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Higher Numbers of Blood CD14$^+$ Cells before Starting Conditioning Regimen Correlate with Greater Risk of Acute Graft-versus-Host Disease in Allogeneic Stem Cell Transplantation from Related Donors

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ABSTRACT

Host antigen-presenting cells (APCs) have been shown to induce acute graft-versus-host disease (aGVHD) in experimental models. In this study, we investigated whether pretransplantation blood levels of host APCs, such as plasmacytoid and myeloid dendritic cells and monocytes, correlate with the development of aGVHD. A total of 89 consecutive patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) from HLA-matched related (n = 48) or unrelated (n = 41) donors were enrolled in the study. Blood samples were analyzed by flow cytometry before initiating the conditioning regimen. In related donor transplants, patient–donor sex mismatch and monocyte levels significantly correlated with aGVHD grade II–IV in both univariate and multivariate analyses. Similar results were not observed in recipients of matched unrelated transplants, possibly due to use of antithymocyte globulin (ATG) or differences in graft source in these patients. In conclusion, pretransplantation recipient monocyte levels are relevant to the development of GVHD in HSCT from related donors.

KEY WORDS

Hematopoietic stem cell transplantation • Graft-versus-host disease • Monocyte • Dendritic cell • Antigen-presenting cell

INTRODUCTION

Dendritic cells (DCs), the most powerful antigen-presenting cells (APCs), comprise myeloid (mDC) and plasmacytoid (pDC) subsets [1]. mDCs reside in peripheral tissues and migrate to T-cell areas of peripheral lymphoid organs on maturation [2], whereas pDCs reside in secondary lymphoid organs. Peripheral blood (PB) contains low numbers of mDCs and pDCs [3]; nevertheless, it has been shown that mDCs residing in peripheral tissues can originate from circulating CD14$^+$ monocytes [4], particularly during infection and inflammation.

In allogeneic hematopoietic stem cell transplantation (HSCT), acute graft-versus-host disease (aGVHD) is caused by donor mature T cells targeting host tissues on alloantigen recognition [5,6]. Host alloantigens can be directly presented to donor lymphocytes by host APCs (direct presentation), or can be captured by donor APCs and then presented to donor T cells (indirect presentation). In experimental models of major or minor histocompatibility antigen-mismatched allogeneic HSCT, direct presentation has been demonstrated to be essential in the development of aGVHD [7-9]. Despite previous studies that have correlated levels of circulating DCs and GVHD [10-12], whether circulating APCs contribute directly to the activation of donor T cells after transplantation has not yet been demonstrated in humans. In particular, the significance of different levels of circulating APCs before the conditioning regimen is not understood. In fact, myeloablative or reduced-intensity pretransplantation conditioning regimens cause a decline in PB

cell numbers, including DCs [13]. However, after preparative chemoradiotherapy, some of the circulating APCs may also migrate to peripheral tissues [14] due to elevated levels of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, or pathogen-derived products, such as lipopolysaccharides [15].

In this study, we tested whether the number of APCs, such as myeloid and plasmacytoid DCs, or CD14$^+$ monocytes measured in the PB of patients before initiating the conditioning regimen for an allogeneic HSCT could predict the development of aGVHD. Our findings show that the level of circulating monocytes correlates with aGVHD in a multivariate analysis.

PATIENTS AND METHODS

Patients

Between January 2002 and December 2004, 89 consecutive adult patients (31 with acute myelogenous leukemia [AML]/myelodysplastic syndrome [MDS], 13 with acute lymphocytic leukemia [ALL], 15 with chronic myelogenous leukemia [CML], 15 with multiple myeloma [MM], and 15 with non-Hodgkin lymphoma [NHL]/Hodgkin disease [HD]) undergoing allogeneic HSCT at the University of Bologna were enrolled in this study after informed consent was obtained. Of these 89 patients, 48 received transplants from HLA-matched related donors (47 peripheral blood stem cell [PBSC], 1 bone marrow [BM]), and 41 received transplants from unrelated donors (3 PBSC, 38 BM). A total of 71 patients received myeloablative regimens, including high-dose busulfan-based (16 mg/kg, n = 39), total body irradiation (TBI)-based (800 cGy, n = 27), and melphalan-based (140 mg/sm, n = 5), as described previously [10]. In 18 patients, reduced-intensity regimens were used, including fludarabine 120 mg/m^2, cyclophosphamide 60 mg/kg, and thiotepa 10 mg/kg (n = 10); fludarabine 60 mg/m^2 and melphalan 140 mg/m^2 (n = 6); and cyclophophamide 100 mg/kg and thiotepa 10 mg/kg (n = 2). In 39 of the 41 patients receiving unrelated transplants and 5 of the 48 receiving related transplants, rabbit-ATG (Fresenius-Kabi, Graz, Austria; 15 mg/kg total dose) was added to the conditioning regimen from day −5 to −1. GVHD prophylaxis included standard cyclosporine and methotrexate in 82 patients and cyclosporine and mofetil mycophenolate in 7 patients. aGVHD and chronic GVHD (cGVHD) were graded according to standard criteria.

Flow Cytometry

Blood samples were obtained from each patient 1 day before initiating the conditioning regimen and were tested by cytofluorimetric analysis. Only samples containing $> 1 \times 10^9$ white blood cells (WBCs)/L (ie, 87 out of 89) were suitable for analysis. The following monoclonal antibodies were used, all from Becton Dickinson (Mountain View, CA): fluorescein isothiocyanate–conjugated antilineage (lin) (a mixture of anti-CD3, -CD14, -CD16, -CD19, -CD20, and -CD56), anti-CD34, and anti-CD45; phycoerythrin-conjugated anti-CD11c, anti-CD123, and anti-CD14; and peridin chlorophyll protein –conjugated anti–HLA-DR. mDCs were identified as lin$^-$CD34$^-$CD11c$^+$HLA-DR$^+$ and pDCs as lin$^-$CD34$^-$CD123$^+$HLA-DR$^+$, as described previously [10]. Monocytes were identified as CD45^{bright} CD14^{bright} in a standard leukogate analysis. Sample acquisition and analysis were performed on a FACSCalibur analyzer (BD Biosciences, San Jose, CA) using Cell Quest software. Absolute numbers were defined by multiplying percentages by the WBC count.

Statistical Analysis

The Wilcoxon rank-sum test was used to compare pre-DC numbers in transplant recipients and healthy controls (Figure 1), as well as to compare pre-DC numbers in patients with distinct gender. The Kruskal-Wallis test was used to compare pre-DC numbers in patients with different diseases. To correlate pre-DC numbers and patient age, disease duration, and number of previous treatments, a linear regression analysis was performed and Pearson's correlation coefficient was determined. Analysis of vari-

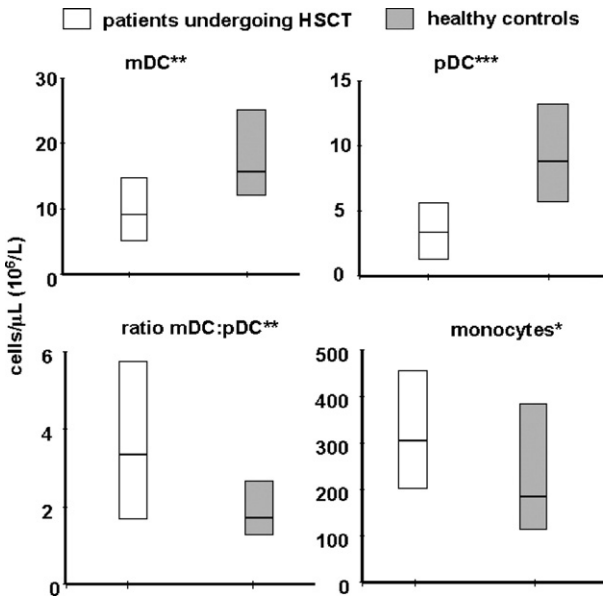

Figure 1. PB APC counts in allogeneic HSCT patients and healthy controls. PB mDC, pDC, and monocyte counts, as indicated, were determined in patients undergoing allogeneic HSCT before the start of pretransplantation chemoradiotherapy (n = 82 for mDCs and pDCs; n = 88 for monocytes) (white boxes) and in healthy age-matched controls (n = 27) (gray boxes), as described in Materials and Methods. Horizontal lines indicate median cell counts in each group. Boxes indicate interquartile ranges. *$P < .05$; **$P < .01$; ***$P < .001$ as determined by Wilcoxon's rank-sum test.

Table 1. *Risk Factors for aGVHD in Related and Unrelated Donor Transplantations*

	Related (n = 44)*				Unrelated (n = 39)			
aGVHD grade (n)	0-I (n = 29)	2-4 (n = 15)	P†	P‡	0-I (n = 31)	2-4 (n = 8)	P†	P‡
Recipient age, years (range)§	42 (36-49)	52 (39-54)	NS		33 (26-44)	36 (25-44)	NS	
Donor								
Age§	39 (32-49)	46 (38-53)	NS		35 (22-56)	27 (20-45)	NS	
Female into male	4 (14%)	7 (47%)	.01	.04	2 (6%)	2 (25%)	NS	
Source								
PBSC	28 (97%)	15 (100%)			3 (10%)	0 (0%)		
BM	1 (3%)	0 (0%)	NS		28 (90%)	8 (100%)	NS	
Disease								
AML/ALL	11 (38%)	5 (33%)			19 (61%)	5 (63%)		
CML	7 (24%)	0			5 (16%)	2 (25%)		
MM	5 (17%)	5 (33%)			4 (13%)	1 (13%)		
NHL/HD/CLL	6 (21%)	5 (33%)			3 (10%)	0		
Aadvanced¶	20 (69%)	12 (80%)	NS		19 (61%)	7 (87%)	NS	
Disease duration§‖	10 (9-24)	28 (13-55)	NS		17 (11-26)	11 (7-27)	NS	
Conditioning								
RIC	7 (24%)	6 (40%)	NS		5 (16%)	1 (12%)	NS	
TBI	7 (24%)	3 (20%)	NS		13 (42%)	3 (38%)	NS	
ATG	4 (14%)	0 (0%)	NS		30 (97%)	8 (100%)	NS	
Graft §								
TNC**	765 (543-985)	848 (755-1160)	NS		300 (225-379)	250 (202-338)	NS	
CD34+**	4.1 (3.5-5.6)	5.2 (4.8-5.7)	NS		2.2 (1.5-3)	1.6 (1.2-3)	NS	
CD3+**	182 (153-248)	170 (154-253)	NS		25 (18-27)	26 (20-28)	NS	
Recipient PB§								
WBCs††	5.1 (3.8-6.1)	5 (4.4-5.6)	NS	NS	4.4 (3.3-7.2)	4.9 (3.2-7.6)	NS	
mDC‡‡	9.6 (4.5-13.8)	8.6 (6.7-14.1)	NS	NS	8.1 (5.1-19.1)	9.1 (3.8-11.7)	NS	
pDC‡‡	3.6 (2.2-5.4)	2.6 (1.2-5.4)	NS	NS	3.1 (1.5-5.9)	1 (0.4-3.5)	NS	
CD14+ pre-DC‡‡	238 (207-322)	380 (292-507)	.004	.001	323 (189-463)	211 (146-497)	NS	
Ratio mDC:pDC	1.8 (1.3-4.4)	5.2 (1.8-7.5)	NS	NS	3.1 (1.5-5.9)	1 (0.4-3.5)	NS	

Abbreviations: CLL, chronic lymphocytic leukemia; RIC, reduced-intensity conditioning; TNC, total nucleated cells; NS, not significant.
*Six patients (4 related and 2 unrelated) were not evaluable due to death before engraftment.
†Univariate analysis (*P* values as determined by analysis of variance [ANOVA] as described in Materials and Methods).
‡Multivariate analysis (*P* values as determined by ANOVA as described in Materials and Methods).
§Median values are indicated (interquartile ranges in parentheses); because absolute counts had a skewed distribution, statistical analysis was performed on ranked counts.
¶Advanced disease was defined as AML and ALL beyond first CR, CML not in chronic phase, and NHL, HD, CLL, and MM at whatever stage.
‖Expressed in months.
**Values are expressed as 10^6cells/kg recipient's body weight.
††Values are expressed as 10^3cells/μL.
‡‡Values are expressed as cells/μL.

ance (SAS version 8.0 [SAS Institute, Cary, NC]) was used to examine the association between aGVHD and the variables listed in Table 1. GVHD was divided into 2 categories: grade 0–I and grade II–IV. The preconditioning variables of $CD14^+$ monocyte numbers, mDCs, pDCs, total WBCs, and mDC:pDC ratio had a skewed distribution; therefore, statistical analysis was performed on ranked counts. Patients were analyzed in 2 separate groups, those receiving matched related donor transplants (n = 48) and those receiving matched unrelated donor transplants (n = 41). Only variables associated with GVHD ($P < .1$) in univariate analysis were entered into multivariate analysis. The comparison of monocyte numbers in patients with distinct GVHD grades (Figure 2A) was done using the Kruskal-Wallis test. Curves comparing aGVHD and cGVHD, overall survival, and relapse rate depending on pre-DC counts were plotted according to the Kaplan and Meier method and analyzed according to the log-rank test.

RESULTS

Absolute numbers of circulating DCs and monocytes were initially compared in 89 patients before allogeneic HSCT and in 27 normal controls comparable in terms of age and sex. The patients schedule for HSCT had lower mDC counts (8.8×10^6/L [interquartile range, 5.1–14.7 $\times$ 10^6/L] vs 15.5 $\times$ 10(e)6/L [12.1–25.1 $\times$ 10(e)6/L]; $P = .0006$) and pDC counts (3.3×10^6/L [1.4–5.5 $\times$ 10^6/L] vs 8.6 $\times$ 10^6/L

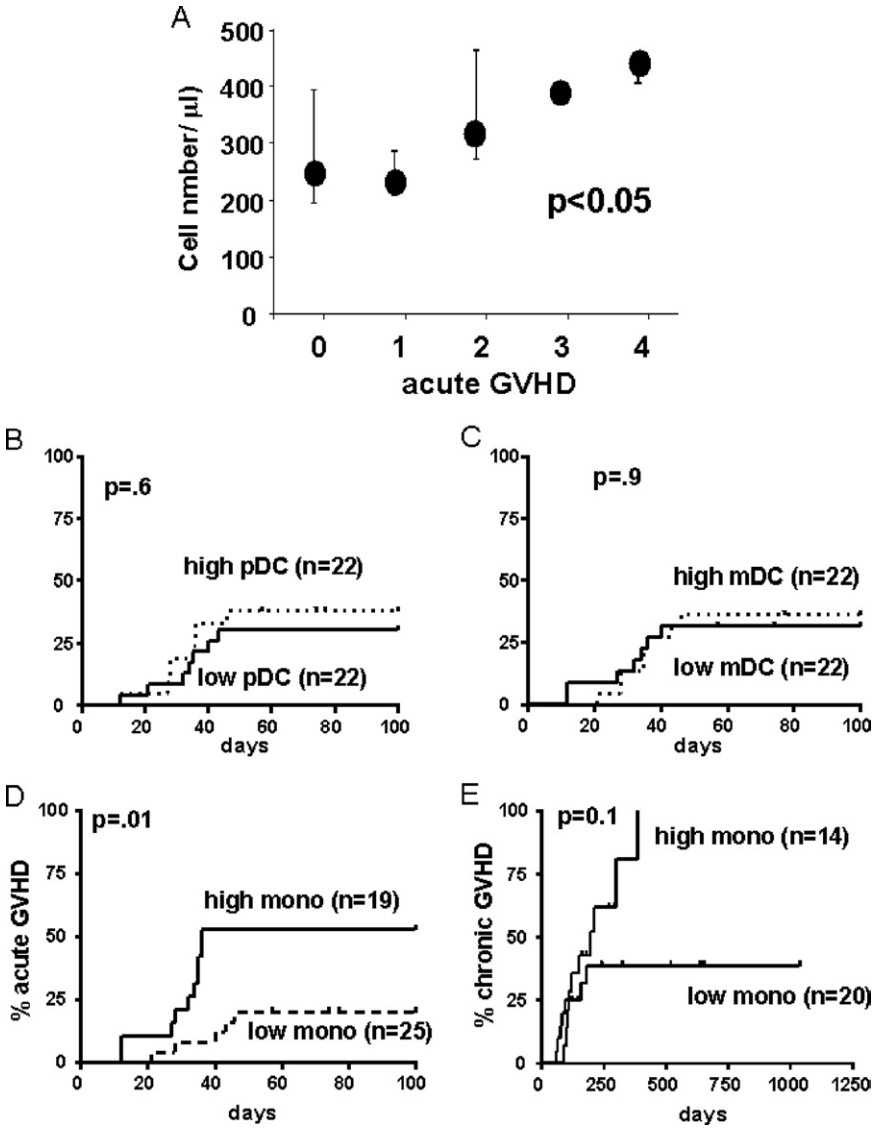

Figure 2. Correlation between recipient PB monocyte counts before transplantation and increased aGVHD after allogeneic HSC transplantation from HLA-identical related donors. A, Evaluable patients were classified depending on aGVHD grade (grade 0, n = 19; grade 1, n = 10; grade 2, n = 7; grade 3, n = 6; grade 4, n = 3). Circles indicate median monocyte counts in each group. Error bars indicate interquartile ranges. $P = .01$ as determined by the Kruskal-Wallis test. B–E, Patients were classified according to whether they had pDC counts (B), mDC counts (C), or monocyte counts (D, E) higher or lower than the median value, as determined on the whole patient population. Time to a GVHD (B–D) or cGVHD (E) GVHD was determined using the Kaplan-Meier method. The significance of the differences between the curves, as determined by the log-rank test, is indicated.

[5.8–13.1 × 10^6/L]; $P < .00001$), but higher monocyte counts (308/µL [204–450 /µL) vs 177/µL [116–377/µL]; $P = .04$) (Figure 1). Nevertheless, the pretransplantation mDC:pDC ratio was significantly higher in patients than in controls (3.3 [1.7–5.7] vs 1.7 [1.3–2.6]; $P = .0005$), due to much lower levels of pDCs than mDCs in the patients compared with normal controls (Figure 1). Blood mDC and monocyte counts did not correlate with age, sex, disease type, disease duration, or the number of previous treatments (not shown). Instead, pDC counts were lower in patients who had received more previous treatments ($P = .005$) or steroid-containing treatments ($P = .002$), and in those with MM ($P = .01$).

aGVHD grade II–IV was observed in 15 patients who received related transplants and in 8 patients who received unrelated transplants. Six patients (4 in the related donor group and 2 in the unrelated donor group) died before engraftment and could not be evaluated for aGVHD. Because factors potentially affecting aGVHD, including stem cell source, graft composition, and ATG use, were unevenly distributed in the related and unrelated transplant groups, the 2 groups were analyzed separately (Table 1).

In the related transplant group, univariate analysis found an association among aGVHD grade II–IV, the presence of sex-mismatched (female into male) donors, and pretransplantation monocyte levels (Table 1). Other potential risk factors for aGVHD, including donor and recipient age, disease type, and status, conditioning regimen, GVHD prophylaxis, and graft composition, did not correlate with aGVHD. In multivariate analysis, both sex mismatch (female into male) and monocyte levels were associated with aGVHD grade II–IV (Table 1). When early death was combined with acute GVHD to exclude its role as a competing factor, a correlation between both factors and the composite end point of death and aGVHD was still seen ($P = .04$ and .003, respectively; data not shown).

Comparable median numbers of monocytes were observed in patients with aGVHD grade I and in those with no GVHD (224/μL [interquartile range, 209–286/μL] vs 238/μL [193–393/μL], whereas monocyte counts were higher in patients with grade II (308/μL [271–464/μL]), grade III (382/μL [374–398/μL]), and grade IV (431 [406–1962]) aGVHD ($P = .01$) (Figure 2A). After dividing the patients into 2 groups according to the median number of monocytes, mDCs, or pDCs observed in the study, we found that patients with a monocyte count greater than the median value (308/μL) had a higher incidence (52%) of aGVHD than the others (23%) ($P = .01$) (Figure 2D). However, the risk of aGVHD was not related to pDC (Figure 2B) or mDC counts (Figure 2C).

Among patients receiving transplants from family donors, 35 patients (73%) were alive in CR after a median follow-up of 309 days (range, 28–1460 days). Among the 34 patients that could be evaluated (> 100 days follow-up), 17 developed cGVHD, including 7 (35%) in the low-monocyte group and 10 (63%) in the high-monocyte group. A trend toward a higher actuarial probability of chronic GVHD was observed in patients with higher monocyte counts (Figure 2E). Relapse rate was similar in patients with higher and lower monocyte counts (11% vs 12%, respectively). However, due to disease heterogeneity and short follow up, the lack of relationship between monocyte counts and relapse cannot be considered conclusive.

In transplants from unrelated donors with ATG added to the conditioning regimen, none of the cell types analyzed correlated with aGVHD grade II–IV, cGVHD, overall survival, or relapse rate (data not shown).

DISCUSSION

In this study, we have shown that patients with higher levels of $CD14^+$ circulating cells before initiation of a conditioning regimen for allogeneic HSCT from a related donor are more likely to develop aGVHD grade II–IV. Although aGVHD often has been associated with the cellular composition of the graft, our data suggest for the first time in a clinical setting a correlation between blood APCs in the recipient and the risk of developing aGVHD after allogeneic HSCT. However, of the various types of APCs analyzed, $CD14^+$ cells correlated with a greater incidence of aGVHD but circulating mDCs and pDCs did not, as was also reported previously [11]. A possible explanation for this finding is that PB contains more monocytes than DCs, and these monocytes are capable of migrating to lymphoid organs or tissues and rapidly differentiating into DCs on release of cytokines in inflammatory sites.

The relevance of recipient APCs in the induction of aGVHD has been previously demonstrated in animal models of GVHD [3-6]. Nevertheless, the mechanism through which higher recipient monocyte counts predispose to aGVHD remains unclear and may not depend solely on antigen presentation. In murine studies, circulating host monocytes are recruited to tissues after pretransplantation chemoradiotherapy through CCR2–CCL2 interactions [16,17]. This may be one reason, together with direct toxicity, why we found an association between pretransplantation chemoradiotherapy and a rapid reduction of PB monocyte numbers. In fact, 90% of our patients had < 100 monocytes per μL on the day of transplantation (data not shown).

In humans, aGVHD is known to be associated with a more rapid recovery of monocytes producing interleukin-12 and TNF-α [18,19]. However, in this study, posttransplantation recovery of monocytes, mDCs, and pDCs at 1 and 3 months did not correlate with pretransplantation numbers of circulating monocytes (data not shown). Moreover, monocyte recovery did not correlate with aGVHD, as also described previously [10]. These data are in agreement with the previously reported observation that 100% circulating dendritic cells and monocytes are of donor origin as early as 7 days posttransplantation [20]. However, these data do not exclude a correlation between pretransplantation circulating monocyte numbers and APC numbers in peripheral tissues and lymphoid organs early after transplantation. Thus our hypothesis is that the number of circulating monocytes of the recipient detected before initiation of the conditioning regimen might predict the amount of myeloid APCs that will be found in peripheral tissues and/or lymphoid organs after transplantation. Experiments designed to test this hypothesis in humans will need to take into consideration multiple variables, such as the degree of HLA compatibility, type of conditioning regimen, and the immunosuppressive therapy used to prevent aGVHD, which may affect the kinetics of

activation, migration, and maturation of monocytes and of T cells.

The lack of correlation between monocyte levels and aGVHD in persons receiving unrelated transplants can be partially explained by the use of thymoglobulin, which potentially depletes graft T cells as well as recipient APCs [21]. Interestingly, patients receiving ATG before transplantation had a trend toward lower monocyte counts on the day of transplantation (data not shown). Alternatively, the difference between related and unrelated transplants in our study may possibly be related to the type of graft (BM in unrelated donors vs PBSC in related donors) used in these settings. In fact, the lower T-cell dose in BM compared with PBSC could potentially reduce monocyte migration and activation [17]. In addition, T cells from granulocyte colony- stimulating factor–mobilized PBSC grafts are less sensitive to B7-dependent costimulation [22] and may be selectively committed to Th2 polarization [23].

Although larger studies addressing the role of monocytes in transplant are warranted, our initial observation of a correlation between recipient PB monocyte levels before transplant and the risk of aGVHD after transplantation suggest a possible role of host accessory cells in aGVHD in human allogeneic HSCT as well. These results may further support the hypothesis, previously demonstrated in mice experiments [7], that depletion of host APCs before transplantation may prevent aGVHD. To address this hypothesis, we will test whether new drugs such as thymoglobulin, alemtuzumab [13], and bortezomib [24] at doses specifically targeting monocytes and DCs may be useful in experimental designs aimed at preventing or decreasing the incidence of severe aGVHD.

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