

## CD34+ Cell Enrichment

# High incidence of graft failure in children receiving CD34+ augmented elutriated allografts for nonmalignant diseases

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### Summary:

**T-cell depletion of the marrow graft using counterflow centrifugal elutriation reduces the risk of graft-versus-host disease (GVHD). However, because of high rates of graft failure and relapse, elutriation alone has not improved survival. We have carried out a phase II clinical trial in 54 pediatric patients to determine if CD34+ selection to rescue pluripotent stem cells from the small lymphocyte fraction improves engraftment. The processed grafts contained a mean of  $5.5 \times 10^7$  cells/kg IBW,  $4.7 \times 10^6$  CD34+ cells/kg IBW, and  $6.3 \times 10^5$  CD3+ cells/kg IBW. Patients achieved an ANC >500 at a median of 16 days and platelet count >20 000 at a median of 28 days. The incidence of clinically significant GVHD was 19%. In total, 10 patients enrolled in this study experienced graft failure, with eight of the 14 patients transplanted for nonmalignant indications failing to engraft stably. Graft failure was statistically significantly associated with nonmalignant diagnosis ( $P < 0.001$ ), but was not associated with CMV seropositivity, donor gender, or cell counts of the allograft. We conclude that although time to engraftment is similar to that seen with unmanipulated grafts, graft failure remains a significant problem in patients with hereditary, nonmalignant diseases. Future efforts will seek to preserve the benefits of elutriation with CD34+ selection by increasing immune ablation of the preparative regimen and/or increasing posttransplant immune suppression.**

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Allogeneic bone marrow transplant (BMT) is a therapeutic option for a wide variety of hematological and inherited metabolic disorders. However, the successful treatment of these diseases has been limited by the severe toxicities associated with allogeneic BMT, most prominently graft-versus-host disease (GVHD). Strategies employed to reduce

the incidence and severity of GVHD include modification of the preparative regimen, alterations in the type and timing of immunosuppressive therapy, and graft manipulation.

T-cell depletion (TCD) of the donor marrow has been used clinically since the late 1970s.<sup>1</sup> Numerous methods of TCD have been employed, with most dependent on negative selection techniques by physical separation or antibody-based purging.<sup>2</sup> However, although each of these methods has been successful in reducing rates of severe GVHD, virtually no improvement in overall survival has been observed because of the increased incidence of graft failure and disease relapse associated with T-cell depletion. Although intensification of the preparative regimen may reduce the rate of graft failure by minimizing the number of residual host T-cells,<sup>3–5</sup> this approach carries the potential risk of increased transplant-related morbidity and mortality.

Counterflow centrifugal elutriation (CCE) separates bone marrow cells on the basis of sedimentation properties, and has been shown to yield almost complete recovery of cells in several size fractions.<sup>6</sup> The lymphocytes separate to the small- and intermediate-cell fractions, while the large cell fraction contains most of the CFU-GM in the bone marrow harvest and is 100 to 1000-fold depleted of lymphocytes.<sup>7,8</sup> Early trials at this institution demonstrated that when the large cell fraction is used as the sole component of the graft, the incidence of clinically significant GVHD can be significantly reduced.<sup>9</sup> However, the incidence of graft failure increased to 10%. Patients who received lower lymphocyte doses ( $5 \times 10^5$  vs  $1 \times 10^6$  lymphocytes/kg ideal body weight) experienced delayed engraftment of granulocytes and platelets and a high incidence of mixed hematopoietic chimerism as determined by RFLP analysis of bone marrow. Although other researchers have reported a graft failure rate of only 5% with CCE alone,<sup>10</sup> this rate nonetheless exceeds that observed in unmanipulated marrow transplants.<sup>11,12</sup>

In 1990, Jones *et al*<sup>13</sup> showed in a murine model that two classes of cells are necessary for successful engraftment: committed progenitors that provide initial but unsustainable engraftment, and pluripotent hematological stem cells (PHSC) that provide delayed and durable engraftment. These PHSC copurify with lymphocytes in the small cell elutriation fractions and are thus excluded from the TCD allograft. On this basis, a phase I trial was developed employing CD34+ selection to recover PHSC from the elutriated small lymphocyte fraction.<sup>14</sup> By rescuing the

small CD34+ cells, the time to engraftment can be significantly shortened, while the low incidence of severe GVHD observed with standard TCD by elutriation is preserved.

Phase II clinical trials were then undertaken to more clearly elucidate the role of T-cell depleted, CD34+ augmented bone marrow in matched sibling and unrelated transplants. Results of the trial in adults<sup>15,16</sup> showed that both acute and chronic GVHD were significantly decreased and that CD34+ stem cell selection was associated with rapid engraftment and shorter in-patient hospital stays. The 3.6% incidence of graft failure was higher than that observed in unmanipulated marrow transplants,<sup>11,12</sup> but was explainable by known risk factors in four out of five patients. Three patients with AML had received platelet transfusions from the marrow donor prior to transplant and were thereby specifically alloimmunized. The fourth patient had a 3-year history of aplastic anemia that had evolved into myelodysplastic syndrome (MDS). The fifth patient experienced late graft failure at >155 days after BMT in the absence of known infection or GVHD and the reasons for this failure remain unknown.

The technique of elutriation with CD34+ stem cell selection has now been studied in pediatric patients undergoing allogeneic BMT, and the results of that Phase II clinical trial are reported here. Like the adults, these patients showed a significant reduction in clinically significant GVHD and in time to engraftment. However, a much higher incidence of graft failure was observed in this population, specifically in children undergoing BMT for nonmalignant hereditary disorders.

## Materials and methods

### *Patient eligibility*

Between 1 January 1995 and 31 August 2000, pediatric patients between the ages of 6 months and 21 years meeting specific diagnostic and eligibility criteria were enrolled on one of three institutional protocols employing identical graft processing. Patients enrolled in these treatment protocols met specified disease and eligibility criteria: age over 6 months; adequate pulmonary function testing (>75% of predicted value); ejection fraction >50% demonstrated by echocardiogram or MUGA; adequate hepatic function as shown by a bilirubin <2.0 and SGOT and SGPT <two times normal; and absence of serious infection. Patients were considered for unrelated donor transplantation only when a suitable sibling donor was not available. Patients were considered for a one antigen-mismatched transplant only if their expected disease-free survival using autologous stem cell transplantation was <40%. Patients eligible for matched, unrelated BMT included those with (1) chronic myeloproliferative or lymphohistiocytic disorder, (2) myelodysplastic syndromes, or (3) inherited metabolic disorders. Patients with the following diagnoses were eligible for a one antigen-mismatched BMT when a fully matched donor was not available: (1) AML in relapse or >CR1, (2) ALL, (3) myelodysplastic syndromes, (4) inherited disorders, (5)

lymphoma in resistant relapse, or (6) Hodgkin's disease in resistant relapse. Two antigen-mismatched transplants were considered only for patients with (1) relapsed or refractory leukemia, (2) resistant aggressive lymphoma, or (3) accelerated phase/blast crisis CML. Patients were analyzed as of 1 October 2001.

### *Informed consent*

The protocols for CCE, selection of CD34+ cells, and the myeloablative preparative regimen were reviewed and approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions. Informed consent was obtained from all patients or from their legal guardian.

### *Transplant procedure*

For confirmatory purposes, all patients and donors were HLA typed in our laboratory. Serologic typing was employed for class I HLA antigens (HLA-A, -B, -C) and molecular typing for class II antigens (HLA-DRB1, -DRB3, -DRB4, -DPQ1, -DQB1).

The BMT preparative regimen was not mandated by the research protocol and varied according to diagnosis and donor. Patients with HLA-identical sibling donors undergoing BMT for acute leukemia, Hodgkin's disease, beta thalassemia major, or autoimmune disease received a preparative regimen of busulfan and cyclophosphamide. Busulfan was administered orally at a dose of 4 mg/kg/day in four divided doses from day -9 to day -6 pretransplant (total dose 16 mg/kg). Pharmacokinetic targeting was employed after the first dose to achieve an AUC between 800 and 1400 (ng/ml)·min. Cyclophosphamide was given at a dose of 50 mg/kg/day i.v. from day -5 to day -2 pretransplant (total dose 200 mg/kg). Patients receiving marrow donation from an unrelated donor or an HLA-nonidentical relative underwent conditioning with cyclophosphamide 50 mg/kg/day i.v. for 4 days and total body irradiation (TBI) 300 cGy/day for 4 days (total dose 1200 cGy). Diagnoses included acute leukemia, CML, adrenoleukodystrophy (ALD), and congenital pancytopenia

Patients with Fanconi anemia receiving an allograft from an unrelated or HLA-non-identical relative were conditioned with ATG (15 mg/kg/day for 5 days) given concurrently with cyclophosphamide at a dose of 10 mg/kg/day for 4 days (total dose 40 mg/kg) and a single 400 cGy dose of TBI. The one patient with severe combined immunodeficiency disease (SCID) and intact natural killer cell function received ATG in addition to full busulfan and cyclophosphamide conditioning. Two other SCID patients received no preparative regimen and one child received fludarabine at a dose of 1 mg/kg/day for 5 days prior to transplant.

Cyclosporine A (CSA) was administered to all patients for GVHD prophylaxis, with dosing based on ideal body weight. Additional immunosuppression with methylprednisolone was administered to patients whose donors were not genotypically HLA-identical. The following dosing schedule was employed: CSA at 5 mg/kg/day i.v. on days -2 to +2; 3.75 mg/kg/day i.v. on days 3 to 14; and 2.5 mg/kg/day

i.v. from day 14 until the patient was able to absorb oral medications. A transition was made from i.v. to oral dosing of CSA, which was continued until day 180. All CSA was given in divided doses. Prophylactic steroids were administered according to the following regimen:<sup>17</sup> methylprednisolone at 10 mg/kg on pretransplant days -3, -2, and -1 to eliminate residual host lymphocytes; methylprednisolone at 0.5 mg/kg/day i.v. from days 7-14 and at 1.0 mg/kg/day i.v. from days 15-28; oral prednisone at 0.8 mg/kg/day from days 29-42, at 0.5 mg/kg from days 43-56, at 0.2 mg/kg/day from days 57-71, and at 0.2 mg/kg every other day from days 72-79. Steroids were discontinued after day 79 when an ACTH stimulation test showed endogenous steroid production.

#### *CCE and CD34+ selection*

Cell separation by CCE has been previously described.<sup>18</sup> Bone marrow buffy coat cells were prepared on a COBE Spectra apheresis system set for bone marrow processing (COBE BCT, Lakewood, CO, USA). Cells consisting of 96% of harvested mononuclear cells were then loaded into a Beckman JE-10x elutriation rotor and chamber (Beckman Instruments, Palo Alto, CA, USA) at 20°C, a flow rate of 50 ml/min, and a rotor speed of 3000 rpm. Three fractions were collected: (1) a small cell fraction composed of erythrocytes and small lymphocytes; (2) an intermediate cell fraction composed of large lymphocytes, granulocytes, and monocytes; and (3) a large rotor-off fraction composed of granulocytes, monocytes, blasts, and <0.2% lymphocytes. To ensure a standard lymphocyte dose of  $5 \times 10^5$  cells/kg IBW, differential cell counts were performed on 500 leukocytes from the unseparated marrow buffy coat, the elutriation large cell fraction, and the CD34+ stem cell product.

The CD34+ cell selection procedure has been published in detail.<sup>11</sup> Following elutriation, the loading fraction and small- and intermediate-size fractions were combined and incubated with murine anti-CD34 antibody. Cells were then concentrated, washed, and resuspended, and the CD34+ cells selected using an automated Ceparate SC stem cell concentrator (Cellpro, Bothell, WA, USA). The purity of the CD34+ cells was verified by flow cytometry using phycoerythrin-conjugated monoclonal antibody to CD34 (HPCA-2) and to CD-3, and fluorescein isothiocyanate-conjugated monoclonal antibody recognizing CD45 (Becton Dickinson, San Jose, CA, USA).

#### *Evaluation of donor engraftment*

Initial engraftment was determined by daily evaluation of hematologic parameters and by bone marrow examination if dictated by clinical course. The day of neutrophil engraftment was defined as the first of three consecutive days with an ANC > 500. Platelet engraftment was defined as the first day with a platelet count > 20 000 without platelet transfusion in the preceding 7 days. Graft durability was evaluated on days +30, +60, +90, +180, and at 1 and 2 years posttransplant. Chimerism analysis was with XY FISH (sex mismatched donor) or with RFLP/DNA probe analysis (same sex donor). Engraftment was

defined as count recovery by 30 days with >95% donor chimerism. All patients who failed to meet this criterion were included as patients with graft failure. Secondary graft failure was defined as loss or persistent decline of donor chimerism after achieving primary engraftment.

#### *Statistical methods*

Cell counts were approximately normally distributed after logarithmic transformation. Therefore, geometric mean cell counts are reported, and logarithmic transformed cell counts were used in all statistical analyses. Event times were measured from the date of BMT with patients censored at the time of death or date of last follow-up. Event time distributions were estimated using the product limit method of Kaplan and Meier<sup>19</sup> and confidence intervals determined using Greenwood's<sup>20</sup> formula. Date and stage of GVHD were determined by the treating team or the GVHD team and were reported according to published criteria.<sup>21</sup> Patients were evaluable for acute GVHD if they survived beyond day 30 after BMT or developed GVHD prior to day 30. Patients who survived 90 days post-BMT were evaluable for chronic GVHD. Fisher's exact test and Wilcoxon's rank-sum tests were used to determine heterogeneity. Student's *t*-test was used to compare two means. Analysis was performed by using STATA statistical software.

## **Results**

#### *Clinical characteristics*

Between January 1995 and August 2000, 79 pediatric patients underwent initial allogeneic BMT at the Johns Hopkins Children's Center. Of these 79 patients, 58 were enrolled on one of three institutional protocols employing identical methods of TCD and CD34+ stem cell selection. Four of the enrolled patients ultimately received unmanipulated grafts because of inadequate cell counts ( $< 2.5 \times 10^8$  nucleated cells/kg IBW) or inadequate volume ( $< 750$  ml) of the harvested marrow. In all, 21 patients were not considered for TCD because of diagnoses of acute leukemia with active disease, or aplastic anemia or CML with matched sibling donors. Clinical characteristics of the 54 recipients of processed grafts are described in Table 1. Patients ranged in age from 7 months to 20 years and the male:female ratio was 1.25 to 1. In total, 14 patients were treated for nonmalignant diseases and the remaining 40 had leukemia or lymphoma.

#### *Bone marrow graft*

Characterization of the large cell fraction, the CD34+ augmented fraction, and the engineered graft is given in Table 2. On average, the harvest contained  $8.1 \times 10^8$  mononuclear cells/kg IBW before processing. The infused large cell and CD34+ selected fractions contained an average of  $6.0 \times 10^7$  mononuclear cells/kg IBW, and  $3.6 \times 10^6$  mononuclear cells/kg IBW, respectively. The large cell fractions contained an average of 0.64% CD3+ cells (range 0.1-2.2%) and 5.6% CD34+ cells (range 1.2-

**Table 1** Patient characteristics

Number of patients	54
Median age in years (range)	
Donor	23 (4–53)
Recipient	9 (6–21)
Gender, number (%)	
Male	30 (56%)
Female	24 (44%)
Diagnosis, number (%)	
Malignancies	40 (74%)
ALL	15 (28%)
AML	16 (30%)
CML	3 (6%)
T-cell disease	4 (7%)
HD	1 (2%)
Biclonal leukemia	1 (2%)
Nonmalignant diseases	14 (26%)
Beta thalassemia	4 (7%)
SCID	4 (7%)
ALD	2 (4%)
FA	2 (4%)
Congenital pancytopenia	1 (2%)
Autoimmune disease	1 (2%)

13.4%). The CD34+ fractions contained an average of 13% CD3+ cells (range 1.8–36.8%) and 73.4% CD34+ cells (range 43.1–91.4%).

There was no correlation between total mononuclear cell count, CD34+ cell count, CD3+ cell count and time to engraftment of neutrophils or platelets (Figure 1).

### Engraftment

The use of elutriated allografts augmented by CD34+ selection in this patient population resulted in median neutrophil engraftment (ANC > 500) in 16 days (95% CI: 15–17 days) and platelet engraftment above 20 000 in 28 days (95% CI: 22.5–32 days). The pediatric patients who received unmanipulated grafts during this time period had a median time to neutrophil recovery of 19 days (95% CI: 17–21.5) and median time to platelet recovery of 24.5 days (95% CI: 21–28). Time to neutrophil recovery was statistically significantly faster in recipients of elutriated grafts ( $P=0.009$ ), but there was no statistically significant difference in speed of platelet recovery ( $P=0.37$ ). There was no association between time to engraft and cell dose (Figure 1).

### Failure of engraftment

Of the 54 pediatric patients who received elutriated, CD34+ augmented allografts, 10 children (18.5%) experienced graft failure. The graft characteristics of the patients with graft failure are detailed in Table 3. None of the children treated with unprocessed marrow during this time period experienced graft failure. Graft failure was statistically significantly associated with a nonmalignant underlying diagnosis ( $P<0.0001$ ), with eight of the 14 children transplanted for nonmalignant diseases (57%) vs two of 40 children with malignancies (5%) experiencing graft failure. Graft failure was not associated with CMV seropositivity

of the patient ( $P=0.49$ ) or donor ( $P=1.0$ ), GVHD ( $P=0.85$ ), or donor gender ( $P=0.17$ ). The association between graft failure and gender mismatch approached statistical significance ( $P=0.07$ ), with seven of the 10 patients with graft failure having marrow donors of the opposite gender. Six of these seven patients were male patients with female donors. HLA disparity was also associated with graft failure, with half of the patients with related donors mismatched at two or more HLA alleles experiencing graft failure (Table 4).

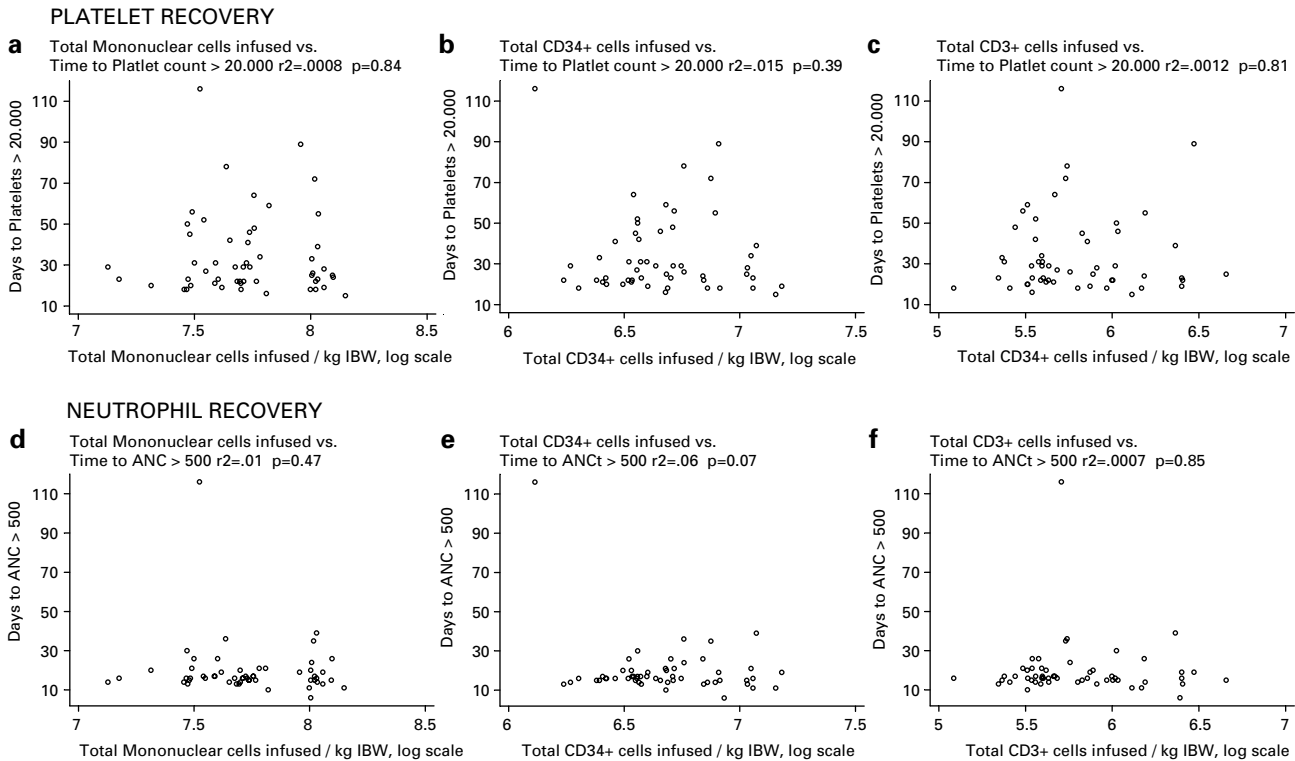
The mean total mononuclear cell count, mean CD34+ cell count, and mean CD3+ cell count in the subset of patients with graft failure did not differ significantly from the mean cell counts for patients who engrafted fully (Table 5). The difference in CD3+ cell count approached statistical significance ( $P=0.06$ ), with stably engrafted patients receiving a lower mean CD3+ cell dose than those with graft failure. Of the 10 children with graft failure, only two had malignant diseases. Indications for BMT included beta thalassemia major (3), ALD (1), multisystem autoimmune disease (1), and SCID (3). The three thalassemic patients were heavily transfused, but had not received red cells from the marrow donor or other relatives, and none had received platelet transfusions.

Seven of the 10 patients with graft failure did not engraft after initial donor marrow infusion and were classified as primary graft failure. Three patients, including one patient with thalassemia, one patient with ALD, and one patient with multisystem autoimmune disease, experienced late graft failure after initial donor engraftment. These three patients were successfully converted to full donor chimerism with donor lymphocyte infusions. The two patients with thalassemia and primary graft failure experienced autologous recovery with stable partial chimerism of red blood cells and both remain alive and transfusion independent.

Two of the patients with SCID received no preparative regimen and did not become cytopenic. XY FISH performed in one patient 19 days after transplant on peripheral blood showed no donor cells. He died on day 19 of overwhelming infection and ARDS. The second patient developed warm IgM agglutinin autoimmune hemolytic anemia and died of this complication 40 days post-BMT.<sup>22</sup> The third patient with SCID received fludarabine at a dose of 1 mg/kg/day for 5 days prior to haploidentical marrow infusion. Initial engraftment was complicated by steroid-refractory Stage III GVHD of the liver and gut, but after salvage therapy with pentostatin he had no evidence of donor engraftment. He underwent second BMT following busulfan/cyclophosphamide conditioning 8 months later and remains alive and well with full donor hematopoiesis 16 months after his second BMT.

### GVHD

In all, 10 of the 52 evaluable patients (19%) in the study developed clinically significant acute GVHD (Stage II or greater). The incidence was greatest in patients undergoing mismatched related transplants (four of 10 patients), while those with matched sibling donors and unrelated donors had rates of clinically significant GVHD of 11 and 15%,



**Figure 1** Relation between time to engraftment and cell dose. (a) Correlation between total mononuclear cell dose and platelet recovery; (b) Correlation between CD34+ cell dose and platelet recovery; (c) Correlation between CD3+ cell dose and platelet recovery; (d) Correlation between total mononuclear cell dose and neutrophil recovery; (e) Correlation between CD34+ cell dose and neutrophil recovery; (f) Correlation between CD3+ cell dose and neutrophil recovery.

**Table 2** Characteristics of the CD34(+) augmented, elutriated allograft

	Total cells/kg IBW Mean (range)	CD34+ cells/kg IBW Mean (range)	CD3+ cells/kg IBW Mean (range)
Large cell fraction	$7.0 \times 10^7$ ( $1.2 \times 10^7$ – $3.7 \times 10^8$ )	$3.4 \times 10^6$ ( $6.9 \times 10^5$ – $1.7 \times 10^7$ )	$3.4 \times 10^5$ ( $4.3 \times 10^4$ – $3.7 \times 10^6$ )
CD34+ Selected fraction	$3.2 \times 10^6$ ( $7.2 \times 10^5$ – $8.3 \times 10^8$ )	$2.3 \times 10^6$ ( $3.1 \times 10^5$ – $5.7 \times 10^8$ )	$3.4 \times 10^5$ ( $5.6 \times 10^4$ – $8.1 \times 10^7$ )
Total infused graft	$5.5 \times 10^7$ ( $1.3 \times 10^7$ – $1.4 \times 10^8$ )	$4.7 \times 10^6$ ( $1.3 \times 10^6$ – $1.5 \times 10^7$ )	$6.3 \times 10^5$ ( $1.2 \times 10^5$ – $4.5 \times 10^6$ )

Mean values represent the geometric mean of the sample.

respectively. In total, eight patients developed Stage I, three patients Stage II, four Stage III, and three Stage IV GVHD. Severe GVHD occurred only in recipients of unrelated and mismatched related transplants. Three of the patients with high-stage GVHD responded completely to therapy and remained long-term survivors. One patient had a complete response to GVHD therapy but died of an infection 8 months later. The deaths of two patients were directly attributable to GVHD, while a third patient died of complications of acute GVHD with active leukemia. Of the 44 patients evaluable for chronic GVHD, six were affected (13.6%). Two of the six cases were extensive, but both patients responded well to therapy with tacrolimus, mycophenolate mofetil, steroids, PUVA, and, in one case, pentostatin.

### Mortality

Overall mortality was 50% for patients with both malignant (20/40) and nonmalignant (7/14) diseases.

Patients with malignant diseases had a median survival time of 15 months, while those with nonmalignant diseases had a median survival time of 8.5 months. The median follow-up time for survivors was 38 months (range 4–62 months). A total of 11 deaths were classified as peritransplant (occurring before day 100), and 16 occurred later. Transplant-related mortality, defined as nonrelapse deaths before day 100, was 17%. Six of the seven deaths attributable to disease relapse occurred after day 100. Survival for the patients with graft failure (60%) was not inferior to that of the group as a whole. Both patients with leukemia and two of the patients with SCID died, while the other six patients, including all three with thalassemia, remain long-term survivors.

### Discussion

This study illustrates the effective use of T-cell depletion by elutriation and CD34+ stem cell augmentation in children

**Table 3** Characteristics of patients with graft failure

Diagnosis	Time of graft failure <sup>a</sup> ( $\times 10^7$ )	Total cell dose ( $\times 10^6$ )	CD34+ cell dose ( $\times 10^6$ )	CD3+ cell dose ( $\times 10^5$ )	Donor type	Preparative regimen	Donor-recipient gender	GVHD	Infections	Allo-immunization <sup>b</sup>	Outcome
Beta-Thalassemia	180	6.7	4.8	7.8	Sibling 6/6	Bu/Cy	Male to female	None	None	Positive	Alive (autologous recovery)
Beta-Thalassemia	30	4.3	7.7	5.5	Sibling 6/6	Bu/Cy	Male to male	Stage I	HSV, Staph Bacteremia	Negative	Alive (autologous recovery)
Beta-Thalassemia	30	3.0	3.6	10.6	Sibling 6/6	Bu/Cy	Male to male	None	Klebsiella Bacteremia	Negative	Alive (autologous recovery)
SCID	20	11.4	15.1	25.1	Parent 3/6	None	Female to male	None	Influenza A	Negative	Died (ARDS)
SCID	30	10.1	8.6	24.6	Parent 3/6	Fludarabine	Female to male	Stage III	None	Negative	Alive (second BMT)
SCID	30	10.7	11.8	23.2	Parent 4/6	None	Female to female	None	Staph Bacteremia	Not done	Died (IgM AIHA)
Multisystem autoimmune disease	90	10.5	3.4	10.0	Sibling 6/6	Bu/Cy	Female to male	None	HSV, Staph Bacteremia	Negative	Alive (DLI)
Adrenoleukodystrophy	180	3.9	2.6	4.6	Unrelated 6/6	Cy/TBI	Female to male	None	CMV	Negative	Alive (DLI)
T-cell ALL, Ph+, CR1	30	10.8	15.2	25.2	Parent 5/6	Cy/TBI	Female to male	Stage I	BK Virus, EBV	Negative	Died (EBV LPD)
AML, CR1	30	3.3	1.3	5.1	Unrelated 6/6	Cy/TBI	Female to male	None	BK Virus	Negative	Died (infection)

<sup>a</sup>Days after BMT.

<sup>b</sup>Alloimmunization to HLA antigens screened for using solid-phase ELISA.

**Table 4** Patient characteristics associated with graft failure

Donor HLA compatibility (% with graft failure)	
Related	28
3/6	4 (50%)
4/6	2 (50%)
5/6	4 (25%)
6/6	18 (22%)
Unrelated	26
5/6	6 (0%)
6/6	20 (10%)
Donor-recipient gender (% with graft failure)	
Male to male	17 (12%)
Male to female	10 (10%)
Female to male	12 (50%)
Female to female	14 (7%)
Unknown	1

undergoing allogeneic BMT for the treatment of hematologic malignancies. However, in patients with nonmalignant diseases in whom avoidance of GVHD is most desirable, an unacceptably high rate of graft failure was observed. Of the 14 pediatric patients who received TCD allografts for nonmalignant indications, eight experienced graft failure. An increased risk of graft failure is well recognized with TCD and is in part attributable to a reduction in the number of pluripotent hematopoietic stem in the graft.<sup>13</sup> However, in 1998 O'Donnell *et al*<sup>15</sup> reported that this complication had been largely overcome by augmentation of the allograft with CD34+ stem cells. Of the 110 adult patients treated in his study with matched sibling transplants and graft manipulation identical to that described here, only five patients, (4.5%) experienced graft failure. In four of the five patients, the graft failure was explainable by a known risk factor, including aplastic anemia, extensive pretransplant platelet transfusions, or prior sensitization to donor histocompatibility antigens. These risk factors did not explain the cases of graft failure observed in our pediatric patients.

Of the 10 pediatric patients with graft failure reported here, eight underwent BMT for nonmalignant diseases. The seven cases of early graft failure were not explainable by inadequate cell counts in the graft. The mean mononuclear cell count in this subset of patients was higher than that of the entire group of eluted grafts ( $6.38 \times 10^7$  vs  $5.5 \times 10^7$ ), and the smallest cell dose received was  $2.95 \times 10^7$  mononuclear cells/kg IBW. Likewise, both the mean CD34+ and mean CD3+ cell counts were higher in the patients with graft failure than the study group as a whole. Nor were any of the cases of graft failure explainable by prior HLA sensitization. Although both patients with thalassemia had received numerous red blood cell transfusions prior to transplant, none was from the bone marrow donor. Furthermore, none of the four patients had ever received a platelet transfusion before BMT.

All 10 of the patients who experienced graft failure received GVHD prophylaxis with CSA, and the four patients with an unrelated or HLA mismatched family donor received prophylactic steroids. Therapeutic CSA levels were maintained in all patients. Only one patient developed clinically significant GVHD. An imbalance of

**Table 5** Characteristics of infused allograft in patients with graft failure vs stable engraftment

	Patients with stable engraftment (n = 44)	Patients with graft failure (n = 10)	P-value
Total mononuclear cells/kg IBW mean (range)	$5.3 \times 10^7$ ( $1.3 \times 10^7$ – $1.4 \times 10^8$ )	$6.4 \times 10^7$ ( $3.0 \times 10^7$ – $1.1 \times 10^8$ )	0.34
CD34+ cells/kg IBW mean (range)	$4.6 \times 10^6$ ( $1.7 \times 10^6$ – $1.4 \times 10^7$ )	$5.4 \times 10^6$ ( $1.3 \times 10^6$ – $1.5 \times 10^7$ )	0.45
CD3+ cells/kg IBW mean (range)	$5.7 \times 10^5$ ( $1.2 \times 10^5$ – $4.5 \times 10^6$ )	$9.6 \times 10^5$ ( $4.6 \times 10^5$ – $2.5 \times 10^6$ )	0.06

Mean values represent the geometric mean of the sample.

alloreactivity in the direction of host-versus-graft would predispose to rejection, while GVHD would favor donor engraftment. This patient had only lymphoid chimerism and salvage therapy for refractory GVHD was highly lymphotoxic.

In our study, six of the 12 donor–recipient pairs with male patients and female donors experienced graft failure. The association between gender mismatch and nonengraftment approached statistical significance with a *P*-value of 0.07. Other authors have found an association between graft failure and gender mismatch in TCD transplants,<sup>23,24</sup> but the association was more pronounced in female patients with male donors. Similarly, a study of BMT for severe aplastic anemia performed at this institution found an increased risk of rejection when female patients received marrow from male donors.<sup>25</sup> A report of the European Group for Blood and Marrow Transplantation on unrelated donor transplants for Fanconi anemia<sup>26</sup> did find a higher incidence of primary graft failure with female donors, but this finding was attributed to low cell counts of female marrow grafts. This was not the case in our patients with graft failure and female donors.

Viral infection can be associated with graft failure, and one patient with SCID did have documented influenza A from a sputum culture obtained 1 day post transplant. Two of the three patients who received high-dose cytoxan conditioning pretransplant developed BK virus positive hematuria, but there is no reported association between BK virus infection and graft failure. CMV early antigen was detected in the patient with ALD 1 month prior to graft rejection and was adequately treated with ganciclovir without development of cytopenias or CMV disease.

Graft failure in thalassemia is a well-described risk, with incidences ranging from 7% in class 1 and class 2 patients to 22% in class 3 patients.<sup>27–30</sup> Although the number of thalassemic patients undergoing BMT in our series is too small to reach statistical significance, the occurrence of graft failure in three of four patients is clinically important and much higher than anticipated given the current literature. Furthermore, all three thalassemic patients who experienced graft failure were class 1 and would therefore have an expected risk of graft failure of only 7%. Interestingly, the one patient in our series who achieved sustained engraftment was categorized as class 3.

Using CCE to deplete the marrow graft of T-cells and flow cytometry to rescue CD34+ stem cells from the lymphocyte-rich fraction, we were able to achieve rapid engraftment of donor marrow while maintaining low rates of clinically severe GVHD. This method of graft manipulation led to a 200-fold decrease in T-cells compared to the harvests before processing and a two-fold increase in

CD34+ stem cells when compared the elutriated rotor-off fractions. With the addition of CD34+ stem cells to the allograft, the delayed engraftment observed with simple elutriated marrow was avoided. Our patients showed a median time to neutrophil (ANC > 500) and platelet (> 20 000 without transfusion for 7 days) recovery of 16 days and 25 days, respectively. These results are similar to those reported by O'Donnell *et al*<sup>15</sup> in adult patients undergoing matched sibling transplants with similarly prepared allografts.

The incidence of clinically significant GVHD (19%) observed in this study was low. In patients undergoing HLA-matched unrelated BMTs, the rates of clinically significant GVHD exceed 75% with unmanipulated grafts and CSA/methotrexate prophylaxis.<sup>31</sup> Although some degree of GVHD is desirable because of its associated graft vs leukemia effect, precise titration of GVHD is difficult. GVHD therefore remains a major cause of morbidity and mortality in unrelated transplants. At this institution, the use of simple TCD decreased the incidence of clinically significant GVHD to below 25%.<sup>9</sup> However, simple TCD was associated with a higher incidence (10%) of graft failure and thus did not lead to an overall improvement in survival.<sup>32</sup> The concomitant use of CD34+ augmentation with elutriation has allowed us to achieve fast and durable engraftment while maintaining low rates of severe GVHD. In a simultaneous study of adults undergoing matched sibling transplants, O'Donnell *et al* reported a rate of clinically significant GVHD of only 11%.<sup>15</sup> These results are consistent with the rate of 19% observed here in pediatric patients undergoing related and unrelated transplant. Both experiences represent a significant improvement over unmanipulated graft BMT.

In this study of 54 pediatric patients, TCD with CD34+ augmentation resulted in rapid engraftment and low rates of clinically significant GVHD in patients with hematologic malignancies. However, in patients with nonmalignant diseases, the subset of patients in whom prevention of GVHD is most desirable because it carries no associated graft vs leukemia benefit, this technique of graft manipulation was associated with a significant risk of graft failure. Possible explanations for this finding include more intact immunity compared to heavily pretreated leukemia patients and alloimmunization from multiple red blood cell transfusions. For children with nonmalignant diseases, it will be necessary to reduce the risk of graft rejection by increasing immune ablation by the preparative regimen and/or by improving posttransplant immune suppression. The goal of these modifications is to preserve the benefits of TCD with CD34+ augmentation, including rapid time to engraftment and low incidence of GVHD, while reducing the risk of graft failure.

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