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Chimerism Studies in HLA-Identical Nonmyeloablative Hematopoietic Stem Cell Transplantation Point to the Donor CD8$^+$ T-Cell Count on Day +14 as a Predictor of Acute Graft-versus-Host Disease

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ABSTRACT

Chimerism analysis of hematopoietic cells has emerged as an essential tool in nonmyeloablative hematopoietic stem cell transplantation. We have investigated the development of donor chimerism in granulocytes and CD4$^+$ and CD8$^+$ T cells in blood and bone marrow of 24 patients with hematologic malignancies who received HLA-identical sibling peripheral blood stem cell grafts after conditioning with fludarabine and 2 Gy of total body irradiation. The T-cell chimerism of blood and bone marrow was tightly correlated. Complete donor chimerism was reached earlier in the granulocytes than in the T cells. Mixed T-cell chimerism was common at the time of onset of acute graft-versus-host disease (aGVHD), and both CD4$^+$ and CD8$^+$ donor T-cell chimerism increased with the occurrence of aGVHD grades II to IV (P = .0002 and P = .019, respectively). The rate of disappearance of recipient CD8$^+$ T cells was faster in patients with aGVHD grades II to IV than in patients without clinically significant aGVHD (P = .016). This observation indicates a role of graft-versus-lymphohematopoietic tissue reactions in creating complete donor T-cell chimerism. A donor CD8$^+$ T-cell count above the median on day +14 increased the risk of subsequent development of aGVHD grades II to IV (P = .003).

KEY WORDS

Nonmyeloablative hematopoietic stem cell transplantation • Donor chimerism • CD8$^+$ T-cell count • Acute graft-versus-host disease

INTRODUCTION

Conditioning regimens that are nonmyeloablative or have reduced intensity have extended the use of allogeneic hematopoietic stem cell transplantation (HSCT) to patients who, because of older age or comorbidity, were previously ineligible for this potentially curative treatment [1-10]. In nonmyeloablative HSCT (NST), the eradication of the malignant disease is dependent on a graft-versus-tumor (GVT) effect, and there is a gradual change from recipient to donor hematopoiesis. Determination of the level of donor chimerism of subpopulations of leukocytes sequentially after transplantation is therefore an integrated part of NST, and clinical interventions such as changes in the pharmacologic immunosuppression or donor lymphocyte infusions (DLI) may depend on chimerism status [11]. Graft-versus-host disease (GVHD) is a significant complication of NST and in some studies is a major cause of nonrelapse mortality [6,7,9,12]. Furthermore, GVHD may occur as a complication of DLI. If the occurrence of GVHD could be predicted, individually tailored immunosuppression might subsequently improve the treatment results. Whether complete donor T-cell chimerism is needed before alloresponses such as GVHD or the

Table 1. *Patient Characteristics*

Patient No.	Diagnosis	Age, R/D (y)	Sex (R/D)	No. Previous Chemotherapy Regimens	No. Auto Tx	$CD3^+$ Cells ($\times 10^8$/kg)	Graft $CD4^+/CD8^+$ T-Cell Ratio	$CD34^+$ Cells ($\times 10^6$/kg)	CSP Taper before Day +100 (Start Day)	aGVHD Grade/ Day
628	NHL + t-MDS, RA	45/44	F/F	4	1	5.9	2.7	2.8		II/38
633	NHL	52/55	M/F	7	0	3.8	2.0	8.9	35	II/77
636	CLL	56/52	M/M	5	0	5.0	3.4	11.6	35	II/40
645	NHL	46/52	M/M	5	2	3.1	2.5	5.9	35	II/41
650	MM	42/40	M/M	2	1	5.1	2.3	5.7		II/34
656	t-MDS, RA	56/53	M/M	1	0	3.6	2.2	8.9		IV/33
662	NHL	55/51	M/M	6	0	3.2	1.9	7.1		IV/21
666	NHL	57/60	F/F	7	1	ND	ND	3.1		I/18
669	SLL	49/53	M/M	2	0	3.6	2.5	14.2		I/35
674	AML	27/29	F/M	3	0	3.7	1.2	28.8		III/7
682	CLL	57/58	M/F	7	0	2.2	3.8	4.7	68	I/45
688	T-NHL	34/41	M/F	3	0	4.3	2.4	4.5		0/—
692	T-NHL	37/43	M/F	2	0	3.0	ND	9.5	47	0/—
694	NHL	51/57	M/F	10	0	3.6	1.2	11.4		III/54
708	MM	59/57	M/M	4	1	3.7	2.0	6	56	0/—
715	t-MDS, RAEB	51/49	F/F	1	0	2.8	2.7	4.8	63	0/—
723	NHL + t-MDS, RA	49/43	M/M	6	1	4.9	2.7	4.7	56	0/—
733	MM	63/52	M/F	3	1	4.4	3.4	7.7		II/47
741	HD	34/36	F/M	5	1	6.8	2.4	21.7	73	II/31
749	MM	47/41	M/M	2	1	3.2	1.9	3.8	91	0/—
760	MM	51/45	F/F	4	2	ND	ND	6.5		II/31
766	CLL	57/68	M/F	5	0	ND	ND	2.6		II/31
773	MM	51/56	F/M	4	2	5.1	2.7	14.4	63	0/—
780	HD	42/44	M/F	7	2	3.3	1.9	4.7		II/29

NHL indicates non-Hodgkin lymphoma; t-MDS, therapy-related myelodysplastic syndrome; RA, refractory anemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; SLL, small lymphocytic lymphoma; AML, acute myeloid leukemia; T-NHL, T-cell non-Hodgkin lymphoma; HD, Hodgkin disease; RAEB, refractory anemia with excess of blasts; R/D, recipient/donor; Auto Tx, autologous hematopoietic stem cell transplantation; CSP, cyclosporine; aGVHD, acute graft-versus-host disease; ND, not done.

GVT effect can occur is a matter of controversy. Childs et al. [3] found that complete donor T-cell chimerism preceded the occurrence of GVHD and disease responses, whereas Mattsson et al. [13] observed both GVHD and disease responses in the context of mixed chimerism. We have analyzed the kinetics of the development of donor chimerism in granulocytes and $CD4^+$ and $CD8^+$ T cells in both peripheral blood (PB) and bone marrow (BM) in patients conditioned with a nonmyeloablative regimen of fludarabine 30 mg/m^2 for 3 days and 2 Gy of total body irradiation (TBI) [6,14]. We observed that most of the patients were mixed T-cell chimeras when they experienced acute GVHD (aGVHD), and an increase in donor T-cell chimerism was observed during the period when the patients had aGVHD. In addition, we found that the day +14 donor $CD8^+$ T-cell count had predictive value for the subsequent development of aGVHD.

MATERIALS AND METHODS

Patients

Twenty-four patients received an NST with PB stem cells (PBSC) from HLA-identical sibling donors between March 2000 and October 2002. Patients aged 18 to 70 years with hematologic malignancies were eligible for NST if they—because of diagnosis, comorbidity, or age—were considered to have a high treatment-related mortality with conventional HSCT. The median age of the patients was 51 years at the time of transplantation. They were generally heavily pretreated and had received a median of 4 chemotherapy regimens before the NST. Twelve patients had previously undergone autologous stem cell transplantation (Table 1). Written, informed consent was obtained from all patients and donors enrolled in the study. The study was approved by the local ethics committee.

Conditioning Regimen and GVHD Prophylaxis

The patients underwent transplantation with a conditioning regimen that consisted of 2 Gy of TBI on day 0 (patients 628, 636, and 674) or fludarabine 30 mg/m^2 intravenously once daily on day −4 to day −2 and 2 Gy of TBI on day 0 (for the remaining 21 patients) [6,14]. Oral mycophenolate mofetil 30 mg/kg/d was initiated on day 0 and discontinued on day +27 in the absence of GVHD. Oral cyclosporine 12.5 mg/kg/d was initiated on day −3. In the absence of

GVHD, cyclosporine taper was planned to begin on day +35 in patients 628 to 674 and on day +56 in patients 682 to 780. DLI in increasing doses starting at 1×10^3 $CD3^+$ cells per kilogram could be given after day +56 to increase the donor T-cell chimerism or as treatment of progression or relapse of the malignant disease. GVHD was graded according to standard criteria [15].

Mobilization of PBSC

The donors received filgrastim 16 μg/kg/d from day −4 to day 0. Leukapheresis was performed on days −1 and 0, and both leukapheresis products were given to the patient. The graft contents of $CD3^+$ T cells, $CD4^+$ T cells ($CD3^+$, $CD4^+$, and $CD8^-$), $CD8^+$ T cells ($CD3^+$, $CD8^+$, and $CD4^-$), and $CD34^+$ cells were quantified by use of monoclonal antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA) and 3-color flow cytometry. The median cell numbers infused per kilogram of recipient weight were as follows: 3.7×10^8 $CD3^+$ T cells, 2.5×10^8 $CD4^+$ T cells, 1.15×10^8 $CD8^+$ T cells, and 6.25×10^6 $CD34^+$ cells (Table 1).

T-Cell Counts

TruCOUNT tubes and monoclonal antibodies obtained from Becton Dickinson were used to quantify the absolute numbers of $CD4^+$ T cells and $CD8^+$ T cells in the PB by 3-color flow cytometry. Five thousand beads were analyzed in each sample. The normal values were obtained from 51 healthy donors with a median age of 43 years (range, 16-62 years) [16].

Cell Separation and DNA Extraction

Ten milliliters of PB or 3 ml of BM was used for chimerism analysis. BM samples were obtained monthly during the first 4 months after transplantation and thereafter at longer intervals. The separation of T-cell subsets for DNA extraction was performed either by isolation of the mononuclear cells by gradient centrifugation (Lymfoprep; Pharmacia, Peapack, NJ) and purification of the T-cell subsets from the mononuclear cells with anti-CD4 and anti-CD8 immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway) or by direct isolation of $CD4^+$ T cells and $CD8^+$ T cells from whole blood with the immunomagnetic beads. The chimerism analysis of granulocytes was performed either on DNA extracted from the pellet obtained from gradient centrifugation or on $CD15^+$ cells isolated directly from whole blood with anti-CD15 immunomagnetic beads (Dynabeads). Potential contamination of the separated T cells with $CD4^+$ monocytes ($CD4^+$, $CD14^+$, and $CD3^-$) or $CD8^+$ natural killer cells ($CD8^+$, $CD16^+$, $CD56^+$, and $CD3^-$) was examined by 3-color flow cytometry and was <3% in the $CD4^+$ T-cell population and <1% in the $CD8^+$ T-cell population with both separation methods (data not shown). DNA was isolated from each cell population by NaCl extraction [17].

Chimerism Analysis

The level of donor chimerism was determined by fluorescence-based polymerase chain reaction (PCR) of short tandem repeats [18]. Separation and detection of the amplified PCR products was performed on an automated 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), and the results were analyzed with GeneScan software (Applied Biosystems). The detection limit of the method was generally 1%, but varying signal intensity of the primers increased the detection limit to 5% in 26 of 823 analyzed DNA samples and to 10% in 3 samples. Complete donor chimerism was defined as the absence of detectable PCR fragments of patient origin. This implied that in the vast majority of samples, mixed chimerism was defined as 1% to 99% donor cells, and complete donor chimerism was defined as >99% donor cells.

Statistical Methods

Time-dependent variables were analyzed by the method of Kaplan and Meier, and the cumulative incidences were compared by using the log-rank test. Categorical variables were compared by using the Fisher exact test. Continuous variables were compared by using paired and unpaired nonparametric tests as appropriate. The calculated numbers of T cells of recipient origin after transplantation were log-transformed, and linear regression analysis was performed to compare the rate of decrease in patients with and without aGVHD. Nonparametric correlation (Spearman) was used to correlate the T-cell chimerism in PB and BM and to correlate the graft CD4/CD8 T-cell ratio with the ratio of the donor T cells after transplantation. All calculations, regressions, and comparisons were performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). A P value <.05 was considered significant.

RESULTS

Immunosuppression, GVHD, and Follow-up

The tapering regimen for cyclosporine was not strictly followed in all patients (Table 1). In 3 patients, cyclosporine was changed to tacrolimus before day +100 because of nausea and vomiting (patient 656; day +16), liver toxicity of cyclosporine (patient 662; day +27), and thrombotic thrombocytopenic purpura (patient 760; day +49). Acute GVHD grades II to IV occurred in 59% of the patients, with a median onset on day +33 (Table 1). Eight patients (patients 636, 656, 662, 666, 674, 688, 715, and 780) died on day

+297, +125, +72, +267, +38, +659, +178, and +142, respectively. As analyzed on September 12, 2003, the median follow-up in the surviving patients was 670 days (range, 319-1263 days).

Donor Lymphocyte Infusion

In 3 patients, DLI (1×10^7 $CD3^+$ cells per kilogram) was given within the first year after transplantation. The reasons for DLI were progression of the malignant disease (patient 682; day +116), low donor T-cell chimerism (patient 692; day +115), and the combination of disease progression and low donor T-cell chimerism (patient 715; day +84).

Chimerism

The kinetics of donor chimerism development differed in the 3 cell populations studied (Figure 1). The donor $CD4^+$ T-cell chimerism was higher than the donor $CD8^+$ T-cell chimerism on day +7 ($P = .007$; Wilcoxon matched pairs test). From day +14, no significant differences in $CD4^+$ and $CD8^+$ T-cell chimerism were observed. Both the $CD4^+$ and the $CD8^+$ T-cell chimerism were higher than the granulocyte chimerism on day +7 and +14 ($P = .0001$; Wilcoxon matched pairs test), but complete donor chimerism was generally reached earlier in the granulocyte compartment, with a median of 42 days (range, 14-104 days), than within the T cells, where the medians were 154 days (range, 14-378 days) for the $CD4^+$ T cells and 120 days (range, 14-378 days) for the $CD8^+$ T cells. No patients rejected their grafts, but patient 715 had a relapse of acute myeloid leukemia on day +56 and subsequently had a decrease in the level of donor granulocyte chimerism (Figure 1c). Tapering of cyclosporine, DLI, or both was used in several cases, with the purpose of converting a low level of donor T-cell chimerism to complete donor chimerism. In patient 708, a decrease in the donor $CD8^+$ T-cell chimerism led to tapering of cyclosporine, and subsequently both T-cell subsets became of complete donor origin (Figure 2a). In patient 692, the conversion to complete donor T-cell chimerism occurred only after DLI (Figure 2b). In patient 715, the discontinuation of cyclosporine or DLI had no effect either on the level of donor T-cell chimerism or on the disease burden (Figure 2c). The value for T-cell chimerism in the PB very accurately reflected the value in the BM and showed a strong correlation both on day +28 ($R = 0.91$ and $P < .0001$ for the $CD4^+$ T cells; $R = 0.82$ and $P = .0002$ for the $CD8^+$ T cells) and on day +56 ($R = 0.87$ and $P < .0001$ for the $CD4^+$ T cells; $R = 0.96$ and $P < .0001$ for the $CD8^+$ T cells; data not shown). The donor granulocyte chimerism in most of the BM samples showed >95% donor cells, but in patient 715, the donor granulocyte

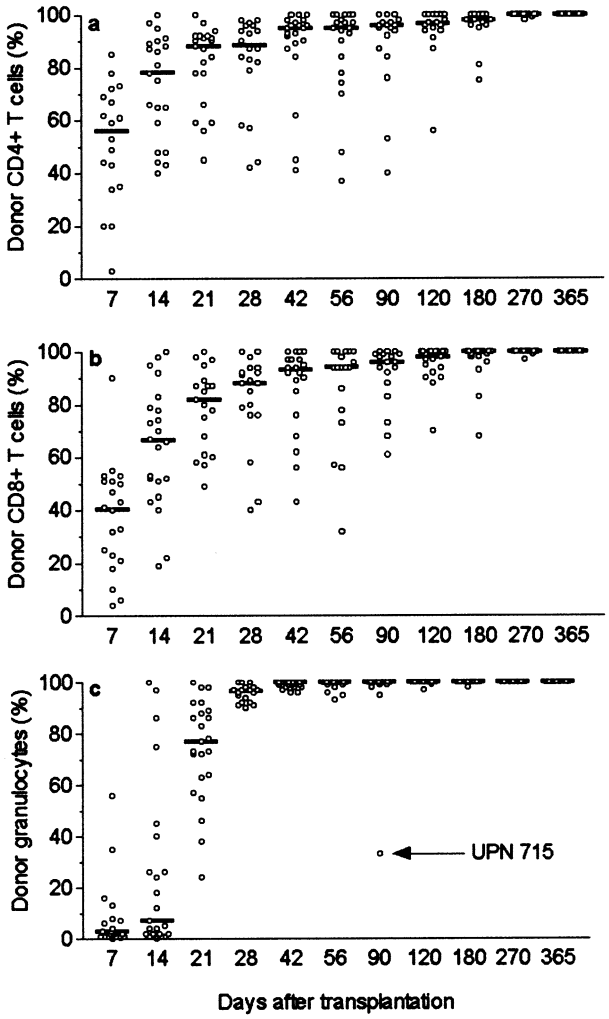

Figure 1. Donor T-cell and granulocyte chimerism after nonmyeloablative HSCT. Chimerism analysis of $CD4^+$ T cells (a), $CD8^+$ T cells (b), and granulocytes (c). The horizontal bar in each group of data points represents the median.

chimerism on the day of relapse was markedly lower in the BM (77%) than in the PB (99%).

The occurrence of aGVHD did not seem to influence the donor chimerism development in the granulocyte compartment (Figure 3a), whereas patients who developed clinically significant aGVHD (grades II-IV) seemed to have a faster increase in the degree of donor T-cell chimerism when compared with patients who did not experience aGVHD (Figure 3b and 3c). The relationship between aGVHD and donor T-cell chimerism was further studied in 13 of 14 patients who experienced aGVHD grades II to IV and for whom data on the donor T-cell chimerism before and after the onset of aGVHD were available (Figure 4). In most patients, aGVHD occurred in the context of mixed chimerism, and both the $CD4^+$ and $CD8^+$ donor T-cell chimerism increased significantly during aGVHD ($P = .0002$ and $P = .019$, respectively; Wilcoxon matched pairs test).

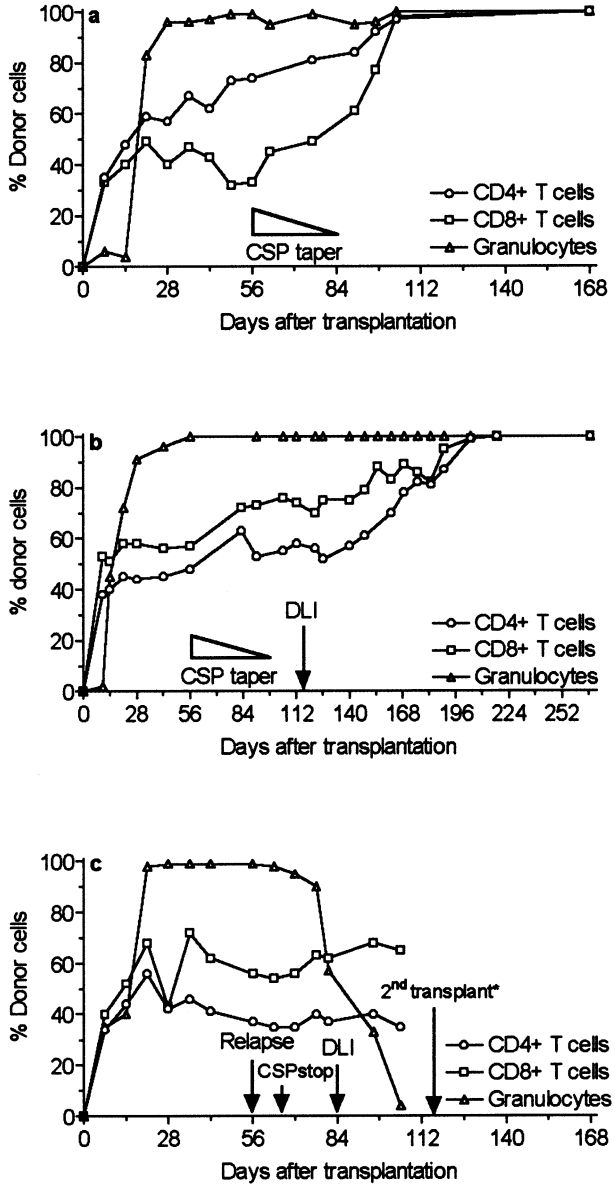

Figure 2. Effect of cyclosporine taper and DLI on T-cell chimerism. Chimerism development of $CD4^+$ T cells, $CD8^+$ T cells, and granulocytes in patient 708 (a), patient 692 (b), and patient 715 (c). CSP, cyclosporine; DLI, donor lymphocyte infusion. *Patient 715 received a second NST with cells from the original donor. The patient died 62 days after the second transplantation because of a new relapse of acute myeloid leukemia.

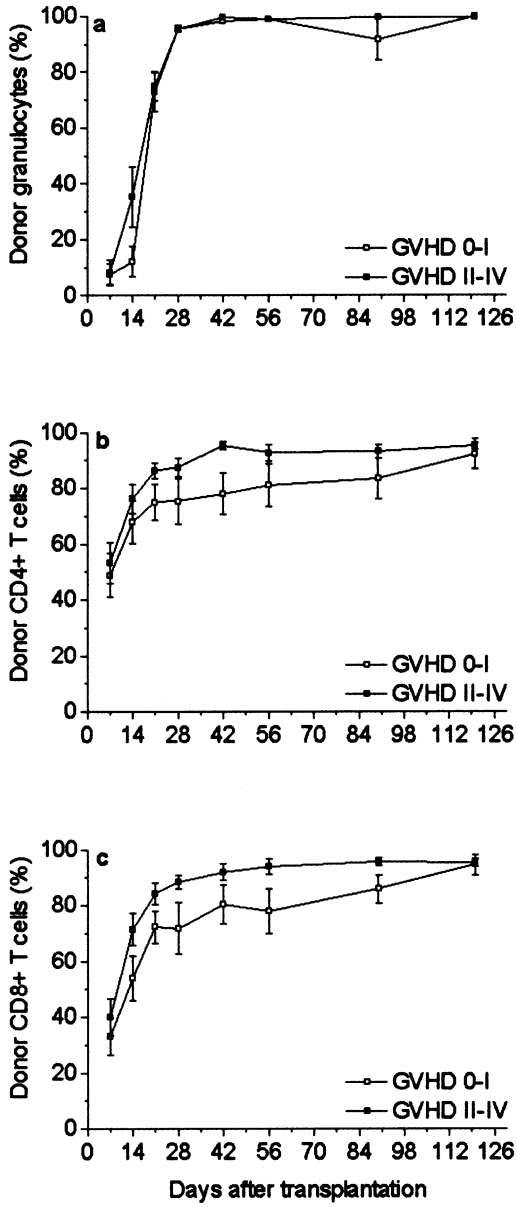

Figure 3. Donor chimerism in patients with and without acute GVHD. Donor chimerism (mean and SEM [error bars]) of granulocytes (a), $CD4^+$ T cells (b), and $CD8^+$ T cells (c) in patients with aGVHD grades 0 to I and grades II to IV.

Patient and Donor T-Cell Counts

Measurement of both chimerism and absolute T-cell subset counts enabled us to calculate the absolute number of $CD4^+$ and $CD8^+$ T cells of recipient and donor origin after transplantation. Because the studies of donor T-cell chimerism had suggested a more rapid increase in the patients who developed aGVHD grades II to IV, we investigated whether the same relationship was present for the absolute numbers of donor T cells. The patients were divided on the basis of the occurrence of aGVHD, and the increase in donor T-cell counts and decrease in patient T-cell counts were studied (Figure 5a and 5b). In the first 4 weeks after transplantation, both the $CD4^+$ and the $CD8^+$ donor T-cell counts resembled the observations performed for the chimerism data, with a tendency toward higher counts in the patients who developed aGVHD (Figure 5a and 5b). However, when the data for the individual time-points were analyzed, this difference was significant only for the $CD8^+$ T cells on day +14 ($P = .01$; Mann-Whitney test; Figure 5c). There was a clear correlation between the CD4/CD8 ratio of the grafts and the CD4/CD8 ratio of the donor T cells on day +7 ($R = 0.75$; $P = .0005$) but not

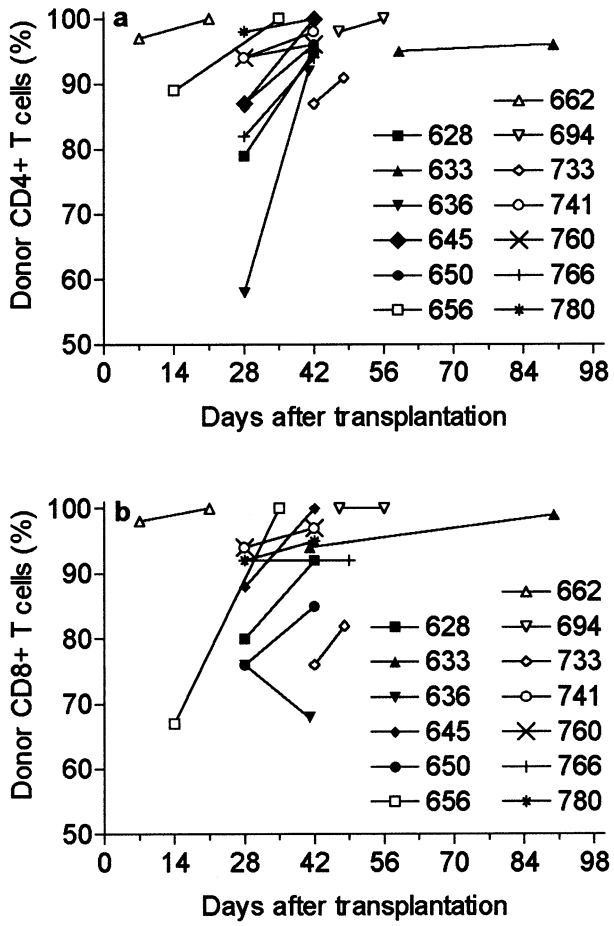

Figure 4. Donor T-cell chimerism before and after the onset of acute GVHD. Donor $CD4^+$ T-cell chimerism (a) and $CD8^+$ T-cell chimerism (b) before and after the onset of aGVHD grades II to IV in 13 patients. The unique patient number for each patient is shown.

on day +14 ($R = 0.25$; $P = .35$; data not shown). We compared the recipient T-cell counts obtained before conditioning with the recipient-derived T-cell counts on day +7 (Figure 5a and 5b). Both subsets were reduced ($P = .0006$ for the $CD4^+$ T cells and $P < .0001$ for the $CD8^+$ T cells; Wilcoxon matched pairs test), but the $CD4^+$ T-cell counts were less reduced (median, 32%; range, 6%-139% of the pretransplantation value) than the $CD8^+$ T-cell counts (median, 11%; range, 2%-63%; $P = .0003$; Wilcoxon matched pairs test). No difference in this reduction was observed between patients who did or did not develop aGVHD ($P > .9$ for both subsets; Mann-Whitney test). Beyond day +7, we analyzed the rate of decrease of T cells of recipient origin in the 2 groups of patients by linear regression analysis of the log-transformed recipient T-cell counts. In the period when most patients experienced aGVHD, ie, between days +7 and +56, the slopes of the regression lines were compared. We observed that there was a trend toward a higher rate of decrease of $CD4^+$ T cells of recipient origin (Figure 5d) and that the $CD8^+$ T cells of recipient origin disappeared faster in patients with aGVHD grades II to IV than in patients without aGVHD ($P = .016$; Figure 5e).

Predictive Value of Early Measurements of Chimerism and T-Cell Counts

As mentioned previously and as shown in Figure 5c, the $CD8^+$ donor T-cell count on day +14 was higher in patients who developed aGVHD when compared with patients who did not experience this complication. To analyze the predictive value of the $CD8^+$ donor T-cell count on day +14 for the subsequent occurrence of aGVHD, we divided the patient population in 2 by using the median value of this variable (0.0429×10^6 cells per milliliter). The cumulative incidence of aGVHD in the 2 groups of patients was then estimated by use of Kaplan-Meier statistics and compared by using the log-rank test (Figure 5f). Three patients were not included in this analysis: in patient 633 and patient 666, no chimerism data on day +14 were available, and patient 674 had the onset of aGVHD on day +7. These patients were therefore excluded. The same analysis was performed for the donor $CD8^+$ T-cell chimerism and the total $CD8^+$ T-cell count on day +14. We found that whereas neither donor $CD8^+$ T-cell chimerism alone nor the total $CD8^+$ T-cell count on day +14 alone could predict the occurrence of aGVHD ($P = .25$ and $P = .08$, respectively), the combined effect of these 2 factors, ie, the donor $CD8^+$ T-cell count, predicted aGVHD development ($P = .003$; Figure 5f). Factors such as the infused $CD3^+$ cell number and recipient age are known risk factors for the development of aGVHD. We did not observe any relationship between the donor $CD8^+$ T-cell count on day +14 and other possible risk factors for the development of aGVHD (Table 2). A multivariate analysis of a larger cohort of patients is, however, needed to examine whether the donor $CD8^+$ T-cell count on day +14 is an independent risk factor for the development of aGVHD in this setting.

DISCUSSION

In HLA-identical NST, the curative principle is the GVT effect mediated by the donor T cells. The donor T cells may, however, also mediate GVHD, which is a serious complication of this treatment. In this study, we investigated the development of donor chimerism in blood and BM after NST and focused on relations between aGVHD; donor granulocyte, $CD4^+$, and $CD8^+$ T-cell chimerism; and the absolute numbers of donor and recipient T cells. With use of a nonmyeloablative regimen developed at The Fred Hutchinson Cancer Research Center [6,14], 24 pa-

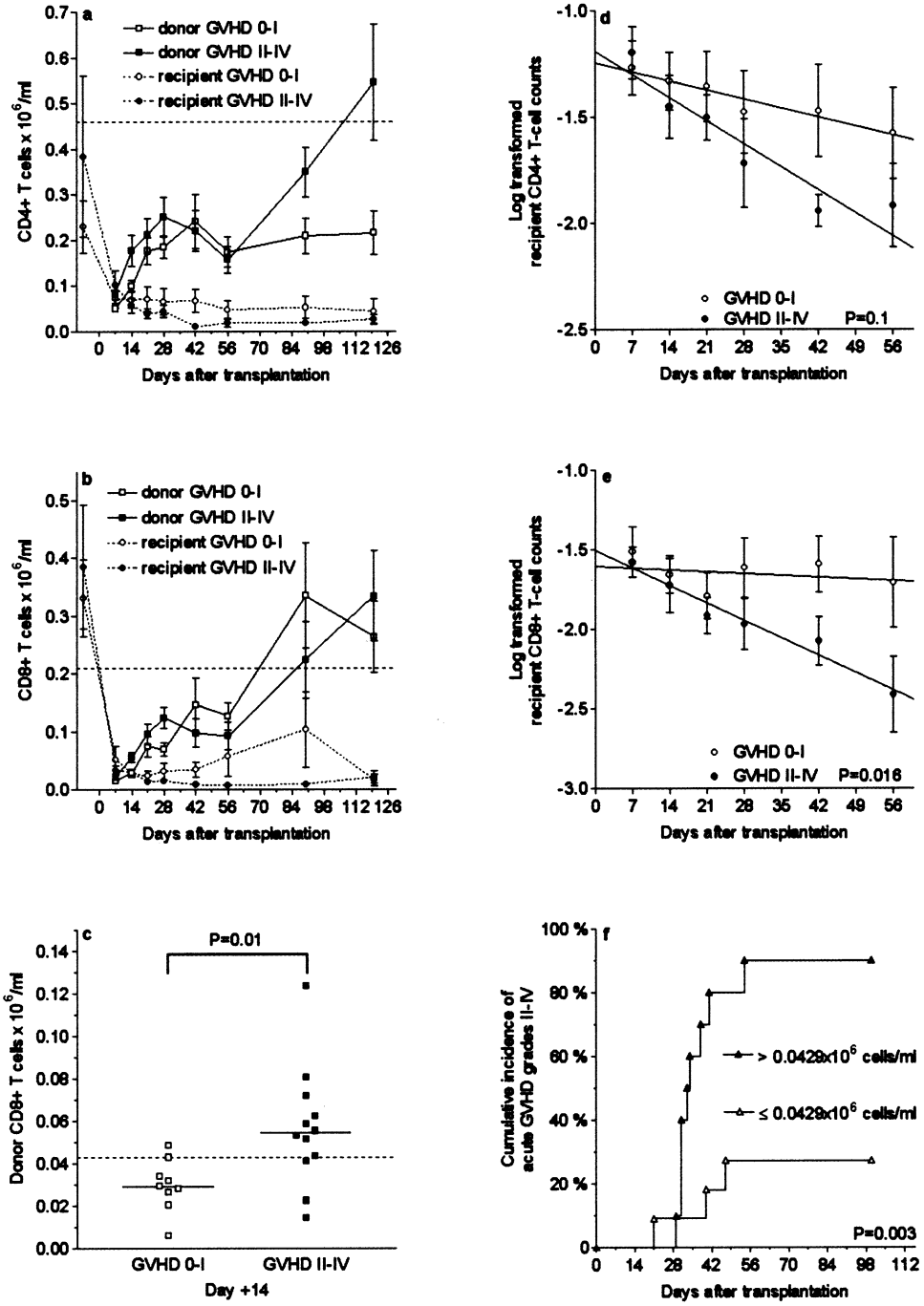

Figure 5. T-cell counts in patients with and without acute GVHD. Absolute numbers (mean and SEM [error bars]) of $CD4^+$ T cells (a) and $CD8^+$ T cells (b) of donor and recipient origin in patients with aGVHD grades 0 to I and grades II to IV are shown. The horizontal dotted lines in (a) and (b) represent the 5th percentile of the counts in 51 healthy donors. c, Comparison of the donor $CD8^+$ T-cell count on day +14 in 21 patients with aGVHD grades 0 to I and grades II to IV. The horizontal bar in each group of data points represents the median. The dotted line represents the median of the 21 patients (0.0429×10^6 donor $CD8^+$ T cells per milliliter). The *P* value of the Mann-Whitney test is shown. Linear regression analysis of the log-transformed recipient $CD4^+$ T-cell counts (d) and $CD8^+$ T-cell counts (e) from day +7 to day +56 in patients with aGVHD grades 0 to I and grades II to IV is shown. Mean values and SEM (error bars) are shown. The slopes of the regression lines were compared, and the *P* values are shown. f, Kaplan-Meier plot of the cumulative incidences of aGVHD grades II to IV in patients with a donor $CD8^+$ T-cell count less than or equal to or greater than the median on day +14. The cumulative incidences of aGVHD were compared by using the log-rank test, and the *P* value is shown.

tients underwent transplantation with PBSC from HLA-identical sibling donors. Acute GVHD grade II to IV occurred in 59% of the patients. Similar or slightly lower incidences of aGVHD were reported by the Seattle consortium when this regimen was used in the HLA-identical setting [6,14,19,20].

Table 2. *Possible GVHD-Associated Factors versus the Day +14 Donor $CD8^+$ T-Cell Count*

Variable	Day +14 $CD8^+$ Donor T-Cell Count ≤0.0429 × 10^6/mL (n = 11), Median (Range)	Day +14 $CD8^+$ Donor T-Cell Count >0.0429 × 10^6/mL (n = 10), Median (Range)	*P* Value*
Pretransplantation characteristics			
Recipient age (y)	51 (34-63)	49 (34-57)	.34
Donor age (y)	52 (41-63)	47 (36-68)	.67
Previous chemotherapy regimens	4 (2-7)	5 (1-10)	.89
Previous autotransplantation	5	6	.67
Female donor to male recipient	4	3	.99
Graft composition			
Infused $CD3^+$ T cells × 10^8/kg	3.7 (2.2-5.1)	3.6 (2.8-6.8)	.74
Infused $CD4^+$ T cells × 10^8/kg	2.8 (1.7-3.9)	2.4 (2.0-4.8)	.97
Infused $CD8^+$ T cells × 10^8/kg	1.1 (0.5-1.4)	1.3 (0.8-2.0)	.41
Infused $CD34^+$ cells × 10^6/kg	7.1 (3.8-14.4)	5.8 (2.6-21.7)	.55

*The categorical variables previous autotransplantation and female donor to male recipient were compared by using the Fisher exact test. The other possible risk factors were considered as continuous variables and were compared by using the Mann-Whitney test.

The granulocyte donor chimerism was lower than the T-cell chimerism for the first 2 weeks. After that, a rapid increase in donor granulocyte chimerism occurred, with the result that complete donor granulocyte chimerism was reached earlier than complete donor T-cell chimerism. A similar pattern of the development of donor granulocyte chimerism has been reported previously by others who have used the same transplantation regimen [21-23], and it may reflect the short life span of the infused granulocytes. An increase in donor granulocyte chimerism in the PB must await the generation of new granulocytes from the engrafted precursors in the BM. In contrast, the infused donor T cells may undergo peripheral expansion and thereby increase the donor chimerism early after transplantation. The delay in the T cells to become entirely of donor origin when compared with the granulocytes has been reported previously [13,24]. The kinetics of the development of donor chimerism in PB cell subpopulations may be affected by the conditioning regimen used. Using a regimen based on cyclophosphamide and fludarabine, Childs et al. [3] observed that 100% donor T-cell chimerism generally occurred before complete donor myeloid chimerism. Valcarcel et. al. [25] found that patients conditioned with melphalan and fludarabine were complete donor chimeras in both T cells and granulocytes on day +30, whereas patients conditioned with busulphan and fludarabine were mixed T-cell chimeras and complete donor granulocyte chimeras at the same time-point.

Canine and murine studies have shown that a state of mutual graft/host tolerance and absence of GVHD can be created by the induction of stable mixed chimerism [26-28]. In humans, it is still an open question whether mixed chimerism per se protects the recipient against GVHD. Childs et al. [3] reported that aGVHD occurred only in patients who were 100% donor T-cell chimeras, whereas Mattsson et al. [13] found that mixed chimerism was common at the time of onset of aGVHD. In our study, most of the patients experienced aGVHD when they were still mixed T-cell chimeras, but a significant increase in the degree of donor T-cell chimerism was observed when the patients had aGVHD.

The toxicity of the conditioning regimen and the alloreactivity of the donor T cells may both lead to the elimination of the recipient T cells after NST. Storb et al. [29] and Georges and Storb [30] have proposed a model in which alloreactive donor T cells eliminate the recipient lymphohematopoietic tissues during subclinical or clinical graft-versus-host reactions, which results in an increase in the donor T-cell chimerism. In this study, we observed a reduction in the T cells of recipient origin; this was most pronounced in the $CD8^+$ T-cell subset when we compared the pretransplantation values and the values on day +7 (Figure 5a and 5b). Because of the lack of difference in this reduction between patients who developed aGVHD and patients who did not, we find it likely that the reduction in T cells of recipient origin until day +7 is mainly due to the toxicity of the conditioning regimen and that the $CD8^+$ T cells may be more sensitive to this toxicity than the $CD4^+$ T cells. However, both the finding that the donor T-cell chimerism increased during the occurrence of aGVHD (Figure 4) and the observation of a faster rate of disappearance of recipient $CD8^+$ T cells after day +7 in patients who experience aGVHD grades II to IV (Figure 5e) support the hypothesis by Storb et al.

In allogeneic HSCT with myeloablative conditioning, the onset of aGVHD is generally observed within the first month after transplantation, and in a large study of HLA-identical BM transplantation (BMT), aGVHD occurred with a median onset of 16 days after transplantation [31]. In contrast, we found that aGVHD occurred rather late, with a median onset of 33 days after transplantation. Similarly, in a larger cohort of recipients of HLA-identical sibling

NST for which the same postgrafting immunosuppression regimen was used, the median day of onset of aGVHD was day +40 [6]. In that report, the level of donor T-cell chimerism in the PB on day +28 predicted the subsequent occurrence of aGVHD grades II to IV. This relationship was, however, not seen when more patients were analyzed [19]. In this study, the donor T-cell chimerism on day +28 could not predict the occurrence of aGVHD; instead, we showed that the donor $CD8^+$ T-cell count on day +14 had predictive value for the subsequent occurrence of aGVHD grades II to IV. The ability to predict aGVHD on the basis of a blood sample on day +14 and the late occurrence of aGVHD with this regimen is important for the clinical usefulness of the obtained information, because this leaves ample time for sample analysis and possible intervention. The relationship between early T-cell reconstitution and aGVHD has been studied previously in myeloablative BMT in different clinical settings. Gratama et al. [32] found a correlation between a low CD4/CD8 T-cell ratio on days +18 to +25 and the occurrence of aGVHD in 24 patients who received HLA-identical BMT with methotrexate as GVHD prophylaxis. When the authors subsequently analyzed a cohort of 23 recipients of HLA-identical BMT with cyclosporine as GVHD prophylaxis, they did not observe this correlation [33]. Soiffer et al. [34] reported in a study of 136 recipients of $CD6^+$ T-cell depleted BMT, in whom no immunosuppressive agents were used as GVHD prophylaxis, that a high percentage of $CD8^+$ T cells among the PB mononuclear cells in week 2 after transplantation identified patients with a high risk of aGVHD. In our study, the donor $CD8^+$ T-cell count on time-points other than day +14 could not predict the occurrence of aGVHD. The finding of a strong correlation between the CD4/CD8 ratio of the grafts and the CD4/CD8 ratio of the donor T cells on day +7 but not on day +14 could indicate that in the first week after transplantation, the donor T cells undergo lymphopenia-induced homeostatic proliferation at a similar rate in most patients. In the second week after transplantation, differences in the rate of expansion of alloreactive donor T cells could then explain why the day +14 donor T-cell count predicts aGVHD.

To reduce the occurrence of GVHD, the Seattle consortium has extended the full-dose cyclosporine treatment in all patients from the original 35 days after transplantation to the presently used 56 days [30]. The regimen for cyclosporine taper depends on the risk of disease progression. In patients with aggressive disease, the taper is rapid, with discontinuation on day +75, whereas in patients with indolent disease, cyclosporine is discontinued on day +180 [30]. Individualization of the immunosuppression on the basis of a combination of the risk of aGVHD and the risk of disease progression could improve the treatment results if the incidence of aGVHD in patients with high risk could be reduced. We think, however, that our results need to be confirmed in a larger cohort of patients before they can be used in the clinical setting. One way to reduce the incidence of aGVHD in high-risk patients could be to increase the duration and possibly the dose of mycophenolate mofetil. The Seattle consortium has already modified the immunosuppressive regimen in this way for all patients in the unrelated donor setting [10].

In conclusion, we found that chimerism analysis of subpopulations of leukocytes is important in the treatment of patients undergoing NST. Chimerism studies may also contribute to the understanding of the relationship between clinical GVHD, graft-versus-lymphohematopoietic tissue reactions, and the GVT effect.

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REFERENCES

1. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood.* 1998;91:756-763.
2. Khouri IF, Keating M, Korbling M, et al. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol.* 1998;16:2817-2824.
3. Childs R, Clave E, Contentin N, et al. Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. *Blood.* 1999;94:3234-3241.
4. Sykes M, Preffer F, McAfee S, et al. Mixed lymphohaemopoietic chimerism and graft-versus-lymphoma effects after nonmyeloablative therapy and HLA-mismatched bone-marrow transplantation. *Lancet.* 1999;353:1755-1759.
5. Spitzer TR, McAfee S, Sackstein R, et al. Intentional induction of mixed chimerism and achievement of antitumor responses after nonmyeloablative conditioning therapy and HLA-matched donor bone marrow transplantation for refractory hematologic malignancies. *Biol Blood Marrow Transplant.* 2000;6:309-320.
6. McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood.* 2001;97:3390-3400.
7. Giralt S, Thall PF, Khouri I, et al. Melphalan and purine analog-containing preparative regimens: reduced-intensity

conditioning for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. *Blood.* 2001;97: 631-637.
8. Chakraverty R, Peggs K, Chopra R, et al. Limiting transplantation-related mortality following unrelated donor stem cell transplantation by using a nonmyeloablative conditioning regimen. *Blood.* 2002;99:1071-1078.
9. Corradini P, Tarella C, Olivieri A, et al. Reduced-intensity conditioning followed by allografting of hematopoietic cells can produce clinical and molecular remissions in patients with poor-risk hematologic malignancies. *Blood.* 2002;99:75-82.
10. Niederwieser D, Maris M, Shizuru JA, et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. *Blood.* 2003;101:1620-1629.
11. Antin JH, Childs R, Filipovich AH, et al. Establishment of complete and mixed donor chimerism after allogeneic lymphohematopoietic transplantation: recommendations from a workshop at the 2001 Tandem Meetings of the International Bone Marrow Transplant Registry and the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant.* 2001;7:473-485.
12. Schetelig J, Kroger N, Held TK, et al. Allogeneic transplantation after reduced conditioning in high risk patients is complicated by a high incidence of acute and chronic graft-versus-host disease. *Haematologica.* 2002;87:299-305.
13. Mattsson J, Uzunel M, Brune M, et al. Mixed chimaerism is common at the time of acute graft-versus-host disease and disease response in patients receiving non-myeloablative conditioning and allogeneic stem cell transplantation. *Br J Haematol.* 2001;115:935-944.
14. Feinstein LC, Sandmaier BM, Hegenbart U, et al. Non-myeloablative allografting from human leucocyte antigen-identical sibling donors for treatment of acute myeloid leukaemia in first complete remission. *Br J Haematol.* 2003;120:281-288.
15. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation.* 1974;18: 295-304.
16. Petersen SL, Ryder LP, Björk P, et al. A comparison of T-, B-, and NK-cell reconstitution following conventional or nonmyeloablative conditioning and transplantation with bone marrow or peripheral blood stem cells from human leucocyte antigen identical sibling donors. *Bone Marrow Transplant.* 2003;32:65-72.
17. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988;16:1215.
18. Thiede C, Florek M, Bornhauser M, et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. *Bone Marrow Transplant.* 1999;23:1055-1060.
19. Sandmaier BM, Maloney DG, Gooley T, et al. Nonmyeloablative hematopoietic stem cell transplants (HSCT) from HLA-matched related donors for patients with hematologic malignancies: clinical results of a TBI-based conditioning regimen [abstract]. *Blood.* 2001;98:742a-743a.
20. Mielcarek M, Martin PJ, Leisenring W, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood.* 2003;102:756-762.
21. Auffermann-Gretzinger S, Lossos IS, Vayntrub TA, et al. Rapid establishment of dendritic cell chimerism in allogeneic hematopoietic cell transplant recipients. *Blood.* 2002;99:1442-1448.
22. Little MT, Baker JE, Sandmaier BM, et al. Kinetics of mixed chimerism in peripheral blood hematopoietic subpopulations from 120 patients after nonmyeloablative hematopoietic stem cell transplant [abstract]. *Blood.* 2002;100:39a.
23. Fernandez-Aviles F, Urbano-Ispizua A, Aymerich M, et al. Low-dose total-body irradiation and fludarabine followed by hematopoietic cell transplantation from HLA-identical sibling donors do not induce complete T-cell donor engraftment in most patients with progressive hematologic diseases. *Exp Hematol.* 2003;31:934-940.
24. Niiya H, Kanda Y, Saito T, et al. Early full donor myeloid chimerism after reduced-intensity stem cell transplantation using a combination of fludarabine and busulfan. *Haematologica.* 2001;86:1071-1074.
25. Valcarcel D, Martino R, Caballero D, et al. Chimerism analysis following allogeneic peripheral blood stem cell transplantation with reduced-intensity conditioning. *Bone Marrow Transplant.* 2003;31:387-392.
26. Storb R, Yu C, Wagner JL, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood.* 1997;89:3048-3054.
27. McSweeney PA, Storb R. Mixed chimerism: preclinical studies and clinical applications. *Biol Blood Marrow Transplant.* 1999;5: 192-203.
28. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J Exp Med.* 1989;169:493-502.
29. Storb R, Yu C, Barnett T, et al. Stable mixed hematopoietic chimerism in dog leukocyte antigen-identical littermate dogs given lymph node irradiation before and pharmacologic immunosuppression after marrow transplantation. *Blood.* 1999;94: 1131-1136.
30. Georges GE, Storb R. Review of "minitransplantation": nonmyeloablative allogeneic hematopoietic stem cell transplantation. *Int J Hematol.* 2003;77:3-14.
31. Nash RA, Pepe MS, Storb R, et al. Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. *Blood.* 1992;80:1838-1845.
32. Gratama JW, Naipal A, Oljans P, et al. T lymphocyte repopulation and differentiation after bone marrow transplantation. Early shifts in the ratio between T4+ and T8+ T lymphocytes correlate with the occurrence of acute graft-versus-host disease. *Blood.* 1984;63:1416-1423.
33. Gratama JW, Wursch AM, Nissen C, et al. Influence of graft-versus-host disease prophylaxis on early T-lymphocyte regeneration following allogeneic bone marrow transplantation. *Br J Haematol.* 1986;62:355-365.
34. Soiffer RJ, Gonin R, Murray C, et al. Prediction of graft-versus-host disease by phenotypic analysis of early immune reconstitution after CD6-depleted allogeneic bone marrow transplantation. *Blood.* 1993;82:2216-2223.