Leukemia (2005) 19, 822–828

www.nature.com/leu

# High doses of transplanted CD34$^+$ cells are associated with rapid T-cell engraftment and lessened risk of graft rejection, but not more graft-versus-host disease after nonmyeloablative conditioning and unrelated hematopoietic cell transplantation

F Baron[1,3,5], MB Maris[1,2,5], BE Storer[1,2], BM Sandmaier[1,2], JP Panse[1], TR Chauncey[1,2,4], M Sorror[1], M-T Little[1], DG Maloney[1,2], R Storb[1,2] and S Heimfeld[1]

[1]*Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA;* [2]*University of Washington School of Medicine, Seattle, WA, USA;* [3]*Department of Hematology, University of Liège, Belgium; and* [4]*VA Puget Sound Health Care System, Seattle, WA, USA*

**This report examines the impact of graft composition on outcomes in 130 patients with hematological malignancies given unrelated donor granulocyte-colony-stimulating-factor-mobilized peripheral blood mononuclear cells (G-PBMC) ($n=116$) or marrow ($n=14$) transplantation after nonmyeloablative conditioning with 90 mg/m$^2$ fludarabine and 2 Gy TBI. The median number of CD34$^+$ cells transplanted was $6.5\times10^6$/kg. Higher numbers of grafted CD14$^+$ ($P=0.0008$), CD3$^+$ ($P=0.0007$), CD4$^+$ ($P=0.001$), CD8$^+$ ($P=0.004$), CD3$^-$CD56$^+$ ($P=0.003$), and CD34$^+$ ($P=0.0001$) cells were associated with higher levels of day 28 donor T-cell chimerism. Higher numbers of CD14$^+$ ($P=0.01$) and CD34$^+$ ($P=0.0003$) cells were associated with rapid achievement of complete donor T-cell chimerism, while high numbers of CD8$^+$ ($P=0.005$) and CD34$^+$ ($P=0.01$) cells were associated with low probabilities of graft rejection. When analyses were restricted to G-PBMC recipients, higher numbers of grafted CD34$^+$ cells were associated with higher levels of day 28 donor T-cell chimerism ($P=0.01$), rapid achievement of complete donor T-cell chimerism ($P=0.02$), and a trend for lower risk for graft rejection ($P=0.14$). There were no associations between any cell subsets and acute or chronic GVHD nor relapse/progression. These data suggest more rapid engraftment of donor T cells and reduced rejection rates could be achieved by increasing the doses of CD34$^+$ cells in unrelated grafts administered after nonmyeloablative conditioning.**
*Leukemia* (2005) **19,** 822–828. doi:10.1038/sj.leu.2403718
Published online 17 March 2005
**Keywords:** cell dose; CD34; T-cell; transplantation; nonmyeloablative

## Introduction

To extend allogeneic hematopoietic cell transplantation (HCT) from unrelated donors (URD) to older patients and those with comorbid conditions, reduced intensity or truly nonmyeloablative conditioning regimens have been developed[1–6] in which the burden of tumor eradication has been shifted from the high-dose chemoradiotherapy agents to cell-based graft-versus-tumor effects.[7] Based on experimental canine studies[8] and subsequent clinical trials in HLA-identical sibling recipients,[9] a nonmyeloablative regimen was developed for URD HCT that utilized minimal host conditioning with fludarabine and 2 Gy total body irradiation (TBI),[5,10,11] combined with postgrafting immunosuppression consisting of mycophenolate mofetil (MMF) and cyclosporine (CSP). We previously reported early data from 89 patients with advanced hematologic malignancies given unrelated grafts after nonmyeloablative conditioning.[5] Sustained engraftment was observed in 60 of 71 (85%) of G-CSF-mobilized peripheral blood mononuclear cell (G-PBMC) recipients and in 10 of 18 (56%) of marrow recipients.[5] Over 40% of patients with measurable disease at the time of HCT achieved complete remissions, and 2-year progression-free survival (PFS) was 42% for G-PBMC recipients.

The focus of the current study was to determine whether the dose of total nucleated cells (TNC) and/or specific cell subtypes in the unrelated grafts were associated with clinical outcomes in 130 consecutive patients given unrelated grafts after nonmyeloablative conditioning for hematologic malignancies. The eventual aim was to determine whether prospective manipulations of the graft composition might be useful to improve HCT outcomes in patients with hematologic malignancies given URD HCT after nonmyeloablative conditioning.

## Patients and methods

### *Patients*

Included in the study were results from 130 consecutive patients with hematologic malignancies given allogeneic HCT after nonmyeloablative conditioning on prospective research protocols between January 2000 and December 2003 at the Fred Hutchinson Cancer Research Center (FHCRC), the University of Washington Medical Center, the Children's Hospital and Regional Medical Center and the Veterans Affairs Puget Sound Health Care System (all in Seattle, WA, USA). Data were analyzed as of 31 March 2004. The median follow-up for surviving patients was 531 (range, 93–1475) days. Patients were considered ineligible for conventional allogeneic HCT because of age (>50 years) and/or comorbidities, or preceding extensive therapies such as a myeloablative autologous or allogeneic HCT.[5] Reasons for nonmyeloablative conditioning for the 46 patients younger than 50 years (including five patients younger than 16 years) were previous high-dose myeloablative allogeneic ($n=3$), syngeneic ($n=1$) or autologous ($n=27$) HCT, abnormal liver function tests with or without chronic renal failure ($n=5$), cardiomyopathy ($n=2$), active mycotic infections ($n=2$), chronic renal failure ($n=1$), morbid obesity ($n=1$), chronic leg ulcerations ($n=1$), cavernous arteriovenous malformation ($n=1$), hypoxemia ($n=1$), and patient decision ($n=1$). Characteristics of the patients are summarized in Table 1.

Median patient age was 54 (range, 5–71) years. Patients were classified as being at standard-risk, high-risk or very high risk for disease progression after HCT as described in Table 1. Pretransplant comorbidities were determined from the patients'

Correspondence: Dr MB Maris, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, D1-100, PO Box 19024, Seattle, WA 98109-1024, USA; Fax: +1206 667 6124; E-mail: mmaris@fhcrc.org
[5]FB and MBM contributed equally to this work
Received 17 December 2004; accepted 31 January 2005; Published online 17 March 2005

**Table 1** Patients ($n=130$)

| *Characteristic* | *All patients (n = 130)* | *G-PBMC recipients (n = 116)* |
|---|---|---|
| Median patient age, year (range) | 54 (5–71) | 54 (9–71) |
| Recipient gender, #M (%)/#F (%) | 85 (65)/45 (35) | 74 (64)/42 (36) |
| Female donor/male recipient, # pts (%) | 31 (24) | 28 (24) |
| *Diagnosis, # pts (%)* | | |
| Acute myeloid leukemia | 24 (18.5) | 20 (17.2) |
| Acute lymphoblastic leukemia | 9 (6.9) | 8 (6.9) |
| Chronic myeloid leukemia | 11 (8.5) | 10 (8.6) |
| Chronic lymphocytic leukemia | 10 (7.7) | 10 (8.6) |
| Myelodysplastic syndrome[a] | 20 (15.4) | 17 (14.7) |
| Multiple myeloma | 12 (9.2) | 12 (10.3) |
| Non-Hodgkin's lymphoma | 26 (20.0) | 22 (19.0) |
| Hodgkin's disease | 12 (9.2) | 12 (10.3) |
| Myeloproliferative syndrome other than chronic myeloid leukemia | 6 (4.6) | 5 (4.3) |
| *Disease risks*[b]*, # pts (%)* | | |
| Standard risk | 52 (40.0) | 46 (39.7) |
| High risk | 67 (51.5) | 61 (52.6) |
| Very high risk | 11 (8.5) | 9 (7.7) |
| Tandem autologous–allogeneic HCT | 5 (3.8) | 5 (4.3) |
| Failed previous myeloablative HCT, # pts (%) | 51 (39.2) | 47 (40.5) |
| *Charlson comorbidity index (CCI)*[12]*, # pts (%)* | | |
| Score 0 | 59 (45.4) | 54 (46.5) |
| Score >2 | 18 (13.8) | 15 (12.9) |
| *Donor, # pts (%)* | | |
| HLA-identical | 109 (83.8) | 96 (82.8) |
| One HLA antigen-mismatch | 10 (7.7) | 10 (8.6) |
| One HLA allele-mismatch | 11 (8.5) | 10 (8.6) |
| *Stem cell source, # pts (%)* | | |
| G-PBMC | 116 (89.2) | 116 (100) |
| Marrow | 14 (10.8) | 0 |
| *Graft composition, median (range)* | | |
| Total nucleated cells | 909 (153–2958) | 966 (235–2958) |
| $CD3^+$ | 247 (16–934) | 261 (31–934) |
| $CD4^+$ | 150 (8–586) | 155 (20–586) |
| $CD8^+$ | 72 (6–341) | 80 (10–341) |
| $CD3^-CD56^+$ | 26 (2–68) | 27 (11–68) |
| $CD3^+CD56^+$ | 14 (1–88) | 14 (3–88) |
| $CD20^+$ | 46 (4–184) | 48 (26–184) |
| $CD14^+$ | 171 (9–517) | 193 (47–517) |
| $CD34^+$ | 6.5 (0.8–26.8) | 6.8 (0.8–26.8) |

[a]Including patients who received chemotherapy for RAEB, RAEBT, or AML now either in CR or in RA.
[b]Standard risks were defined as acute myeloid leukemia in first complete remission, acute lymphoblastic leukemia in first complete remission, myelodysplastic syndrome-refractory anemia, chronic myeloid leukemia in first chronic phase, chronic lymphocytic leukemia, low-grade non-Hodgkin's lymphoma, high or intermediate grade non-Hodgkin's lymphoma in complete remission, Hodgkin's disease in complete remission, multiple myeloma in complete remission or with minimal residual disease; very high-risks were defined as acute leukemia above second complete remission, chronic myeloid leukemia in accelerated phase or blast crisis, and myelodysplastic syndrome-refractory anemia with blast excess or above; all other diagnoses were classified as high risk.

pretransplant evaluation notes and scored using a template adapted from the Charlson Comorbidity Index, as previously reported.[12,13]

Compatibility between patients and donors for HLA-A, -B, and -C antigens was assessed by intermediate-resolution DNA typing to a level at least as sensitive as serology and for -DRB1 and -DQB1 by high-resolution techniques.[5] In total, 10 patients received 1 HLA-antigen-mismatched (three of these also had single HLA-allele-mismatches) transplants, and 11 had one HLA-allele-mismatched donors.

Prospective research protocols and the current study were approved by the Institutional Review Board of the FHCRC for the participating institutions. All patients signed informed consents for the original prospective research protocols.

## *Conditioning regimen, postgrafting immunosuppression, and HCT collection*

All patients were conditioned with fludarabine 30 mg/m$^2$/day on days −4, −3, and −2, and 2 Gy TBI delivered at 0.07 Gy/min before URD donor HCT (day 0). Postgrafting immunosuppression included MMF, 15 mg/kg orally twice a day ($n=51$)[5] or thrice a day ($n=79$) from the evening of day 0 until day +40,

and CSP, 6.25 mg/kg orally twice a day from day −3 to day +100. In the absence of GVHD, MMF was tapered at day 40 (day 96 in class 1 HLA-mismatched recipients) over 56 days and CSP was tapered at day 100 (180 in class 1 HLA-mismatched recipients) over 180 days.

Donor G-PBMC were generally mobilized using G-CSF (10 μg/kg/day; day −5 to −1), and collected as per the National Marrow Donor Program (NMDP) standard for donors collected in the US ($n=96$), or according to recommendations from other National Donor Programs for donors collected outside of the US ($n=20$). In total, 10% of the collected G-PBMC were set aside for cryopreservation for use as DLI, if needed. The remaining cells were infused intravenously on day 0. In total, 28 patients (including three children <15 years old) received G-PBMC obtained in one apheresis collection. No patients were given product of more than two leukaphereses. In total, 24 liters of apheresis collection was prescribed by the transplant center according to HCT protocols, but the decision to perform one or two collections was at the discretion of the collection centers. A total of 14 patients received donor marrow harvested by multiple aspirations from the posterior iliac crests on day 0.

### *Follow-up*

Diagnosis, clinical grading, and treatment of GVHD were performed as previously reported.[5] Standard prophylaxis against infections was used.[5] Patients were examined by a healthcare provider at least three times a week for the first month and then at least weekly. Disease-dependent restaging evaluations after HCT occurred monthly for the first 3 months and then at 6 months, 1 year, and then yearly thereafter.[5]

### *Treatment of persistent/progressive or relapsed diseases and prevention of graft rejection*

Progressive or relapsed malignancies in the absence of severe manifestations of acute and chronic GVHD were treated by rapid discontinuation of systemic immunosuppression in order to initiate graft-versus-tumor effects. In addition, three patients received DLI[14] for disease progression, poor T-cell chimerism, and Epstein–Barr virus-associated lymphoproliferative disease in donor cells, respectively.

### *Graft cell subsets enumeration*

Enumerations of cell subsets in each graft product were performed by 3-color analyses using a FACScan cytometer (Becton Dickinson [BD], San Jose, CA, USA) by the Cellular Therapy Laboratory at the FHCRC. Samples from grafts were stained with monoclonal antibodies (all from BD) directed against the following surface antigens: CD3, CD4, CD8, CD14, CD20, CD34, and CD56. At least 100 000 events were acquired per sample. Gates were set around forward *vs* side scatter and low 7-amino-actinomycin D staining to include only viable nucleated cells for further analysis. Cell types were defined as follows: $CD3^+$ T cell ($CD3^+CD56^-$), $CD4^+$ T cell ($CD3^+CD4^+$), $CD8^+$ T cell ($CD3^+CD8^+$), natural killer (NK) cell ($CD3^-CD56^+$), NK/T cell ($CD3^+CD56^+$), B cell ($CD19^+$), monocyte ($CD14^+$), and CD34 cells ($CD34^+CD14^-$). List mode data were analyzed with either WinList (Verity Software House Inc., Topsham, ME, USA) or CellQuest (BD) software to determine percentages of cell types. Percentages were multiplied by the TNC counts of the grafts, determined by use of an NE8000 Sysmex (TOA Kobe, Japan) automated cell counter, and volume infused to determine absolute cell numbers, and divided by actual recipient weight in order to determine cell doses per kg.

### *T-cell chimerism analyses*

For T-cell chimerism analyses, T cells were isolated from the peripheral blood by flow cytometry using a Vantage SE cytometer (BD) on days 28, 56, 84, 180, 365, and then yearly after HCT. Percentages of donor–host chimerism for recipients of sex-mismatched HCT were evaluated by fluorescent *in situ* hybridization (FISH) for X and Y chromosomes, or by polymerase chain reaction (PCR)-based amplification of variable-number tandem repeat (VNTR) sequences if patients and donors were sex-matched.[5] Quantification of chimerism using VNTR was by visual inspection or silver-stained agarose gel with a standard error of 5%.[5]

### *Statistical methods*

In order to assess the association of cell-type variables with outcome, non-cell-type variables were first examined using regression models and then used to create a base model for each outcome. The non-cell-type variables used for day 28 T-cell chimerism levels, cumulative incidence of achieving full donor chimerism, and risk for graft rejection were prior chemotherapy or not (agents such as chlorambucil, hydroxyurea, imatinib mesylate or immunomodulators were classified in the 'non-chemotherapy' group), and tandem autologous/allogeneic HCT. For acute and chronic GVHD, the non-cell type variables were patient age, degree of HLA-compatibility, female donor to male recipient, CMV serostatus, myeloid or lymphoid malignancies, MMF twice a day *vs* thrice a day, and Charlson's comorbidity index score at transplantation. For overall survival (OS), PFS, relapse and nonrelapse mortality the non-cell-type variables were Charlson's comorbidity index score at transplantation, disease risk and tandem autologous/allogeneic HCT. Cox regression models were used for the outcomes of achievement of full donor T-cell chimerism, relapse/disease progression, acute and chronic GVHD, PFS, OS, and nonrelapse mortality. A linear regression model was used for the outcome of day 28 T-cell chimerism. In all models, cell doses were modeled as ordinal variables defined by quartile of dose. Means and hazard ratios thus refer to effects per quartile of dose, and *P*-values are interpretable as tests for trend. Spearman rank correlation coefficient was used to estimate the correlation between cell types. All *P*-values are two-sided, and no adjustments were made for multiple comparisons; thus, values between 0.01 and 0.05 should therefore be considered as suggestive rather than definitive evidence of a significant association.

## Results

### *Cellular composition of grafts*

Table 1 shows the absolute numbers of TNC, $CD34^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, $CD3^-CD56^+$ and $CD3^+CD56^+$ cells contained in the grafts. The correlations between $CD34^+$ and TNC, $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, $CD3^-CD56^+$, and $CD3^+CD56^+$ cells were highly

statistically significant ($P<0.0001$), but Spearman coefficients were generally low ($R<0.70$). Similarly, the correlations between $CD3^+$ and $CD14^+$, $CD19^+$, $CD3^-CD56^+$ and $CD3^+CD56^+$ cells were statistically highly significant ($P<0.0001$), and Spearman coefficients were relatively low ($R<0.70$). As expected, the correlations between $CD3^+$ and $CD4^+$ and $CD8^+$ cells were stronger ($R>0.85$). Among G-PBMC recipients, graft compositions were not statistically different among patients who received products from two leukapheresis in comparison to patients who were given cells from one leukapheresis. Specifically, among adult G-PBMC recipients, the mean $\pm$ s.d. number of $CD34^+$ cells transplanted was $8.1+4.9\times10^6$ cells/kg for patients given a 1-apheresis product, *vs* $8.2+6.4\times10^6$ cells/kg for patients given a 2-apheresis product ($P=0.95$). The number of apheresis collections was not associated with post-HCT outcomes (data not shown).

*Associations between graft composition and HCT outcomes in the entire 130 cohort of patients*: Day-28 T-cell chimerism levels were available for 126 of the 130 patients. In multivariate analyses, high numbers of $CD14^+$ ($P=0.0008$), $CD3^+$ ($P=0.0007$), $CD4^+$ ($P=0.001$), $CD8^+$ ($P=0.004$), $CD3^-CD56^+$ ($P=0.003$), and $CD34^+$ ($P=0.0001$) cells in the grafts were associated with high levels of day-28 donor T-cell chimerism, while high numbers of $CD14^+$ ($P=0.01$) and $CD34^+$ ($P=0.0003$) cells were associated with higher probabilities of achieving full donor chimerism levels in time-dependent analyses (Table 2). Six of 14 marrow recipients, and eight of 116 G-PBMC ($P=0.001$) recipients rejected their grafts. One of 11 patients given grafts from a one HLA allele mismatched donor (this patient had CML and was given marrow) and none of 10 patients given grafts from one HLA antigen mismatched donors rejected their grafts, compared to 13 of 109 patients (12%) given grafts from 10 of 10 HLA allele-level matched donors. In multivariate analyses, high numbers of infused $CD8^+$ ($P=0.005$) and $CD34^+$ ($P=0.01$) cells were associated with lower probabilities of graft rejection. There were no associations between any cell subsets and acute or chronic GVHD nor relapse/progression, but there was a trend for better PFS with higher cell doses transplanted for all cell type variables, and the association was significant for TNC (HR 0.8 (95% CI, 0.6–0.9), $P=0.01$). Similarly, there was a trend for better overall survival with higher cell doses transplanted for all cell type variables, and the association was significant for TNC (HR 0.7 (95% CI, 0.5–0.9), $P=0.006$).

## *Low day-28 donor T-cell chimerism levels predicted subsequent graft rejection*

Four patients rejected their HCT (<5% donor T-cell chimerism) before day 28. In the remaining 122 patients studied, day-28 donor T-cell chimerism levels were predictive of the subsequent risk of graft rejection. Seven of 10 (70%) patients with donor T-cell chimerism levels between 5–25%, one of eight (13%) patients with levels between 26 and 50%, two of 17 (12%) patients with levels between 51 and 75% and 0 of 87 (0%) patients with levels between 75 and 100% rejected their grafts ($P<0.0001$) (Figure 1a).

**Table 2** Impact of graft composition on T-cell chimerism and risks of graft rejection among all patients or restricted to G-PBMC recipients

*(a) All patients*

| | *Day-28 T-cell chimerism* | | *Achievement of full donor chimerism* (n = 103) | | *Graft rejection* (n = 14) | |
|---|---|---|---|---|---|---|
| | *Mean*[a] *(s.e.)* | P-*value*[b] | *HR*[c] *(95% CI)* | P-*value*[b] | *HR*[c] *(95% CI)* | P-*value*[b] |
| TNC | 4.3 (2.3) | 0.07 | 1.2 (1.0–1.4) | 0.08 | 0.7 (0.4–1.1) | 0.13 |
| $CD3^+$ | **7.5 (2.1)** | **0.0007** | 1.2 (1.0–1.4) | 0.04 | 0.5 (0.3–0.9) | 0.02 |
| $CD4^+$ | **7.4 (2.2)** | **0.001** | 1.3 (1.0–1.5) | 0.02 | 0.6 (0.3–1.0) | 0.06 |
| $CD8^+$ | **6.4 (2.2)** | **0.004** | 1.2 (1.0–1.4) | 0.08 | **0.4 (0.2–0.7)** | **0.005** |
| $CD3^-CD56^+$ | **6.9 (2.2)** | **0.003** | 1.3 (1.0–1.5) | 0.02 | 0.6 (0.3–1.2) | 0.13 |
| $CD3^+CD56^+$ | 4.9 (2.2) | 0.03 | 1.1 (0.9–1.3) | 0.41 | 0.4 (0.2–0.9) | 0.03 |
| $CD20^+$ | 2.3 (2.2) | 0.31 | 1.0 (0.9–1.2) | 0.82 | 0.8 (0.5–1.5) | 0.55 |
| $CD14^+$ | **8.3 (2.4)** | **0.0008** | **1.3 (1.1–1.5)** | **0.01** | 0.5 (0.3–0.9) | 0.03 |
| $CD34^+$ | **8.7 (2.2)** | **0.0001** | **1.4 (1.2–1.7)** | **0.0003** | **0.5 (0.2–0.8)** | **0.01** |

*(b) G-PBMC recipients*

| | *Day-28 T-cell chimerism* | | *Achievement of full donor chimerism* (n = 97) | | *Graft rejection* (n = 8) | |
|---|---|---|---|---|---|---|
| | *Mean*[a] *(s.e.)* | P-*value*[b] | *HR*[c] *(95% CI)* | P-*value*[b] | *HR*[c] *(95% CI)* | P-*value*[b] |
| TNC | −1.5 (2.1) | 0.50 | 1.0 (0.9–1.3) | 0.64 | 1.2 (0.7–2.2) | 0.51 |
| $CD3^+$ | 4.4 (2.0) | 0.03 | 1.2 (1.0–1.4) | 0.09 | 0.6 (0.3–1.2) | 0.15 |
| $CD4^+$ | **5.6 (2.0)** | **0.007** | 1.2 (1.0–1.4) | 0.07 | 0.7 (0.4–1.4) | 0.37 |
| $CD8^+$ | 2.2 (2.1) | 0.29 | 1.0 (0.9–1.3) | 0.66 | 0.5 (0.3–1.2) | 0.12 |
| $CD3^-CD56^+$ | 4.3 (2.1) | 0.04 | 1.2 (1.0–1.4) | 0.14 | 0.9 (0.4–2.0) | 0.85 |
| $CD3^+CD56^+$ | 3.2 (2.1) | 0.13 | 1.1 (0.9–1.3) | 0.46 | 0.6 (0.3–1.3) | 0.18 |
| $CD20^+$ | 0.0 (2.1) | 0.99 | 1.0 (0.8–1.2) | 0.75 | 1.1 (0.6–2.0) | 0.84 |
| $CD14^+$ | 4.5 (2.3) | 0.05 | 1.2 (1.0–1.4) | 0.05 | 0.8 (0.4–1.7) | 0.60 |
| $CD34^+$ | **5.2 (2.1)** | **0.01** | 1.3 (1.0–1.5) | 0.02 | 0.6 (0.3–1.2) | 0.14 |

TNC = total nucleated cells.
[a]Mean chimerism increase per quartile of dose; adjusted for prior chemo or not, and tandem autologous/allogeneic HCT.
[b]All *P*-values were two-sided, and no adjustments were made for multiple comparisons; values between 0.01 and 0.05 should be considered as suggestive for a difference rather than definitive.
[c]HR per quartile of dose; adjusted for prior chemo or not, and planned autologous–allogeneic HCT or not.
bold = statistically significant.

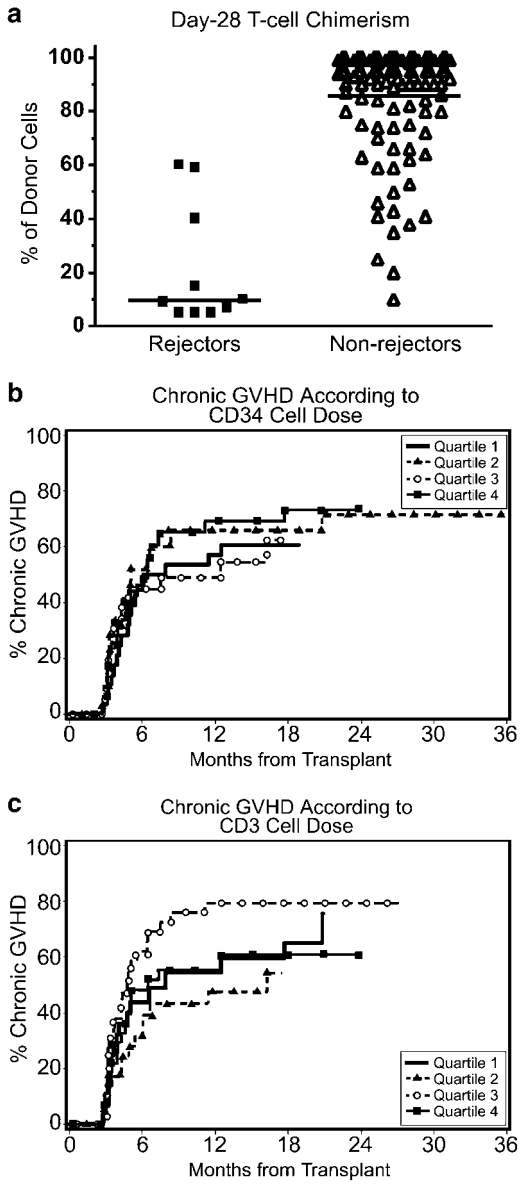


**Figure 1** (a) Day-28 T-cell chimerism levels in patients with or without subsequent graft rejection. Data from patients with graft rejection before day 28 were not included. Impact of $CD34^+$ (b, $P=0.36$) and $CD3^+$ (c, $P=0.52$) cells transplanted on cumulative incidence of chronic extensive graft-versus-host disease among G-PBMC recipients. The cutpoints for quartile doses were $4.2\times10^6$, $6.8\times10^6$, and $12.0\times10^6$ $CD34^+$ cells/kg, and $190\times10^6$, $261\times10^6$ and $363\times10^6$ $CD3^+$ cells/kg.

## *Analyses restricted to G-PBMC recipients* (n = *116*)

*Associations between graft composition and donor T-cell engraftment*: In multivariate analyses, high numbers of $CD4^+$ ($P=0.007$) and $CD34^+$ ($P=0.01$) cells in the grafts were associated with high levels of day-28 donor T-cell chimerism (Table 2). Full donor T-cell chimerism levels (defined as $\geqslant$95% donor T-cells) were achieved in 97 G-PBMC recipients between 22 and 105 (median 35) days after HCT. In multivariate analyses, there was a suggested association between high number of $CD34^+$ cells and achievement of full donor chimerism ($P=0.02$).

*Associations between graft composition and graft rejection*: In multivariate analysis, high numbers of infused $CD8^+$ (HR 0.5, $P=0.12$) and $CD34^+$ (HR 0.6, $P=0.14$) cells were suggestively associated with lower probabilities of graft rejection, although these associations lacked statistical significance, probably as a result of the small number of graft rejections among G-PBMC recipients ($n=8$) (Table 2). In addition, patients given G-PBMC containing more than the median $CD34^+$ cells dose ($6.8\times10^6$/kg) had a strong trend for a lower risk of graft rejection in comparison to patients given less than the median number of $CD34^+$ cells (HR 0.2 (95% CI, 0.0–1.4, $P=0.10$)).

*Associations between graft composition and graft-versus-host disease (GVHD)*: Acute GVHD of grades II, III, and IV occurred in 64 (55.2%), 16 (13.8%), and 2 (1.7%) patients, respectively. In multivariate analyses, none of the cell-type variables were statistically significantly associated with grades II–IV or III–IV acute GVHD (*P*-values ranged from 0.48 to 0.98).

Chronic GVHD requiring systemic therapy was seen in 70 patients. In multivariate analyses, none of the cell-type variables were statistically significantly associated with extensive chronic GVHD (*P*-values ranged from 0.12 to 0.69) (Figure 1b and c).

*Relapse/progression and nonrelapse mortality*: In total, 42 of 116 patients (36%) relapsed/progressed after HCT. Only one of the 42 patients was given DLI. In the remaining patients, DLI were contraindicated due to graft rejection ($n=5$), or because of GVHD preceding ($n=14$) or occurring soon after ($n=3$) disease progression. Further, DLI was not offered to eight patients with florid relapse, two patients who relapsed $<50$ days after HCT, and five patients with CML ($n=2$), CLL ($n=2$) and MDS, respectively, who achieved complete remissions after withdrawal of immunosuppression. Finally, two patients refused DLI, and two patients were given either imatinib or thalidomide instead of DLI for CML and myeloma relapses, respectively, after HCT. In multivariate analysis, none of the cell variables was significantly associated with relapse/progression.

In total, 19 patients (16%) died of nonrelapse causes. In multivariate analysis, none of the cell variables was significantly associated with nonrelapse mortality.

## *Progression-free and overall survival*

The probabilities of PFS at 1, 2, and 3 years were 49, 43, and 33%, respectively. In multivariate analyses, there was a trend for better PFS in patients given higher number of TNC (HR 0.8 (95% CI, 0.7–1.1), $P=0.13$), although the association was not statistically significant.

The probabilities of OS at 1, 2, and 3 years were 62, 52, and 43%, respectively. In multivariate analyses, there was a trend for better OS in patients given higher number of TNC (HR 0.8 (95% CI, 0.6–1.0), $P=0.06$), although the association was not statistically significant.

## Discussion

The present study asked whether the individual cell subsets in unrelated, unmanipulated hematopoietic grafts affected outcomes among patients given nonablative conditioning. Our key findings were as follows. Higher numbers of grafted T-cells (including $CD4^+$, $CD8^+$, and $CD3^+CD56^+$ T-cell subsets),

$CD14^+$ cells, NK cells, and $CD34^+$ cells were associated with higher levels of donor T-cell chimerism, while high $CD14^+$ and $CD34^+$ contents were associated with more rapid achievement of complete donor T-cell chimerism, and high T-cell (including $CD8^+$ and $CD3^+CD56^+$ T-cell subsets), monocyte, and $CD34^+$ cell numbers reduced the risk of graft rejection. Among T-cell subsets, both $CD4^+$ and $CD8^+$ T cells might promote T-cell engraftment, confirming previous observations in a canine model[15] and in patients given HCT after myeloablative[16,17] or nonmyeloablative conditioning.[18,19] Furthermore, since $CD14^+$ cells contained in G-PBMC grafts secrete large amounts of IL-10 (an immunosuppressive cytokine) and have reduced expression of costimulatory molecules needed for T-cell activation, those cells might provide immunosuppressive effects that reduce host-versus-graft (rejection) reactions.[20]

The role of $CD3^+$ cells for the development of acute and chronic GVHD after allogeneic HCT has been established.[16] However, we found no statistical correlation between $CD3^+$ (including $CD4^+$ and $CD8^+$ subsets) cell doses and the risk of acute GVHD. This was consistent with previous studies showing comparable risks of acute GVHD in patients with aplastic anemia, of whom some did and some did not receive infusions of viable donor buffy coat cells in addition to marrow grafts after conditioning with cyclophosphamide.[21] Also, several studies reported similar incidences of acute GVHD after G-PBMC and marrow grafts despite the infusion of 1 to 2 logs more $CD3^+$ cells in the G-PBMC inocula.[22,23] Perhaps, as suggested by Kernan *et al*,[24] once an initial threshold ($10^5$ $CD3^+$ cells/kg) of transplanted $CD3^+$ cells has been exceeded, further increases in T-cell numbers do not necessarily translate into more acute GVHD for given degrees of genetic disparity between donors and recipients.

In the current study, higher doses of $CD34^+$ and $CD3^+$ cells were not associated with increased risks of chronic extensive GVHD. These results were in agreement with previous observations in unrelated graft recipients after myeloablative conditioning,[25] but contrasted with findings in related ablative[26,27] and nonablative[28] recipients that showed a positive correlation between numbers of $CD34^+$ cells and incidence of chronic extensive GVHD, and also the finding of a higher incidence of chronic GVHD in patients with aplastic anemia given donor buffy coat cells in addition to related marrow grafts.[21] The reasons for the differences among related and unrelated recipients are unclear. Perhaps, greater disparity for minor histocompatibility antigens in the unrelated setting could lead to more chronic GVHD. In addition, it might be that there were thresholds for $CD34^+$ and $CD3^+$ cell doses above which correlations between graft composition and chronic GVHD no longer existed. These thresholds might be lower in unrelated recipients because of greater minor histocompatibility antigen divergences than in related recipients.[29] However, one recent study also failed to find a correlation between high $CD34^+$ cell dose and high incidence of chronic GVHD in patients given related G-PBMC after nonmyeloablative conditioning.[30] The current observations suggested that there is no upper cell dose limit for patients given unrelated G-PBMC after nonmyeloablative conditioning with fludarabine and 2 Gy TBI.[30]

When data from all patients were analyzed, there were trends for better PFS and OS with higher cell doses for all cell type variables, and that association was significant for TNC. This observation agreed with previous reports demonstrating better PFS in unrelated recipients given higher nucleated cell doses after myeloablative conditioning.[31,32] A similar trend for better PFS in patients receiving a higher number of TNC was observed when analyses were restricted to G-PBMC recipients, although the association was no longer statistically significant. However, the current study failed to show a correlation between higher $CD8^+$ cell doses in G-PBMC and better PFS ($HR = 0.9$, $P = 0.42$) or OS ($HR = 0.9$, $P = 0.27$), as recently proposed by Cao *et al*[19] in patients given a similar nonmyeloablative conditioning and postgrafting immunosuppression. That study analyzed combined observations in 25 patients given G-PBMC from URD and 38 recipients of related grafts, which may explain the discrepancy with the current study in unrelated recipients. A recent study from our group analyzed the impact of graft composition in 120 patients given G-PBMC from related donors after nonmyeloablative conditioning, and showed better OS ($P = 0.03$) in patients given a higher number of $CD34^+$ cells in the grafts, but no correlations between $CD8^+$ cells contents and HCT outcomes.[30]

In conclusion, our data suggested that giving higher doses of $CD34^+$ cells helped to promote sustained T-cell engraftment in patients given unrelated grafts after nonmyeloablative conditioning without increasing the risks for acute or chronic GVHD, and therefore, as many $CD34^+$ cells as possible should be collected from the donors.

## Acknowledgements

We thank the data coordinators Heather Hildebrant Debbie Bassuk, and Chris Davis, and the study nurses Steve Minor, Mary Hinds, and John Sedgwick for their invaluable help in making the study possible. We also wish to thank Bonnie Larson and Helen Crawford for help with manuscript preparation, and the physicians, nurses, and support personnel for their care of patients involved in this study. This work was supported in part by Grants CA78902, CA92058, HL36444, CA18029, HD44175, DK56465, and CA15704 from the National Institutes of Health, Department of Health and Human Services (DHHS), Bethesda, MD. DGM was supported by a grant from the Gabrielle Rich Leukemia Foundation, and MS by a grant from the Oncology Research Faculty Development Program of the NCI. RS also received support from the Laura Landro Salomon Endowment Fund. FB is research associate of the National Fund for Scientific Research (FNRS) Belgium and supported in part by postdoctoral grants from the Fulbright Commission and from the Centre Anticancéreux près l'Université de Liège.

## References

1 Girgis M, Hallemeier C, Blum W, Brown R, Lin H-S, Khoury H *et al.* Chimerism and clinical outcomes of 110 unrelated donor bone marrow transplant recipients conditioned with low dose (550 cGy), single exposure total body irradiation and cyclophosphamide. *Blood*, prepublished online May 4, 2004; (in press).
2 Wong R, Giralt SA, Martin T, Couriel DR, Anagnostopoulos A, Hosing C *et al.* Reduced-intensity conditioning for unrelated donor hematopoietic stem cell transplantation as treatment for myeloid malignancies in patients older than 55 years. *Blood* 2003; **102**: 3052–3059.
3 Nagler A, Aker M, Or R, Naparstek E, Varadi G, Brautbar C *et al.* Low-intensity conditioning is sufficient to ensure engraftment in matched unrelated bone marrow transplantation. *Exp Hematol* 2001; **29**: 362–370.
4 Chakraverty R, Peggs K, Chopra R, Milligan DW, Kottaridis PD, Verfuerth S *et al.* Limiting transplantation-related mortality following unrelated donor stem cell transplantion by using a nonmyeloablative conditioning regimen. *Blood* 2002; **99**: 1071–1078.
5 Maris MB, Niederwieser D, Sandmaier BM, Storer B, Stuart M, Maloney D *et al.* HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for

patients with hematologic malignancies. *Blood* 2003; **102**: 2021–2030.
6 Appelbaum FR. Dose intensity and the toxicity and efficacy of allogeneic hematopoietic cell transplantation. *Leukemia* 2005; **19**: 171–175.
7 Baron F, Maris MB, Sandmaier BM, Storer BE, Sorror M, Diaconescu R *et al.* Graft-versus-tumor effects after allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning. *J Clin Oncol*, (in press).
8 Storb R, Yu C, Wagner JL, Deeg HJ, Nash RA, Kiem H-P *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.
9 McSweeney PA, Niederwieser D, Shizuru JA, Sandmaier BM, Molina AJ, Maloney DG *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.
10 Niederwieser D, Maris M, Shizuru JA, Petersdorf E, Hegenbart U, Sandmaier BM *et al.* Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and post-grafting 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 Baron F, Maris MB, Storer BE, Sandmaier BM, Stuart MJ, McSweeney PA *et al.* HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with chronic myeloid leukemia. *Biol Blood Marrow Transplant*, (in press).
12 Sorror ML, Maris MB, Storer B, Sandmaier BM, Diaconescu R, Flowers C *et al.* Comparing morbidity and mortality of HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative and myeloablative conditioning: influence of pretransplant comorbidities. *Blood* 2004; **104**: 961–968.
13 Diaconescu R, Flowers CR, Storer B, Sorror ML, Maris MB, Maloney DG *et al.* Morbidity and mortality with nonmyeloablative compared to myeloablative conditioning before hematopoietic cell transplantation from HLA matched related donors. *Blood* 2004; **104**: 1550–1558.
14 Bethge WA, Hegenbart U, Stuart MJ, Storer BE, Maris MB, Flowers MED *et al.* Adoptive immunotherapy with donor lymphocyte infusions after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. *Blood* 2004; **103**: 790–795.
15 Panse JP, Bastianelli C, Santos EB, Schwarzinger I, Raff RF, Storb R *et al.* Dog leukocyte antigen nonidentical unrelated canine marrow grafts: enhancement of engraftment by both CD4 and CD8T cells. *Transplantation* 2003; **76**: 474–480.
16 Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation (Review). *Blood* 2001; **98**: 3192–3204.
17 Champlin R, Ho W, Gajewski J, Feig S, Burnison M, Holley G *et al.* Selective depletion of CD8+ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. *Blood* 1990; **76**: 418–423.
18 Baron F, Baker JE, Storb R, Gooley TA, Sandmaier BM, Maris MB *et al.* Kinetics of engraftment in patients with hematological malignancies given allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. *Blood* 2004; **104**: 2254–2262.
19 Cao TM, Shizuru JA, Wong RM, Sheehan K, Laport GG, Stockerl-Goldstein KE *et al.* Engraftment and survival following reduced-intensity allogeneic peripheral blood hematopoietic cell transplantation is affected by $CD8^+$ T-cell dose. *Blood* 2005; **105**: 2300–2306.
20 Mielcarek M, Graf L, Johnson G, Torok-Storb B. Production of interleukin-10 by granulocyte colony-stimulating factor-mobilized blood products: a mechanism for monocyte-mediated suppression of T cell proliferation. *Blood* 1998; **92**: 215–222.
21 Storb R, Prentice RL, Buckner CD, Clift RA, Appelbaum F, Deeg J *et al.* Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. *N Engl J Med* 1983; **308**: 302–307.
22 Blaise D, Kuentz M, Fortanier C, Bourhis JH, Milpied N, Sutton L *et al.* Randomized trial of bone marrow versus lenograstim-primed blood cell allogeneic transplantation in patients with early-stage leukemia: a report from the Société Française de Greffe de Moelle. *J Clin Oncol* 2000; **18**: 537–571.
23 Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R *et al.* Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med* 2001; **344**: 175–181.
24 Kernan NA, Collins NM, Juliano L, Cartagenia T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. *Blood* 1986; **68**: 770–773.
25 Remberger M, Ringdén O, Blau I-W, Ottinger H, Kremens B, Kiehl MG *et al.* No difference in graft-versus-host disease, relapse, and survival comparing peripheral stem cells to bone marrow using unrelated donors. *Blood* 2001; **98**: 1739–1745.
26 Zaucha JM, Gooley T, Bensinger WI, Heimfeld S, Chauncey TR, Zaucha R *et al.* CD34 cell dose in granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cell grafts affects engraftment kinetics and development of extensive chronic graft-versus-host disease after human leukocyte antigen-identical sibling transplantation. *Blood* 2001; **98**: 3221–3227.
27 Mohty M, Bilger K, Jourdan E, Kuentz M, Michallet M, Bourhis JH *et al.* Higher doses of CD34+ peripheral blood stem cells are associated with increased mortality from chronic graft-versus-host disease after allogeneic HLA-identical sibling transplantation. *Leukemia* 2003; **17**: 869–875.
28 Perez-Simon JA, Diez-Campelo M, Martino R, Sureda A, Caballero D, Canizo C *et al.* Impact of CD34+ cell dose on the outcome of patients undergoing reduced-intensity-conditioning allogeneic peripheral blood stem cell transplantation. *Blood* 2003; **102**: 1108–1113.
29 Riddell SR, Berger C, Murata M, Randolph S, Warren EH. The graft versus leukemia response after allogeneic hematopoietic stem cell transplantation. *Blood Rev* 2003; **17**: 153–162.
30 Panse JP, Heimfeld S, Guthrie KA, Maris MB, Maloney DG, Baril BB *et al.* Allogeneic peripheral blood stem cell graft composition affects early T-cell chimerism and later clinical outcomes after nonmyeloablative conditioning. *Br J Haematol* 2005; **128**: 659–667.
31 Sierra J, Storer B, Hansen JA, Bjerke JW, Martin PJ, Petersdorf EW *et al.* Transplantation of marrow cells from unrelated donors for treatment of high-risk acute leukemia: the effect of leukemic burden, donor HLA-matching, and marrow cell dose. *Blood* 1997; **89**: 4226–4235.
32 Heimfeld S. Bone marrow transplantation: how important is CD34 cell dose in HLA-identical stem cell transplantation (Keynote Address). *Leukemia* 2003; **17**: 856–858.