 PC DC counts in patients with acute grade (II-IV) and cGVHD was significantly lower compared to those who did not develop GVHD (P = .000 and P = .064, respectively) (Figure 1). When analyzed as continuous variable the day 28 total DC, MC, and PC DC counts were significantly associated with development of aGVHD, whereas graft total DC, MC, and PC DC counts did not show a similar association.

Using ROC curve analysis, cutoff values (day 28 total DC = 10.7/μL, MC = 9.7/μL, and PC = 4.5/μL), which gave the highest likelihood ratios for aGVHD (2.7, 2.14, and 3.29, respectively), was determined (Figure 2). The area under the curve for PC (0.830) was higher when compared to total DC (0.759) and MC (0.712). These cutoff values significantly discriminated patient's probability of developing aGVHD and cGVHD on a univariate analysis.

Using the PC DC count cutoff value of 4.5/μL, patients were categorized into a high (>4.5/μL) (n = 31) and a low (≤4.5/μL) (n = 30) day 28 PC level groups. These 2 groups were comparable with regard to age, sex, female-to-male transplants, and graft characteristics (CD34, CD133, CD3, CD4, CD4CD45RA, CD4CD45RO, CD8, CD8CD45RA, CD8CD45RO, CD19, NK, MC, and PC cell dose) as shown in Table 2. However, significantly more cases in the day 28 high PC group received a myeloablative conditioning regimen. Twelve of 61 of our patients received steroids before day 28 for treatment of GVHD. We excluded these patients to see the effect of steroids on DCs. Low day 28 PC (≤4.5 cells/μL) still retained its significance for development of aGVHD (relative risk [RR] = 5.1 (95% confidence interval [CI] 1.06-24.50), P = .042).

On a multivariate analysis, a low day 28 PC (≤4.5/μL), together with patient age, retained their risk for aGVHD (HR = 65.1 and 1.0, P-values .000 and .036, respectively), whereas for cGVHD only a low day 28 PC remained significant (HR = 11.8, P = .008) (Table 3). Cumulative incidence of aGVHD and cGVHD compared by log-rank test revealed that the low PC group had a significant risk (P = .000 and .006, respectively) of developing aGVHD and cGVHD (Figures 3 and 4).

These results suggest that the day 28 PC count in the peripheral blood is a strong predictor for development of aGVHD and cGVHD in recipients of matched related allogeneic PBSCT.

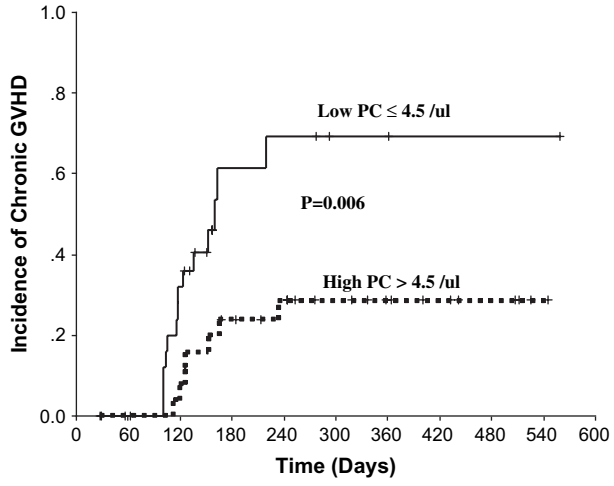


Figure 4. Cumulative incidence of cGVHD by day 28 PC.

DISCUSSION

We hypothesized that graft DC count and early postengraftment DC counts would have an important impact on development of aGVHD and cGVHD, given the central role for DC in the immune response. DC process and present both endogenously and exogenously derived antigens. Therefore, in the setting of a major histocompatibility complex matched allogeneic SCT procedure, DC arising from the donor graft and DC persisting from recipients are expected to present endogenous minor donor antigen peptides that will influence T cell activation and/or induction

of tolerance [15]. Recently, Merad et al. [16] have shown the central role of host DC in recipient antigen presentation to donor T cells that could potentially induce GVHD. Recipient DC are radioresistant and can survive pretransplant conditioning regimens that target cycling or proliferating cells. Persistent host DC can function as initiators, as well as be targets of GVHD reactivity against minor antigens [17]. It has been demonstrated that both residual host and engrafting donor APCs contribute to development of GVHD [16,18]. Our chimerism analysis (peripheral blood MNC by VNTR analysis) done routinely for all transplants on day 28 showed a complete chimera in 90.6% (48 of 53 cases). Although we did not perform a DC subset chimerism on the day 28 samples, from the chimerism data it is likely that the measured PC DCs are donor in origin. It has also been noted that both in myeloablative and RIC the majority of the circulating DCs are of donor origin even in the presence of mixed chimerism in other lineages, including T cells [19,20].

Acute GVHD is usually first treated with corticosteroids. Earlier studies have shown that use of corticosteroids leads to decreased blood DC in various human disorders, including GVHD [14,18,21]. Nine of the 21 patients who developed aGVHD did not receive steroids before day 28 for treatment. Their day 28 PC count was significantly lower than the group that did not develop GVHD, although not significantly different from the group that received steroids (data not shown). Even after excluding patients who received steroids prior to day 28 (12 patients), a low day 28 PC count retained its statistically significant association with the development of aGVHD.

Our categorization of low and high PC patient groups were comparable with regard to recipient age, sex, graft characteristics (CD34, CD133,CD3, CD4, CD4CD45RA, CD4CD45RO, CD8, CD8CD45RA, CD8CD45RO, CD19, NK, MC, and PC cell dose) and engrafted T and B cells (Table 2). The groups were significantly different in the numbers that received a myeloablative conditioning regimen and in the number of NK cells in the peripheral blood on day 28. However, neither of these parameters was significantly associated with GVHD in a univariate analysis and adjusting for these factors in a multivariate analysis, the day 28 PC count still retained its statistically significant association with GVHD. DC cutoff values obtained in this study were comparable to that stated previously [22]. Identifying potential causes of low peripheral blood PC counts after SCT among those who develop a GVHD and cGVHD needs further investigation. It has been suggested that PC might have homed to lymphoid tissues or target organs of GVHD because of allogeneic stimuli or alternatively with the onset of GVHD there is conversion of PC to MC DCs [16].

Waller et al. [23] suggested that donor DC content in the bone marrow graft before transplantation had an impact on clinical outcomes after bone marrow transplantation. In our analysis we were unable to show a similar association with graft DC count. Our observations were consistent with data previously reported by Reddy et al. [22], which suggested that in patients under going a PBSCT the number of DC in the graft has no impact, whereas the number of DC that reconstituted in the recipient early after a transplant appeared to have an impact on the incidence of GVHD and the clinical outcome post transplant.

Recently it was reported that activated circulating DC after a hematopoietic SCT predicts aGVHD and a DC activation marker CMRF-44 was found to be most informative in predicting onset of GVHD [24]. However, in this study as well it was noted that patients with aGVHD (grade II-IV) had a significantly lower level of MC and PC DC count compared to those that did not develop aGVHD [24].

Our data suggests that early post engraftment circulating PC DC count after an allogeneic hematopoietic SCT is an independent predictor for development of aGVHD and cGVHD. Enumerating absolute PC DC count as reported in our study may be a simple, fast, and reproducible method to predict development of aGVHD and cGVHD after PBSCT. Based on the day 28 PC DC counts, it would potentially be possible to stratify patients into risk groups for developing GVHD and further stratify them for therapeutic interventions to enhance or negate the impact of GVHD.

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