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Immune Reconstitution following Myeloablative Allogeneic Hematopoietic Stem Cell Transplantation: The Impact of Expanding CD28^{negative} CD8^{+} T Cells on Relapse

Ibrahim Yakoub-Agha,[1,2,3] Pasquine Saule,[2,3] Leonardo Magro,[1] Pascale Cracco,[2] Alain Duhamel,[4] Valérie Coiteux,[1] Bénédicte Bruno,[1] Françoise Dufossé,[2] Jean-Pierre Jouet,[1,2,3] Jean-Paul Dessaint,[2,3] Myriam Labalette[2,3]

Allogeneic stem cell transplantation has become standard therapy for hematologic malignancies through the positive immunologic graft-versus-leukemia effect. Initial immune recovery relies on peripheral expansion of infused T cells, which switch to a memory-like phenotype. This study prospectively investigated whether changes in subset composition precedes complications after myeloablative HLA-matched transplantation for hematologic malignancies. Of 80 allograft recipients, 18 were still free of clinical complication throughout 395 to 1564 days of follow-up. Compared with this complication-free subgroup, patients who developed chronic graft-versus-host disease (cGVHD) without relapsing recovered similar numbers of circulating T cells with predominance of CD8^{+} T cells lacking CC-chemokine receptor-7 and CD28 expression throughout the first year after transplantation. Conversely, poor CD8^{+} T cell recovery with diminished numbers of CD28^{neg} CD8^{+} T cells ($\sim$1/4th of that of relapse-free patients) preceded occurrence of malignant relapse. In multivariate analysis, lower CD28^{neg} CD8^{+} T cell counts by day 60 postallograft were associated with a greater risk of subsequent relapse (hazard ratio [HR] 0.33; 95% confidence interval [CI]: 0.14-0.76; P = .01). Enumeration of CD28^{neg} CD8^{+} T cells in patients could assist in predicting risk of relapse and help build an algorithm for accelerating the immune recovery by reducing the immunosuppressive treatment and considering the introduction of preemptive donor lymphocyte infusions.

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INTRODUCTION

Improved prophylactic measures, supportive care, and anti-infectious therapies have contributed to reduce treatment-related morbidity and mortality (TRM) in the early period following allogeneic stem cell transplantation (allo-SCT). However, relapse of the underlying hematologic malignancy and chronic graft-versus-host disease (cGVHD) remain major causes of treatment failure. Other cell lineages notwithstanding, mature T cells contained in or derived from the allograft exert powerful antitumor effects through the graft-versus-leukemia (GVL) reaction [1], but T cell-mediated responses can also induce GVHD [2]. Initial T cell recovery is primarily dependent on peripheral expansion of donor mature T cells (termed homeostatic peripheral expansion), because de novo generations of naïve T cells from donor hematopoietic stem cells is compromised following cytoreductive regimens and cannot have a full impact on immune reconstitution for 1 or several years, depending on recipient age [3-7]. Homeostatically expanding T cells show memory-like phenotypes [3-8] and the high homeostatic proliferation following lymphopenia can facilitate expansion of antitumor immunity [7]. Therefore, malignant relapse might result from

From the [1]Service des Maladies du Sang, CHU, Lille, France; [2]Service d'Immunologie, CHU, Lille, France; [3]EA2686, IFR114, Université Lille 2, Lille, France; and [4]Centre d'Etudes et de Recherche en Informatique Médicale, Université Lille 2, Lille, France.

The first 2 authors contributed equally to this work and should be considered as first authors.

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Correspondence and reprint requests: Ibrahim Yakoub-Agha, MD, PhD, Service des Maladies du Sang, UAM allogreffes de CSH, CHRU de Lille, F-59037 Lille, France (e-mail: i-yakoub-agha@chru-lille.fr).

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insufficient homeostatic peripheral expansion, and conversely, peripheral expansion with a too strong immune reaction against host antigens might induce GVHD.

The purpose of this prospective study was to address whether differences in the dynamics of CD4 and CD8 T cell recovery within the first year after allo-SCT can be related to the absence or occurrence of malignant relapse and GVHD. The expression pattern of surface markers in peripheral blood was analyzed longitudinally in 80 patients after myeloablative HLA-matched allo-SCT for hematologic malignancies, to categorize T cells with a naïve phenotype and the phenotypically distinct subsets of memory-type T cells (ie, central memory and effector memory cells) [9]. Expression of the costimulation receptor, CD28, was also analyzed, because CD28 expression is lost after several rounds of cell division, and segregating $CD8^+$ T cells by CD28 staining might be indicative of their functional capacities [10,11]. In a first step, we concentrated on 3 subgroups of recipients according to whether or not they had developed only 1 posttransplant complication: 1 without evidence of infection, GVHD, or relapse throughout the whole period of follow-up; 1 with only cGVHD; and 1 with only evidence of a malignant relapse after transplantation. These subgroups allowed comparing kinetics of T cell recovery in the absence of possible interferences by other clinical complications. Poor recovery of the $CD8^+$ T cell compartment by 2 months postgraft, resulting in reduced numbers of $CD8^+$ T cells that lacked CD28 expression, was significantly correlated with later occurrence of malignant relapse, as verified in a second step by univariate and multivariate analyses of the entire cohort.

MATERIALS AND METHODS

Patients

From October 2003 on, absolute counts and relative frequencies of peripheral blood T cell subsets were determined prospectively in 80 allo-SCT recipients. Their demographic and clinical characteristics are given in Table 1. All patients (and parents of children) provided informed consent, with approval from our institutional ethics committee. All patients were treated for a hematologic malignancy; all were in complete hematologic remission at time of transplantation, and none were at high risk of relapse after allo-SCT. A slight majority of patients received myeloablative conditioning including radiation and chemotherapy instead of myeloablative chemotherapy alone. All patients underwent T cell-replete allo-SCT followed by standard GVHD prophylaxis with cyclosporine (CsA) and short-term methotrexate (MTX). For all patients, it was their first transplantation. Stem cell source was bone marrow in the majority of patients (n = 67) and otherwise granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSCS). Patients were monitored prospectively in terms of clinical complications, including graft rejection, acute GVHD (aGVHD) and cGVHD, infection, and relapse. This includes weekly cytomegalovirus (CMV) pp65 antigenemia assays to monitor for CMV reactivation during the first 3 months postgraft. No prophylaxis or preemptive treatment for antigenemia in the absence of other clinical or biologic CMV-related symptom was given.

Enumeration of $CD4^+$ and $CD8^+$ T Cell Subsets

One milliliter of the final graft product was obtained immediately before infusion for evaluation of graft composition. Peripheral blood samples were collected from recipients 30 ± 2, 60 ± 3, 90 ± 5, 180 ± 10, and 365 ± 15 days after allo-SCT. All living patients had at least 3 samples analyzed. Whole-blood and graft samples were stained without further separation shortly after collection, acquired by multiparameter flow cytometry and categorized by their profiles of CD45RA, CC-chemokine receptor-7 (CCR7), and CD28 expression, as described previously [12,13]. All reagents and instrumentation were from Beckman Coulter (Fullerton, CA), with the exception of the monoclonal antibody (mAb) to CCR7, from R&D systems (Minneapolis, MN). Absolute counts and percentages of naïve ($CD45RA^+$ $CCR7^+$ $CD28^+$) and differentiated T cells with a central memory (T_{CM}, $CD45RA^{neg}$ $CCR7^+$), effector memory (T_{EM}, $CD45RA^{neg}$ $CCR7^{neg}$), and $CD45RA^+$ effector memory ($T_{EM}RA$, $CD45RA^+$ $CCR7^{neg}$) phenotype [9] were determined within the $CD4^+$ and the $CD8^+$ T cell populations. The proportion of cells coexpressing CD28 was determined within each of these subsets. The degree of donor chimerism among $CD3^+$ T cells was assessed by real-time polymerase chain reaction (PCR) with a sensitivity <1% [14].

Statistical Analysis

Patient characteristics are presented as median (range) unless otherwise stated. Normalization of the absolute counts of a given lymphocyte population in the peripheral blood of a patient was defined as reaching the fifth percentile of age-matched reference values of this laboratory [15]. The results were first summarized with descriptive statistics, including medians and interquartile ranges (25th-75th percentiles; interquartile range [IQR]). An initial analysis of the data was performed univariably using rank-order nonparametric tests and log-likelihood ratio G-test or Fisher's exact test, as appropriate. Statistical correlations were performed using Spearman's rank test. For the patient outcome study, probabilities were estimated by the Kaplan-Meier product-limit method, and the log-rank

Table 1. Characteristics of donor/recipient pairs.

	All Patients	Complication Free*	Only Relapse	Only Chronic GVHD†	P‡
No. of patients (male/ female)	80 (43 / 37)	18 (7 / 11)	15 (12 / 3)	13 (9 / 4)	—
Age of recipients: median (range), years	32 (4-56)	24 (4-56)	33 (5-55)	25 (14- 55)	NS
No. <16 years of age	15	6	4	2	NS
Underlying disease, no. of patients:§					
Myelogenous proliferation (AML / CML / MDS)	53 (28/7/18)	14 (9 / 2/3)	9 (8 / — / 1)	6 (3 / 1 / 2)	NS
Lymphoid proliferation (ALL / CLL / NHL)	27 (24/1/2)	4 (4 / — / —)	6 (6 / — / —)	7 (6 / 1 / —)	
Age of donors: median (range), years	35 (2-63)	31 (2–58)	37 (9-55)	30 (9- 57)	NS
Donor-Recipient sex mismatch, no.¶	23	3	3	7	NS
Donor-Recipient CMV-serostatus (either or both positive), no.	49	12	7	9	NS
Donor/recipient pairs, no.:					
HLA-identical sibling	54	14	9	11	
HLA-identical (10/10), unrelated	20	4	4	2	NS
HLA-Cw mismatched (9/10), unrelated	6	—	2	—	
Source of graft, no.:					
Bone marrow	67	17	12	10	
G-CSF-mobilized PBSC	13	1	3	3	NS
Infused $CD34^+$ stem cells $\times 10^7$/ kg recipient's weight: median (range)	2.98 (1.05-13.9)	3.38 (1.23-8.5)	3.44 (1.22-7.4)	2.69 (1.23- 8.5)	NS
Infused T cells $\times 10^6$ / kg recipient's weight: median (interquartile range):					
all $CD3^+$ T cells	24.3 (18.0-35.1)	22.5 (24.3-36.1)	27.9 (20.0-42.1)	27.1 (20.7-35.8)	NS
$CD4^+$ T cells	13.0 (8.8-18.4)	10.6 (6.0-14.8)	17.7 (9.8-22.7)	14.8 (11.2-19.5)	NS
$CD8^+$ T cells	11.0 (8.4-17.2)	10.0 (7.6-15.2)	13.0 (10.2-19.8)	11.0 (9.2-17.5)	NS
Myeloablative conditioning regimens:⊥					
Busulfan- Cyclophosphamide	32	12	4	4	.03
Cyclophosphamide—12-Gray TBI	48	6	11	9	**
Cyclosporine—short-term Methotrexate prophylaxis for acute GVHD, no. of patients:	80	18	15	13	—
Day 60 chimerism: median (interquartile range), percentage of donor T cells	98.5 (93-100)	96 (88- 99)	94 (98-100)	97.5 (96-100)	NS
CMV reactivation, no. of patients: median (range) days postgraft:	15 45 (5-141)	—	—	—	
Acute GVHD, no. of patients: median (range) days postgraft:	23 31 (13- 61)	—	—	—	
Chronic GVHD, no. of patients: median (range) days postgraft:	30 184 (57- 971)	—	—	13 202 (105-971)	
Relapse, no. of patients: median (range), days postgraft:	26 217 (85- 561)	—	15 215 (95- 561)	—	
Whole follow-up period: days postallograft	502 (64-1474)	897 (395-1564)	487 (122-841)	1012 (542-1389)	

AML indicates acute myelogenous leukemia; CML, chronic myelogenous leukemia; GVHD, graft-versus-host dieases; C-GSF, granulocyte-colony stimulating factor; NHL, non-Hodgkin lymphoma; CMV, cytomegalovirus; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukeima; PBSC, peripheral blood stem cells; TBI, total body irradiation.

*Complication-free patients: those who did not develop any transplantation-related complication (severe infection, viral infection, acute or chronic GVHD, graft rejection) and who did not relapse over the whole period of follow-up.

†Chronic GVHD without prior acute GVHD, scored according to standard criteria (10 limited, 3 extensive).

‡Comparisons between the 3 subgroups of patients, using the Kruskal-Wallis test or log-likelihood ratio G-test as appropriate.

§AML, CML in chronic phase and MDS without blast crisis; ALL, NHL (low grade). All patients with acute leukemia were in complete remission at the time of transplantation. Log-likelihood ratio G-test compared AML/CML/MDS versus ALL/NHL.

¶Defined as a male patient undergoing transplantation with cells of a female donor.

⊥Busulfan plus cyclophosphamide (200 mg/kg) or cyclophosphamide (60 mg/kg) plus fractioned TBI (12 Gray).

**Post hoc test revealed a significant difference in the type of myeloablative conditioning regimen between the complication-free patients and those who only relapsed, whereas myeloablative preparative regimen did not differ between patients who relapsed and those who had only cGVHD.

test was used to evaluate differences between stratified patient subgroups. Potential prognostic factors for relapse were brought into a multivariate analysis using Cox proportional-hazards model. Candidate risk factors included in the model were the logarithmic transformation of lymphocyte subset counts, age of the recipient, myeloablative conditioning regimen, source of stem cells, donor-patient histocompatibility, disease type, CMV serostatus, and all other clinical and biologic factors as listed in Table 1. Results were confirmed using a competitive risk regression model [16]. All results were considered statistically significant at the 5% level (2-sided).

RESULTS

Overall, 18 of the 80 myeloablative allo-SCT recipients were free of any clinical complication by the time of last follow-up (395–1564 days postgraft). These patients less commonly received total body irradiation (TBI) containing preparative regimen, and all received grafts from 10 of 10 HLA-matched donors. Two other subgroups were distinguished, 1 with only cGVHD and 1 with only relapse. These 2 subgroups were comparable as regards the proportion of patients younger or older than 16 years of age, of patients receiving preparative TBI, of marrow versus PBSC allografts, and of sibling versus unrelated donors. There were, however, 2 patients who relapsed after receiving a graft with 1 HLA-Cw mismatch, whereas all patients in the subgroup with only cGVHD received a 10 of 10 HLA-matched allograft (Table 1).

Recovery of T Cell Subsets in Long-Term Complication-Free Patients

Inasmuch as immune recovery can be affected by the occurrence of postgraft complications and their treatment, the long-term complication-free patients allowed assessing T cell reconstitution in the absence of interfering factors. Absolute counts of $CD4^+$ T cells remained below the fifth or the 10th percentile of age-matched reference values throughout the first year postgraft, with subsequent reversal of the normal CD4:CD8 ratio (not depicted). Absolute counts of $CD8^+$ T cells increased rapidly, reaching the fifth percentile of age-matched reference values within 3 months in 8 of the 18 complication-free patients and otherwise by 6 months (Figure 1a). Virtually all circulating T cells were of donor origin (Table 1). There were no significant differences in the rate of recovery comparing complication-free patients younger or older than 16 years of age, marrow, and PBSC grafts, sibling and unrelated donor status, and whether myeloablative conditioning regimen included TBI or not.

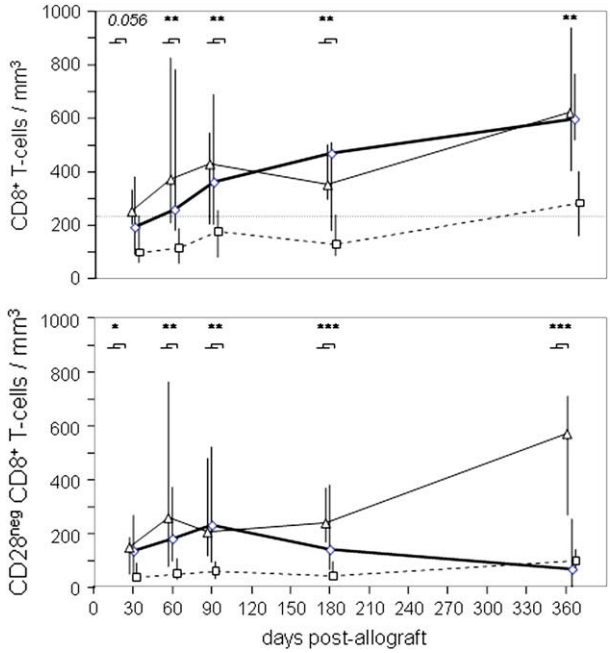

Figure 1. Median absolute counts of peripheral blood $CD8^+$ T cells (a) and their CD28-negative subset (b) by days 30, 60, 90, 180, and 365 after myeloablative HLA-matched allo-SCT, comparing patients free of any clinical complication throughout 395 to 1564 days of follow-up (diamonds and thick solid line) with patients who experienced only cGVHD (triangles and thin solid line) and patients with relapse (squares and dashed line) exclusive of other complication. Error bars indicate the 25th to 75th percentiles. The number of patients studied who were complication-free, who had only cGVHD or only malignant relapse were 17, 13, and 14, respectively, on days 30; 18, 13, and 15 on day 60 and on day 90; 16, 13, and 10 on day 180; and 8, 10, and 8 on day 365. Positions are depicted next to each other around the time point to prevent overlaying 1 subgroup value with another. The horizontal dotted line in (a) denotes the fifth percentile of reference values for the median age of the recipients. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$ by Kruskal Wallis test with Dunn's posttest evaluation to compare complication-free patients with those who relapsed; differences between complication-free patients and patients with only cGVHD were not significant whatever the time point.

At all time points the vast majority of circulating T cells were lacking CC-chemokine receptor-7 (CCR7) expression, indicative of an effector memory-like phenotype. Within the $CD4^+$ population, the $CD45RA^{neg}$ $CCR7^{neg}$ T_{EM} phenotype was disproportionately abundant, resulting in an inversion (median of 0.42 by day 60; IQR 0.30-0.70) of the normal T_{CM}:T_{EM} ratio evidenced in the corresponding graft sample (1.42; IQR: 0.67-2.49) and in age-matched healthy controls. Within $CD8^+$ T cells, T_{EM} represented a median of 25.9% of all $CD8^+$ T cells (IQR 16.6%-39.6%) and $CD8^+$ $T_{EM}RA$ 49.6% (IQR 45.4%-66.1%) by day 60 postgraft.

Circulating $CD8^+$ lacking CD28 expression accumulated steadily over the first year (Figure 1b). Whether in adult or in younger recipients, the percentages of $CD28^{neg}$ $CD8^+$ T cells oversized the percentages determined in paired graft samples, whatever the memory-type subset and whatever the elapsed

time since transplantation ($P \leq .003$ by Friedman test). There was no significant correlation between the total number of $CD8^+$ T cells infused with the graft and absolute counts of peripheral blood $CD28^{neg}$ $CD8^+$ T cells in the recipient ($\rho = .09$; $P = .75$ by day 60). Overall, $CD28^{neg}$ cells predominated within the $CD8^+$ T_{EM} and $T_{EM}RA$ subsets and altogether peaked at a median of 69.4% (IQR 54.5%-78.5%) of all $CD8^+$ T cells by day 60. The proportion of $CD28^{neg}$ $CD8^+$ T cells slightly diminished thereafter to a median of 55.2% (IQR 41.3-56.8) by day 365, still remaining within the upper quartile of reference values in adult recipients, whereas pediatric patients kept throughout a higher percentage than normal for their age. Absolute numbers of $CD28^{neg}$ $CD8^+$ T cells in patients who were younger than 16 years of age at time of transplantation and those 16 years and older were compared, but no statistically significant difference was evidenced ($P = .09$ by a Mann-Whitney test). All these 18 recipients were devoid of clinical and biologic signs of CMV reactivation, and combinations of donor and recipient CMV serostatus were not significantly associated to circulating $CD28^{neg}$ $CD8^+$ T cell counts.

Recovery of T Cell Subsets in Patients with cGVHD

Peripheral blood $CD4^+$ T cell counts increased as slowly and $CD8^+$ T cell counts as rapidly in patients who developed de novo cGVHD as in the complication-free subgroup (Figure 1a). Frequencies of $CD4^+$ and $CD8^+$ T cell subsets, as categorized by their profiles of CD45RA and CCR7 expression, were also substantially similar (data not shown). Absolute numbers and percentages of $CD8^+$ T cells lacking CD28 expression tended to be higher by day 60, but there was substantial overlap with values from the complication-free subgroup, and formal statistical significance was not achieved (Figure 1b). The difference remained nonsignificant when the comparison included only patients who underwent bone marrow transplantation. Individual $CD4^+$ and $CD8^+$ T cell subsets in peripheral blood sampled after cGVHD was diagnosed were compared with values from the complication-free subgroup sampled at similar times. No statistically significant difference was evidenced, even if the patients with ongoing cGVHD tended to have greater numbers of $CD28^{neg}$ $CD8^+$ T cells by day 365 ($P = .09$).

There were 7 patients who had aGVHD preceding cGVHD without other clinical complications. Their median count of $CD28^{neg}$ $CD8^+$ T cells was 180 per mm^3 (range: 140-246) at day 60 ($P = .44$ compared with patients experiencing de novo cGVHD). Including these patients together with those with de novo cGVHD did not evidence a significant difference with the complication-free subgroup.

Recovery of the Main T Cell Subsets in Patients Who Relapsed

Full donor T cell chimerism was achieved among all patients who relapsed exclusive of any other clinical event (Table 1). Relative and absolute counts of $CD4^+$ T cells and their subsets were low and evolved similar to those of complication-free patients (data not shown). Absolute counts of $CD8^+$ T cells increased more slowly (Figure 1a), at around half of the values from complication-free patients, with subsequent higher CD4:CD8 ratios. There was a trend toward a smaller percentage of cells with a $CD8^+$ $T_{EM}RA$ phenotype that achieved formal statistical significance ($P = .01$) only on day 90 postgraft. Absolute numbers and percentages of all $CD8^+$ T cells lacking CD28 expression were significantly diminished from day 30 onward (Figure 1b). The median percentage of $CD28^{neg}$ $CD8^+$ T cells represented around 1/4th that of complication-free patients at all time points. The 3 patients who relapsed after receiving PBSC grafts had as low $CD28^{neg}$ $CD8^+$ T cell numbers as those who relapsed after marrow grafts (range at day 60: 46-165 and 6-227/mm^3, respectively; $P = .78$).

Five additional patients relapsed 71 to 316 days after being diagnosed first with de novo cGVHD. Their $CD8^+$ T cell counts and absolute numbers and percentages of $CD28^{neg}$ $CD8^+$ T cells (median of 94/mm^3 at day 60) ranged intermediate between corresponding values from the patients who relapsed without previous cGVHD (median of 47.5/mm^3) and the patients who were devoid of any clinical complication (median of 179/mm^3).

Univariate and Multivariate Analysis of Factors Affecting Relapse

All the patient and donor factors listed in Table 1 as potential predictors of relapse fell short of significance at the .05 level in univariate analysis comparing the subgroups of patients without complication, with only cGVHD and with only relapse. Although the proportion of patients receiving preparative TBI was lower in the complication-free subgroup, it was comparable between patients who experienced cGVHD and those who relapsed. Likewise, the proportion of patients receiving PBSC instead of bone marrow grafts was similar between patients with cGVHD and those who relapsed (Table 1).

Risk of developing relapse was further examined univariably at day 60 postgraft by categorizing all the 80 patients by the median of $CD28^{neg}$ $CD8^+$ T cell counts from the complication-free subgroup (179 cells/mm^3). Using day 60 values was elected because a complete set of phenotypic analyses was available for all the recipients at this time point, whereas some values were missing on day 30, because of insufficient lymphocyte counts for detailed subset analysis. All

but 5 of the 26 patients who subsequently relapsed had absolute numbers of $CD28^{neg}$ $CD8^+$ T cells <179 cells/mm^3, a significant difference by log-rank test (Figure 2).

Cox regression analysis for relapse as the endpoint was performed to compare the effect of recovering low $CD28^{neg}$ $CD8^+$ T cell counts with the recipient age and disease type, the presence or absence of TBI included in the myeloablative conditioning regimen, the donor type (sibling versus unrelated), the source of stem cells (marrow versus PBSC), and quantities of $CD8^+$ T cells infused into patients. Prior to all analyses, proportional hazard assumptions were tested and met in the subgroups with and without relapse. Considering the entire patient cohort, likelihood of relapse correlated inversely with absolute $CD28^{neg}$ $CD8^+$ T cell counts by day 60 (n = 80, adjusted hazard ratio of 0.33; 95% confidence interval [CI]: 0.14-0.76; $P = .01$). $CD28^{neg}$ $CD8^+$ T cell counts being predictive of relapse was confirmed by competitive risks regression analysis after adjustment for the other factors presented in Table 1 ($P = .01$). The inverse correlation was also verified by day 90 (0.29; 95% CI 0.13-0.65; $P = .003$).

None of the donor and patients characteristics was significantly associated with relapse in multivariate modeling. Marrow graft transferred fewer numbers of mature T cells to the recipient than did G-CSF mobilized PBSC. However, when patients who received PBSC grafts were excluded and only marrow recipients were tested in a multivariate model, the inverse correlation between likelihood of relapse and low absolute $CD28^{neg}$ $CD8^+$ T cell counts by day 60 remained strong (hazard ratio: 0.41; $P = .02$). Likewise, not including in the model the patients who relapsed after receiving a graft mismatched at 1 HLA-Cw locus still evidenced only $CD28^{neg}$ $CD8^+$ T cell counts as associated with relapse (hazard ratio 0.33; $P = .03$). Finally, when pediatric patients were excluded to analyze patients aged 16 years or older at the time of transplantation, the inverse correlation between likelihood of relapse and $CD28^{neg}$ $CD8^+$ T cell counts was still significant (hazard ratio 0.29; $P = .03$).

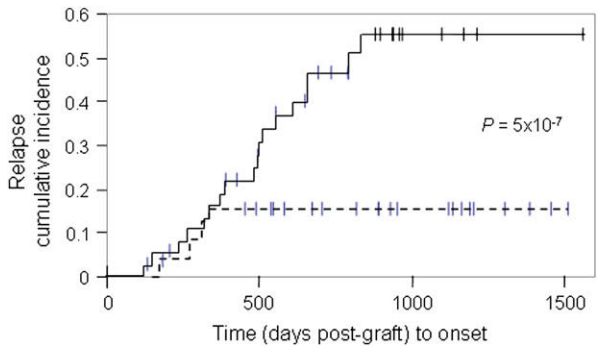

Figure 2. Kaplan-Meier estimate of malignant relapse after myeloablative HLA-matched allo-SCT. The 80 patients were categorized as having absolute $CD28^{neg}$ $CD8^+$ T cell numbers by day 60 postgraft falling lower (solid line) versus equal to or higher than (dashed line) the median (179 $CD28^{neg}$ $CD28^+$ T cells/mm^3 of peripheral blood) determined in the subgroup of 18 patients free of any clinical complication by the time of last follow-up. The *P* value is for the log-rank test.

DISCUSSION

Successful T cell recovery substantially reduces morbidity and mortality following allo-SCT for hematologic malignancies, but disease relapse and cGVHD remain major causes of late morbidity and mortality. This prospective analysis found that poor $CD8^+$ T cell recovery with diminished absolute $CD28^{neg}$ $CD8^+$ T cell counts in the first 2 months after myeloablative allo-SCT was significantly associated with later development of malignant relapse, whereas patients who developed progressive or de novo cGVHD without relapsing recovered T cell counts similar to patients free of any clinical complication.

The subgroup of patients who did not develop any transplantation-related complication and who did not relapse over a period of follow-up longer than 1 year allowed analyzing the kinetics of T cell reconstitution in the absence of possible interferences by infections and GVHD that can affect the number and memory phenotypes of T cells [10,17]. As expected, absolute counts of $CD4^+$ T cells remained low throughout the first year posttransplantation, whereas absolute counts of $CD8^+$ T cells increased rapidly over 3 to 6 months, however, with predominance of cells lacking CCR7 and CD28 expression and persisting low levels of cells with a naïve ($CD45RA^+$ $CCR7^+$ $CD28^+$) phenotype. There were presumably too few pediatric recipients in this series to detect any effect of age on recovery over the first year posttransplantation, but it is known that renewal of thymopoiesis cannot be evidenced before 6 months in young children and is slower in adolescents [3,5]. Accordingly, for the first several months after myeloablative transplantation, the number of circulating T cells depends primarily on homeostatic peripheral expansion rather than on the thymic function. There were also too few PBSC recipients to detect any faster recovery effect of this source of graft. As regards $CD28^{neg}$ $CD8^+$ T cells, however, their absolute counts were reported to be similar in the first year in marrow and in PBSC recipients [18]. Bone marrow grafts transfer around 10 times less mature T cells to the patient, but 3 to 4 more cell divisions would suffice to yield the same count of $CD28^{neg}$ $CD8^+$ T cells as in patients given PBSC grafts. CMV-specific $CD8^+$ T cells predominantly reside in the $CCR7^{neg}$ and $CD28^{neg}$ subsets [10], and we cannot formally exclude that CMV-reactive T cells disproportionately expanded whenever the donor and/or the recipient was

CMV-seropositive. However, this subgroup of patients was devoid of clinical and biologic signs of CMV reactivation, and there was no significant association between levels of $CD28^{neg}$ $CD8^+$ T cells and combinations of donor and recipient CMV serostatus. Homeostatic cytokines can also induce a stable loss of CD28 expression on expanding $CD8^+$ T cells [19,20], which in the absence of CMV disease suggests that homeostatic peripheral expansion directly contributed to the generation and accumulation of $CD28^{neg}$ $CD8^+$ T cells throughout the first year after allo-SCT. Accordingly, sustained accumulation of $CD28^{neg}$ $CD8^+$ T cells has been reported after high-dose chemotherapy or depleting mAb [4,21,22].

Kinetics of T cell reconstitution in patients who developed cGVHD was fairly similar to that in complication-free recipients. It is generally considered that the continuing risks of developing cGVHD may persist until 12 or 15 months postgraft. Our follow-up period was longer, so that incidence of symptomatic cGVHD was not underestimated. Our observation confirms earlier reports showing that $CD8^+$ T cell counts tended to be higher [23] or not significantly associated [24] with cGVHD, and that patients with poor early lymphocyte recovery were not at higher risk of aGVHD or cGVHD [25]. A few cross-sectional studies have variously reported on decreased $CD4^+$ T_{CM} [26,27], elevated $CD4^+$ T_{EM} [26], increased [28] or decreased [29] $CD8^+$ T_{CM}, and increased $CD8^+$ $T_{EM}RA$ [29], in patients who had cGVHD at time of sampling. There also exists uncertainty on whether the proportion of $CD8^+$ T cells lacking CD28 (or the overlapping $CD57^+$ subset) is increased [30] or not [24] during ongoing cGVHD. One possible explanation is that all these reports analyzed mononuclear cells (instead of whole blood as in this study), given that Ficoll purification can induce a bias in subset distribution, particularly among $CD8^+$ T cells [31]. Another difference is that some reports included patients after nonmyeloablative conditioning, who could be generally less lymphopenic, and therefore undergoing lesser homeostatic peripheral expansion.

cGVHD may involve both alloreactivity and autoreactivity against the host [3]. In fully HLA-matched donor/recipient pairs, donor T cells that have gone through homeostatic peripheral expansion while being exposed to ubiquitous minor histocompatibility antigens or self-peptides on host cells could be driven to differentiate into alloreactive effectors or to display pathogenic autoreactivity [32], respectively. Patients who will go on to develop cGVHD might therefore be expected to accumulate memory-like T cells at least as quickly as patients who undergo immune reconstitution without massively expanding pathogenic clones, notwithstanding expected differences in the respective clonal composition of their peripheral T cells [33,34].

Contrary to cGVHD, malignant relapse was consistently preceded by diminished absolute $CD8^+$ T cell counts, at around half of the values from patients who did not relapse. This is in agreement with other reports showing that marrow recipients with a high repopulation of $CD8^+$ T cells have a lower incidence of leukemia relapse [25,35]. Absolute $CD28^{neg}$ $CD8^+$ T cell counts were diminished at 1/4th of that of patients who did not relapse. This difference was significant from 30 days onward, long before the patients went on to relapse (at a median of 215 days in the patients who relapsed without prior cGVHD and after 152 days or more in the patients with cGVHD preceding relapse). Multivariate proportional hazard analyses revealed low absolute $CD28^{neg}$ $CD8^+$ T cell counts by day 60 postgraft as the only significant risk factor of malignant relapse, and their predictive value was confirmed by using competitive risk multivariate modeling. All patients included in this study were transplanted in complete hematologic remission at transplantation, the distribution of underlying diseases was comparable whether or not patients went on to relapse, and no patient was at high risk of relapse. The proportion of PBSC recipients in subgroups that did or did not relapse was also comparable. Furthermore, multivariate proportional hazard analysis including only bone marrow recipients confirmed the inverse correlation between likelihood of relapse and absolute $CD28^{neg}$ $CD8^+$ T cell counts by day 60.

The GVL effect to prevent relapse can be mediated variously by donor-derived T cells and natural killer cells. Missing killer immunoglobulin-like receptor recognition is not expected to predominate after 10 of 10 HLA-matched grafts, and excluding from multivariate analysis the few patients receiving a graft with 1 HLA-Cw mismatch did not ablate the significance of the association between $CD28^{neg}$ $CD8^+$ T cell counts and risk of relapse. Homeostatic proliferation following lymphopenia may facilitate expansion of antitumor immunity. Then, insufficient expansion might result from insufficient interaction of donor T cells with antigens present in the host, including hematopoietic tissue-restricted minor histocompatibility antigens and leukemia-specific antigens that would expand tumor-specific T cell clones [1,6,7]. $CD28^{neg}$ $CD8^+$ T cells are endowed with high cytotoxic capacities and diversified cytokine production [8-11]. Lower absolute $CD28^{neg}$ $CD8^+$ T cell counts might therefore reflect lesser generation of T cells with eradicating potential against the hematologic malignancy, whether directly or through a reduced bystander (cytokine-dependent) effect.

This prospective study is the first to associate a diminished absolute number of circulating $CD28^{neg}$ $CD8^+$ T cells with a greater risk of malignant relapse after a myeloablative conditioning. Enumeration of circulating $CD8^+$ T cells lacking CD28 expression

by day 60 postgraft could assist in predicting risk of relapse. This can help build an algorithm aimed at accelerating the immune recovery by reducing the intensity of posttransplant immunosuppressive regimen [36] and considering the introduction of posttransplant immunotherapy (preemptive donor lymphocyte infusions [DLI]) to overcome the risk of disease relapse after the acute risks of allo-SCT have passed.

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