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Biol Blood Marrow Transplant xxx (2015) 1–6

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

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Article history:
Received 8 February 2015
Accepted 1 April 2015

Key Words:
Plasmacytoid dendritic cell
Allogeneic transplantation
Outcome
GVHD

A B S T R A C T

The rapidly increasing use of allogeneic stem cell transplantation (allo-SCT) emphasizes the need for identifying variables predictive of its outcome. Plasmacytoid dendritic cells (pDCs) play a major role in establishing immune competence and in several autoimmune diseases. Thus, we investigated whether pDCs might influence the outcome of patients after allo-SCT in 79 consecutive patients who underwent this procedure. pDCs were identified in the blood of patients at day 100 after allo-SCT by staining peripheral blood mononuclear cells for surface markers and intracellular cytokines and analyzing them on a flow cytometer. We found the pDC level at day 100 was not influenced by patient or graft characteristics, and only the absence of previous grades II to IV acute graft-versus-host disease was significantly associated with higher levels of blood pDCs after allo-SCT (OR, .67; 95% CI, .54 to .83; $P = .0004$). Using the median value of pDCs at day 100 to divide the patients into 2 distinct groups, we observed that a low pDC level was correlated with a worse overall survival (55% versus 86%, $P = .007$). In a multivariate analysis, only low pDC level (OR, 3.41; 95% CI, 1.19 to 9.79; $P = .02$) and older patient age (OR, 5.16; 95% CI, 1.15 to 23.14; $P = .03$) were significantly predictive of increased risk of death. We conclude that monitoring of pDC may be useful for patient management and may have a significant impact on the probability of a favorable outcome of allo-SCT.

INTRODUCTION

Allogeneic stem cell transplantation (allo-SCT) has evolved into a curative therapy for a variety of hematological and nonhematological malignancies. In the treatment of the former, high-intensity conditioning therapy before allo-SCT eradicates malignant cells, and the infusion of donor stem cells enables reconstitution of the recipient's hematopoietic system and also triggers a graft-versus-leukemia (GVL) effect. However, the main limitation of treating a broader spectrum of diseases and patients with allo-SCT is graft-versus-host disease (GVHD), a reaction closely associated to GVL.

Both GVHD and GVL occur when donor T lymphocytes respond to genetically defined proteins on host cells. Dendritic cells (DCs) are key antigen-presenting cells that present host antigens to donor T lymphocytes. Among DC subsets, plasmacytoid DCs (pDCs) have been proposed to play a major role in induction and regulation of immune responses. pDC activated through Toll-like receptors (TLRs) rapidly secrete massive amounts of IFN-α and induce immune responses through activation of effector T lymphocytes [1]. On the contrary, unstimulated or alternatively stimulated pDCs can alleviate protective immunogenic responses through the induction of T regulatory lymphocytes [2,3]. Previous studies established the role of pDCs in the pathophysiology of several autoimmune diseases [4-6]. The influence of these cell populations in GVHD and GVL is, however, controversial [7-10] and probably depends on their activation state, distribution, and migration patterns.

The rapidly increasing use of allo-SCT emphasizes the need for clinical research aimed at identifying variables predictive of outcome of patients. Therefore, we investigated the impact of circulating pDCs measured at 100 days after allo-SCT in 79 patients to evaluate the role of these cells in GVHD and GVL and their impact on long-term outcome.

Financial disclosure: See Acknowledgments on page 5.

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http://dx.doi.org/10.1016/j.bbmt.2015.04.003
1083-8791/

METHODS

Study Design

Seventy-nine consecutive patients treated with allo-SCT in a single center (Centre Hospitalier Universitaire de Nantes) with a minimal follow-up of 6 months were included in this analysis. Written informed consent was obtained from each patient and donor. The studies were approved by the local ethics committee and performed according to institutional guidelines. Patient, donor, and graft characteristics are summarized in Table 1.

Transplant Procedures

Most patients (89%) were treated with reduced-intensity conditioning allo-SCT. The reduced-intensity conditioning regimen was mainly based on 30 mg/m^2 fludarabine daily over 4 to 6 days, 3.2 mg/kg i.v. busulfan daily over 2 to 3 days, and 5 mg/kg antithymocyte globulin (Thymoglobulin; Genzyme, Lyon, France) infused over 2 days [11]. GVHD prophylaxis was mostly carried out with cyclosporine A alone or with cyclosporine A and mycophenolate mofetil. Most patients received a peripheral blood stem cell allograft mobilized with granulocyte colony-stimulating factor in a dose of 10 mg/kg/day for 5 days.

pDC Analysis

All blood samples were collected in EDTA tubes at day 100 after allo-SCT. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and then cryopreserved in .5-mL aliquots at −80°C.

At the time of analysis, PBMCs were thawed and stimulated for 6 hours with TLR7 ligands (5 μg/mL R848; Invivogen, Toulouse, France) and TLR9 ligands (10 μg/mL CpGA2216; Invivogen), in the presence of 10 ng/mL IL3 (Peprotech, Tebu-bio, Le Perray-en-Yvelines, France), cytokine important for pDC survival. Two hours after the beginning of stimulation, 10 μg/mL Brefeldin A (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to stop the extracellular secretion of cytokines. For surface markers, cells were stained according to manufacturer's protocol with conjugated antibodies Pacific Blue HLA-DR and Pe-Cy7 CD123 (both from Biolegend, Ozyme, Saint-Quentin en Yvelines, France) and APC BDCA2 (Miltenyi, Paris, France). Cells were fixed and permeabilized using Cytofix/Cytoperm reagents (BD Biosciences, Le Pont de Claix, France) and then incubated with PE IFN-α (Miltenyi), FITC IL-6 (Biolegend), and PerCp-Cy5.5 tumor necrosis factor (TNF)-α (Biolegend). Finally, cells were analyzed on a FACSCanto II using DIVA software (BD Biosciences).

Clinical Outcomes and Statistical Methods

Acute GVHD was evaluated according to standard Seattle criteria [12]. Upon diagnosis of grades II to IV acute GVHD, all patients were primarily treated with cyclosporine A and 2 mg/kg/d methylprednisolone. Acute and chronic GVHD were arbitrarily separated by day 100 after allo-SCT. Chronic GVHD was clinically evaluated as limited or extensive. When the latter was diagnosed, patients were treated with 1 mg/kg/d methylprednisolone [13].

Data were computed using the R package (R Foundation for Statistical Computing, http://www.R-project.org) The Mann-Whitney test was used for comparison of continuous variables. Categorical variables were compared by the chi-square or Fisher's exact test. The probability of developing acute and chronic GVHD was calculated by the cumulative incidence treating death as a competitive risk [14]. Cumulative incidence estimates were also used to measure relapse. Probabilities of overall survival (OS) were assessed from the time of transplantation using the Kaplan-Meier product-limit estimates. Differences between groups were tested using the log-rank test. A multiple logistic regression with backward stepwise model selection was used to uncover predictive factors for cell recovery at 100 days after allo-SCT. The association of OS with cell counts and other relevant variables was evaluated in a multivariate analysis using Cox's proportional-hazard regression model.

RESULTS

Transplant related events and outcomes from our study are summarized in Table 2. The OS at 2 years was 74% (95% confidence interval [CI], 64% to 86%), with a median follow up of 592 days (range, 225 to 873 days) for living patients. The

Table 1
Baseline Demographic Characteristics of Low pDC vs. High pDC Group and the Whole Study Population

Characteristic	Low pDC (n = 32)	High pDC (n = 47)	All Patients (n = 79)	*P*
Patient age, median (range)	54 (26-71)	54 (27-69)	54 (26-71)	.65
Patient gender				.65
Male	18 (55)	24 (51)	42 (53)	
Female	14 (45)	23 (49)	37 (47)	
Diagnosis*				.10
Myeloid malignancy	13 (41)	29 (62)	42 (53)	
Lymphoid malignancy	18 (56)	17 (36)	35 (44)	
Aplastic anemia	1 (3)	1 (2)	2 (3)	
Disease risk†				.98
Standard risk	13 (41)	19 (40)	32 (41)	
High risk	19 (59)	28 (60)	47 (59)	
Conditioning regimen				.64
Myeloablative	3 (9)	6 (13)	9 (11)	
RIC	29 (91)	41 (87)	70 (89)	
GVHD prophylaxis				.22
CsA alone	8 (25)	18 (38)	26 (33)	
CsA and MMF/MTX	24 (75)	29 (62)	53 (67)	
Donor gender				.18
Male	17 (53)	32 (68)	49 (62)	
Female	15 (47)	15 (32)	30 (38)	
CMV seronegative pair				.87
Yes	9 (28)	14 (30)	23 (29)	
No	23 (72)	33 (70)	56 (71)	
Donor type				.06
Matched related donor	10 (31)	27 (57)	37 (46)	
Matched unrelated donor	12 (38)	13 (28)	25 (32)	
Mismatched unrelated donor	10 (31)	7 (15)	17 (22)	
Stem cell source				.52
Bone marrow	5 (16)	5 (11)	10 (12)	
Peripheral blood	20 (63)	35 (74)	55 (70)	
Cord blood	7 (21)	7 (15)	14 (18)	
Median cells infused, ×10^6/kg (range)	6 (.06-10.10)	4.98 (.07-10.00)	5.25 (.06-10.10)	.17

RIC indicates reduced-intensity conditioning; CsA, cyclosporine A; MMF, mycophenolate mofetil; MTX, methotrexate; CMV, cytomegalovirus.
Values are number of cases with percents in parentheses, unless otherwise noted.

* Myeloid malignancies in all patients included 28 acute myeloid leukemias, 10 myelodysplastic syndromes, 3 myeloproliferative syndromes, and 2 chronic myeloid leukemias. Lymphoid malignancies included 13 non-Hodgkin lymphomas, 9 acute lymphoblastic leukemias, 7 multiple myelomas, 3 chronic lymphocytic leukemias, and 2 Hodgkin lymphomas.

† Standard risk disease: acute leukemia in first complete remission, chronic myeloid leukemia in chronic phase, and untreated disease; all others-high risk.

Table 2
Transplant-Related Events and Outcome of Low pDC vs. High pDC Groups

Characteristic	Low pDC (n = 32)	High pDC (n = 47)	All Patients (n = 79)	*P*
Neutrophil recovery				
Median ANC > .5 × 10^9/L (range)	17 (11-32)	16 (8-43)	16 (8-43)	.28
Acute GVHD				<.0001
Grades 0-I	13 (41)	39 (83)	52 (65)	
Grades II-IV	19 (59)	8 (17)	27 (35)	
Median acute GVHD onset after transplantation, days (range)	29 (13-97)	32 (8-91)	30 (8-97)	.56
CMV antigenemia before 100 days after allo-SCT				.09
No	24 (75)	42 (89)	66 (84)	
Yes	8 (25)	5 (11)	13 (16)	
Median blood cell counts/μL at day +100 (range)				
Leukocytes	4900 (1700-16,400)	4300 (1000-13,100)	4650 (1000-16,400)	.18
Granulocytes	3200 (800-14,600)	2400 (60-8300)	2570 (60-14,600)	.11
Lymphocytes	800 (100-5200)	1000 (120-2900)	940 (100-5200)	.18
Monocytes	400 (20-1600)	500 (20-1500)	500 (20-1600)	.64
$CD4^+$	90 (1-650)	130 (20-980)	114 (1-980)	.13
$CD8^+$	140 (20-2700)	310 (20-2400)	266 (20-2700)	.24
Chronic GVHD				.26
No or limited	20 (63)	35 (74)	55 (70)	
Extensive	12 (37)	12 (26)	24 (30)	
Median chronic GVHD onset after transplantation, days (range)	127 (100-546)	137 (101-379)	134 (100-546)	.49
Median follow up for surviving patients, days (range)	638 (225-825)	626 (384-873)	592 (225-873)	.72

ANC indicates absolute neutrophil count.
Values are number of cases with percents in parentheses, unless otherwise noted.

median blood pDC proportion at day 100 after allo-SCT after activation with TLR ligands was .2% of PBMCs. We used this value to allocate the patients to a "low pDC" (<.2% of PBMCs) or "high pDC" (≥.2% PBMCs) recovery group. Baseline demographic and transplant characteristic of these 2 groups were comparable and are shown in Table 1. Transplant-related events and patient outcomes of the low pDC and high pDC recovery groups were also similar (Table 2), except for the incidence of acute GVHD. Namely, clinically significant grades II to IV (moderate to severe) acute GVHD occurred in 27 cases (33%) at a median of 30 days after allo-SCT, and this accounted for 19 patients (59%) in the low pDC but only 8 patients (17%) in the high pDC recovery group ($P < .0001$).

Therefore, we built a multivariate logistic regression model for pDC recovery in the blood of patients at day 100 after Allo-SCT. All variables with $P < .20$ in the univariate analysis were included in the model (diagnosis, donor gender and type, cytomegalovirus antigenemia and acute GVHD). A backward stepwise selection was performed and yielded a model with 2 variables: matched unrelated donor (odds ratio, .84; 95% CI, .68 to 1.03; $P = .09$) and occurrence of grades II to IV acute GVHD (odds ratio, .67; 95% CI, .54 to .83; $P = .0004$). Finally, only the presence of clinically significant grades II to IV acute GVHD was significantly associated with an impaired pDC recovery at day 100 after allo-SCT.

Furthermore, we observed that the proportion of pDC producing IFN-α or TNF-α was significantly increased in patients with grades 0 to I acute GVHD in comparison with patients with clinically severe grades II to IV acute GVHD ($P = .002$ and $P = .0005$, respectively) (Figure 1A and B). In contrast, when patients treated with high doses of corticosteroids were excluded from the analysis, we did not observe any significant difference in the secretion of IFN-α and TNF-α from activated pDCs between patients with and without clinically severe GVHD.

The cumulative incidence of extensive chronic GVHD in our study group was 37% (95% CI, 25% to 49%) at 20 months, with this incidence higher in the low pDC (44%; 95% CI, 24% to 63%) than in the high pDC group (30%; 95% CI, 17% to 46%); however, this difference was not statistically

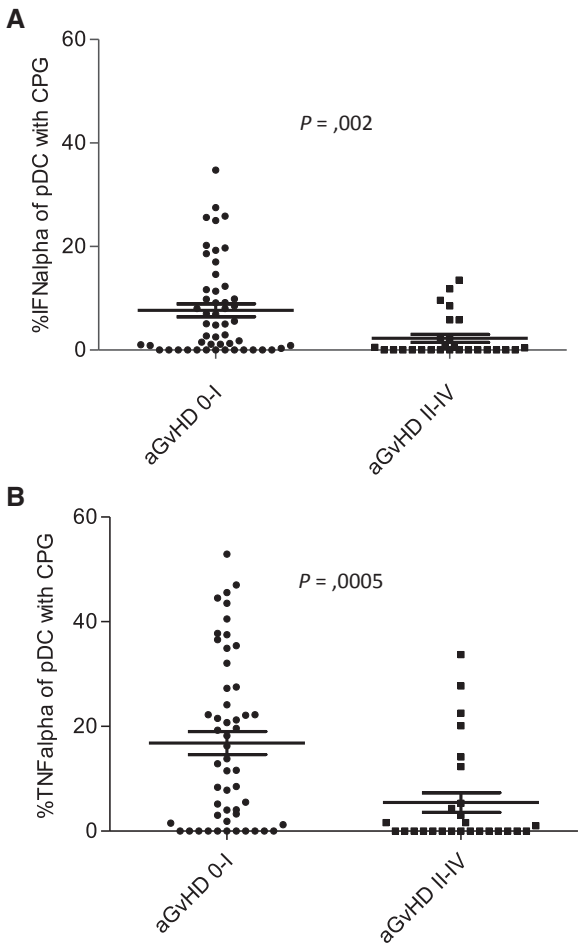

Figure 1. Detection of IFN-α and TNF-α producing pDCs after CpGA stimulation according to acute GVHD (aGVHD) grade. (A and B) Total PBMCs were stimulated with CpG2216, and intracellular IFN-α and TNF-α were analyzed by flow cytometry by gating on BDCA2 and CD123 positive cells. Results are indicated for each individual patient and mean ± SEM of the percentage of IFN-α (A) and TNF-α (B) positive cells is indicated by a bar.

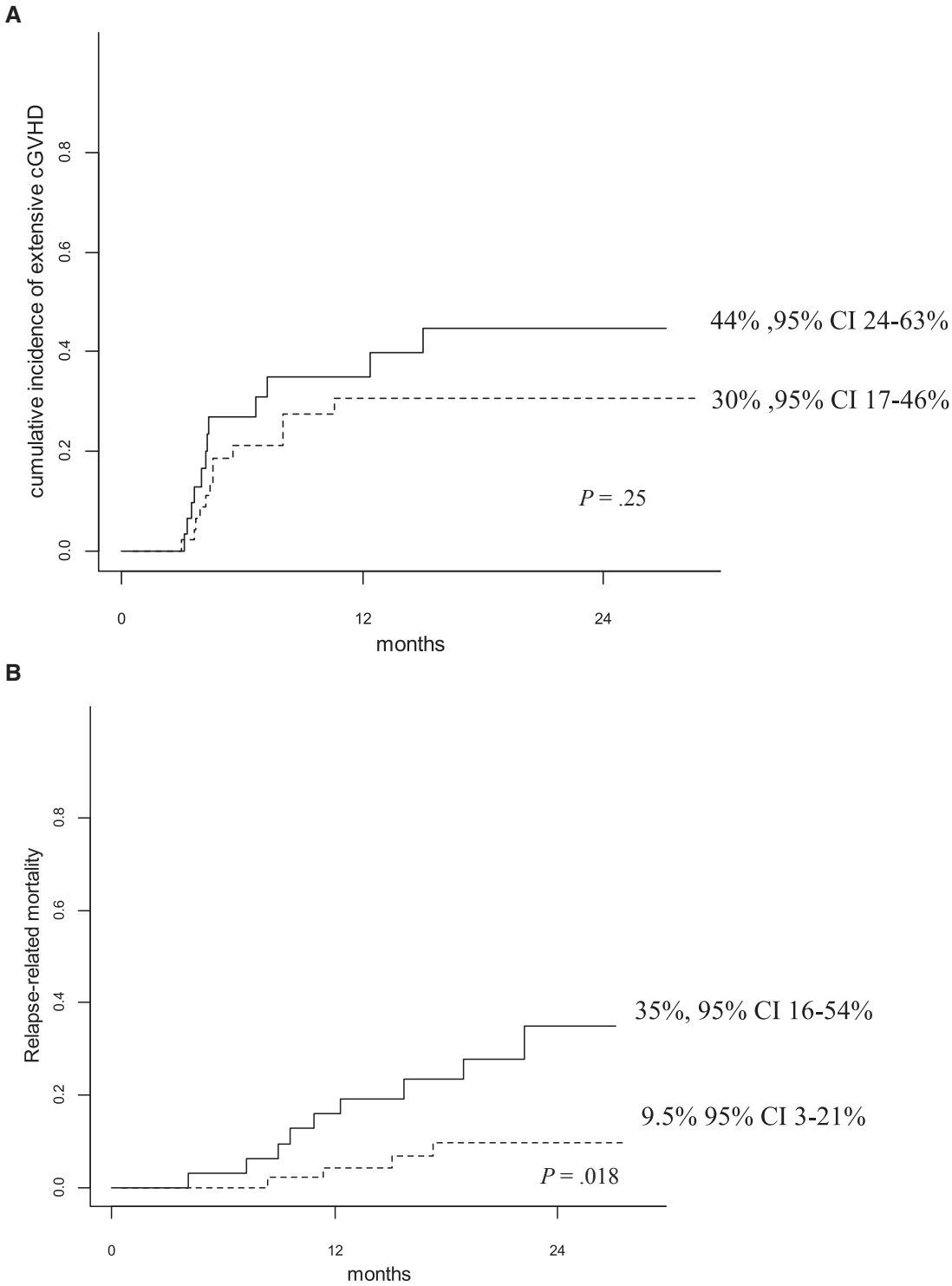

Figure 2. (A) Cumulative incidence of extensive chronic GVHD (cGVHD) at 20 months after allo-SCT according to the low pDC versus high pDC group. (B) Cumulative incidence of relapse-related mortality at 20 months after allo-SCT according to the low pDC versus high pDC group. pDC recovery groups determined at 100 days after allo-SCT. Solid lines indicate low pDC recovery group; dashed lines, high pDC recovery group.

significant ($P = .25$) (Figure 2A). Also, there was a higher number of deaths related to relapse in the low pDC group (35%; 95% CI, 16% to 54%) compared with the high pDC group (9.5%; 95%, CI 3% to 21%; $P = .0018$) (Figure 2B). Moreover, the high pDC group showed significantly better OS (86%; 95% CI, 76% to 97%) than those in the low pDC group (55%; 95% CI, 38% to 80%; $P = .007$) (Figure 3). Finally, a multivariate analysis was done to identify predictors of OS. This included all relevant variables from the univariate analysis with a $P < .20$ (donor, recipient age, pDC count) or previously known risk factors (risk of the disease, grades II to IV acute GVHD). In this analysis, both older age of recipient and low pDC proportion proved to be independent predictive factors of worse OS ($P = .02$ and $P = .03$, respectively) (Table 3).

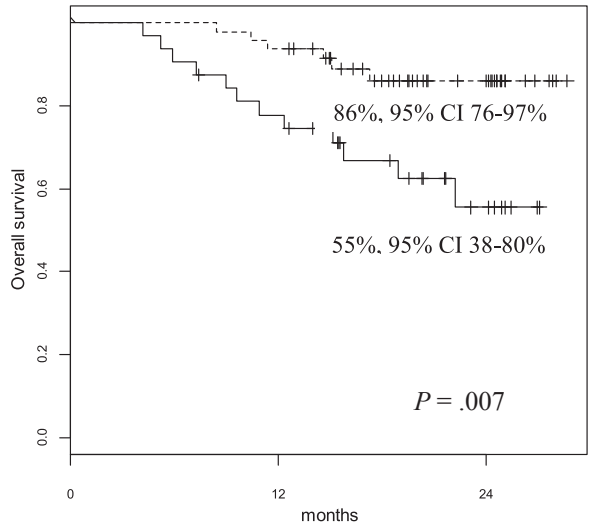

Figure 3. OS according to the low versus high pDC recovery groups determined at 100 days after allo-SCT. Solid lines indicate low pDC recovery group; dashed lines, high pDC recovery group.

DISCUSSION

It was previously shown that DCs recover quickly in the peripheral blood of patients after allo-SCT [9] and that the dynamics of their recovery depends on patient or graft characteristics, conditioning regimen, or infections [15]. However, in our group of patients, only the occurrence of grades II to IV acute GVHD independently impaired pDC recovery. Treatment with corticosteroids has also been shown to efficiently deplete blood pDCs in transplant recipients [16]. Because glucocorticoids represent the standard treatment of grades II to IV acute GVHD in our institution, it is possible that the drop in the pDC level at day 100 in our study was at least partly due to the corticosteroids and not only to acute GVHD itself. Thus, although the proportion of activated pDCs to produce TNF-α and IFN-α was significantly reduced in patients with clinically severe grades II to IV acute GVHD, as compared with patients without acute GVHD, this significance was lost in the same analysis done only in patients without high-dose corticosteroids in the therapy. Previous studies from our group showed a significant increase of pDCs in the intestinal mucosa and skin of patients with acute GVHD compared with those without acute GVHD [17,18]. Therefore, a possible mechanism underlying the decrease of pDCs in the blood of our patients with clinically significant acute GVHD may be the recruitment of pDCs from the peripheral blood to the affected tissues. On the other hand, a tolerogenic potential of human pDCs by inducing regulatory T cells and inhibiting acute GVHD has also been suggested [19]. In this context, a reduced pDC level in the blood of patients with clinically significant acute GVHD could provide evidence for the concept that resting pDCs in the blood are tolerogenic and have the potential to prevent GVHD.

Table 3
Multivariate Analysis of Predictors of OS

Risk Factor	Relative Risk	95% CI	*P*
Low pDC count	3.97	1.29-12.22	.02
Older age of recipient	5.22	1.16-23.44	.03
Donor type: MUD	2.29	.67-7.92	.19
Acute GVHD grades II-IV	.65	.23-1.88	.43
High risk disease	1.21	.46-3.25	.69

MUD indicates matched unrelated donor.

Given the central role of DCs in the immune system, several previous human studies analyzed the relationship between DC recovery after allo-SCT and long-term outcome of patients, particularly GVHD and relapse [20]. Among others, the peripheral blood pDC count at 3 months after allo-SCT was shown to be an independent factor of OS [15].

In our study we allocated patients according to the median value of pDC proportion and obtained 2 comparable groups. In the Kaplan-Meier analysis, the OS was significantly lower in the low pDC as compared with the high pDC group, and this difference was confirmed in the multivariate analysis as well, together with a known factor of worse survival—older age of the recipient. The worse survival of patients in the low pDC group was mostly due to significantly higher cumulative incidence of relapse-related mortality, which suggested that a higher number of pDCs reconstituted after transplantation might reduce relapse. Furthermore, a couple of clinical reports of the relation of levels of DCs in the blood with the development of GVHD yielded conflicting results. Thus, peripheral blood low pDC count on day 28 after allo-SCT independently predicted for both acute and chronic GVHD in 1 study [21], whereas high pDC numbers at 14.5 months predicted chronic GVHD in another [22]. In addition, a recent study demonstrated that pDCs and naive T cells in bone marrow grafts were associated with survival after unrelated-donor allogeneic hematopoietic SCT [23]. However, more functional studies are needed to decipher the exact role of pDCs in antitumor immunity and GVHD.

In addition, the impact of antithymocyte globulin, type of conditioning, and stem cell source on pDC recovery should be investigated in other allo-SCT settings. In the meantime, enumerating pDC count at day 100 after allo-SCT could serve as a simple, fast, and reproducible indicator of adverse clinical outcome of patients, in terms of relapse, death, and GVHD. Monitoring pDC count could allow for early classification of patients according to the risk for these events and for potential early therapeutic interventions to increase the pDC number in the blood of patients to improve their outcomes. Although the group of patients analyzed in our study was rather heterogeneous regarding diagnoses and transplant characteristics, precluding establishing the definitive role and impact of the pDC subsets after allo-SCT, this and similar investigations are important to further understand the precise immunoregulatory properties of pDCs and to pave the way for new targeted therapies in GVHD and hematological malignancies.

ACKNOWLEDGMENTS

The authors thank the nursing staff and attending physicians for their dedicated patient care. M.M. would like to thank Pr. J. V. Melo (University of Adelaide, Australia) for critical reading of the manuscript.

Financial disclosure: Our research activities are supported by educational grants from the Association for Training, Education and Research in Hematology, Immunology and Transplantation. The authors also thank the Association pour la Recherche sur le Cancer (grant 3175 to M.M.), the Fondation de France, the Fondation contre la Leucémie, the Agence de Biomédecine, the Association Cent pour Sang la Vie, the Association Laurette Fuguain, the International Research Group on Hematopoietic Cells Transplantation (IRGHET), and the Ligue contre le Cancer Grand-Ouest for their generous and continuous support for our clinical and basic research work. Our group is supported by several grants from the French National Cancer Institute (Programme Hospitalier de

Recherche Clinique [PHRC], Institut National du Cancer [INCa] to M.M.).

Conflicts of interest statement: There are no conflicts of interest to report.

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