Pre-emptive immunotherapy with CD8-depleted donor lymphocytes after CD34-selected allogeneic peripheral blood stem cell transplantation

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Background and Objectives. To maximize graft-versus-leukemia (GVL) effects while minimizing the risk of graft-versus-host disease (GVHD), we undertook a study of allogeneic CD34-selected peripheral blood stem cell (PBSC) transplantation followed by CD8-depleted donor lymphocyte infusion (DLI).

Design and Methods. Twenty-four patients with advanced hematologic malignancies were included. PBSC were collected in matched (N=16) or one-mismatch (N=8) related donors and CD34-selected. On day 60, donors donated lymphocytes that were CD8-depleted and separated into 3 aliquots containing 2×10^6, 1×10^7 and 5×10^7 $CD3^+$ cells/kg (patients 1-13) or into 2 aliquots containing 1×10^7 and 5×10^7 $CD3^+$ cells/kg (patients 14-24). The 1st aliquot was infused on day 60 and the other 1 (2) cryopreserved and infused on days 100 (and 140).

Results. An average of 100%, 100% and 84% of the scheduled dose could be administered in DLI 1, 2 and 3, respectively. Although the study group was at very high risk of GVHD, the actuarial incidence of grade II-IV acute GVHD was 28% (13% for HLA-identical siblings) with only 1 patient developing grade III-IV GVHD (after DLI). The actuarial 2-year probability of extensive chronic GVHD was similarly low (13% for all patients and 0% for HLA-identical siblings). Individual cases as well as a 30% relapse rate (0% for standard-risk patients versus 55% for high-risk patients) indicated preservation of the GVL effect.

Interpretation and Conclusions. We conclude that allogeneic transplantation of CD34-selected PBSC followed by pre-emptive CD8-depleted DLI is feasible with rapid engraftment and minimizes the risk of severe GVHD. Large prospective trials are required to prove that it preserves the GVL effect fully.

Key words: allogeneic transplantation, GVHD, GVL, CD34 selection, donor lymphocyte infusions.

After allogeneic hematopoietic stem cell transplantation,[1] graft-versus-host disease (GVHD) and its treatment are the major causes of transplant-related morbidity and mortality.[2] Compared with bone marrow transplantation, the use of allogeneic peripheral blood stem cells (allo-PBSC) ensures faster hematopoietic recovery.[3-8] However, the number of T-cells injected is about 1 log higher, leading to an increased incidence of chronic GVHD[3-5,7,9-11] but probably also a decreased risk of relapse.[6,8] The most efficient method for prevention of GVHD consists in T-cell depletion of the graft.[12-15] However this usually leads to greatly increased risks of leukemia relapse and delayed immune reconstitution.[12-18] Positive selection of $CD34^+$ hematopoietic stem cells is a novel way of performing T-cell depletion that results in a 2-3 log elimination of T and NK cells while preserving hematologic reconstitution after transplantation.[17,19-21] As with other T-cell depletion methods, preliminary reports suggest that CD34 selection may reduce the incidence of acute and chronic GVHD[22] but that there could be an increased risk of relapse.

Donor lymphocyte infusions (DLI) have been used increasingly to treat relapses after hematopoietic stem cell transplantation (HSCT).[23-31] The main com-

plication of DLI is GVHD, which is often associated with response to DLI.[23,25,26,31] However, the observation in some patients of complete responses in the absence of GVHD proves that the graft-versus-leukemia (GVL) effect may be separated from the development of clinical GVHD. Moreover, it is possible to reduce the risk of GVHD without impairing the GVL effect by starting with a low dose of T-cells and increasing the dose in a stepwise fashion in case of no response[32,33] or by CD8 depletion of DLI.[29,34,35] As DLI are effective at inducing complete remissions in leukemic patients relapsing after allogeneic bone marrow transplantation, and particularly so if the infusions are performed in early relapse,[23,25,26,29,31,36] it might be even more efficient to give DLI before relapse at a time when minimal residual disease is still present. However, it is well demonstrated that unmanipulated DLI given early after hematopoietic stem cell transplantation are associated with an increased risk of GVHD[23,37] and previous studies of pre-emptive DLI resulted in a high incidence of GVHD despite low numbers of T-cells infused in incremental fashion.[38-40]

In order to take advantage of the engraftment velocity and the GVL effect of allogeneic PBSC while minimizing the risk of GVHD, we undertook a study of allogeneic CD34-selected PBSC transplantation followed by CD8-depleted DLI given in an incremental fashion.

Design and Methods

Patients and donors

Twenty-four patients with hematologic malignancies, 18 males and 6 females, aged 14 to 56 years (median 46 years) were included. Their clinical characteristics are summarized in Table 1. Twelve patients (7 with acute myeloid leukemia-AML, 2 with acute lymphocytic leukemia-ALL, 1 with non-Hodgkin's lymhoma-NHL in first remission and 2 with chronic myeloid leukemia-CML in first chronic phase) were designated as standard risk for relapse although 4 had poor prognostic cytogenetic abnormalities. The remaining 12 patients with more advanced disease were considered as high risk, including patient #3 (ALL in first complete remission) because of the very high number of lymphoblasts and massive cerebral leukostasis at diagnosis. Transplants were carried out from matched (#=16) or one-mismatch (#=8) related donors (Table 1). Written informed consent was

Table 1. Patients and donors.

	Patients				*Donors*		
No.	*Age/Sex*	*Diagnosis*	*Cytogenetic abnormality*	*Status at transplant and other prognostic factors*	*Relationship*	*Age/Sex*	*HLA*
1	14/M	NHL	none	2nd CR	Sibling	17/M	1 mismatch
2	18/M	AML	del7	1st CR	Sibling	23/M	HLA-id
3	15/F	ALL	t(1; 4), del 10	1st CR; 900,000 WBC and cerebral leukostasis at diagnosis	Sibling	13/M	HLA-id
4	50/F	AML	none	1st CR	Sibling	53/M	HLA-id
5	40/M	AML	t(8; 21); -Y	1st relapse	Sibling	16/M	HLA-id
6	32/M	AML	none	2nd relapse; 2nd allogeneic transplant	Parent	70/M	1 mismatch
7	15/M	ALL	t(9; 22)	2nd CR	Sibling	18/F	HLA-id
8	53/M	CML	t(9; 22)	1st CP	Sibling	48/F	1 mismatch
9	47/F	AML	none	1st CR	Child	28/F	1 mismatch
10	42/M	AML	complex karyotype	refractory disease	Sibling	38/M	HLA-id
11	48/M	CML	t(9; 22)	2nd CP	Sibling	47/M	HLA-id
12	27/M	AML	inv16	1st CR	Sibling	30/M	HLA-id
13	51/M	CMML	none	refractory disease	Sibling	43/F	HLA-id
14	26/M	ALL	unsuccessful	1st CR	Parent	57/F	1 mismatch
15	23/M	ALL	not done	2nd CR	Sibling	35/M	HLA-id
16	49/M	CLL	complex karyotype	refractory disease	Sibling	44/M	HLA-id
17	49/F	AML	complex karyotype	refractory disease	Sibling	40/F	HLA-id
18	18/M	ALL	complex karyotype	1stCR	Sibling	14/M	HLA-id
19	56/F	AML	none	1st CR	Sibling	62/F	HLA-id
20	48/F	NHL	none	1st CR	Sibling	51/F	1 mismatch
21	44/F	NHL	none	2nd CR	Sibling	51/F	1 mismatch
22	47/F	CML	t(9; 22)	1st CP	Child	25/F	1 mismatch
23	51/F	AML	+8	1st CR	Sibling	44/F	HLA-id
24	50/F	AML	complex karyotype	1st CR	Sibling	55/M	HLA-id

M = male; F = female; AML = acute myeloid leukemia; CML = chronic myeloid leukemia; NHL = non-Hodgkin's lymphoma; ALL = acute lymphoid leukemia; CMML = chronic myelo-monocytic leukemia; CLL = chronic lymphocytic leukemia; CR = complete remission; CP = chronic phase; del = deletion, t = translocation, inv = inversion, HLA-id = HLA-identical.

obtained from patients and donors and our institution's Ethical Committee approved the protocol.

Clinical management

The conditioning regimen depended on the clinical diagnosis. The following regimens were used: 1) Ara-C (12 g/m^2), cyclophosphamide (120 mg/m^2) and single dose total body irradiation-TBI (8 Gy) (AML and MDS patients); 2) Ara-C (18 g/m^2), melphalan (140 mg/m^2) and fractionated TBI (12 Gy) (ALL patients); 3) busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg) (CML and 2nd transplant patients); 4) cyclophosphamide (120 mg/kg) and fractionated TBI (12 Gy) (NHL and CLL patients). All patients were treated with 5 µg/kg/d lenograstim (Granocyte®) from day +1 until the granulocyte count was > 10^9/L for three consecutive days or > 10^{10}/L for one day. Six early patients (4 not HLA-identical to their donor and 2 AML in CR1) received a short course of methotrexate in addition to cyclosporine (CyA). Because of the low incidence of acute GVHD observed in the first 9 patients, GVHD prophylaxis was carried out with CyA alone for patients 10 to 24. The diagnosis and grading of acute and chronic GVHD was established as previously reported.[41,42] Disease evaluation, including bone marrow aspirations and biopsies, as well as an evaluation of chimerism by fluorescent *in situ* hybridization (FISH) with X and Y probes in patients with donors of the opposite sex, were routinely carried out on days 40, 100, 180 and 365. Mixed chimerism (MC) was defined as between 1% and 94% donor cells and full chimerism (FC) as > 95% donor cells. Polymerase chain reaction (PCR) analyses for cytomegalovirus (CMV) were performed weekly until day 100 and every 2-4 weeks thereafter. Patients with a positive PCR received prophylactic ganciclovir for a minimum of 4 weeks and generally up to day 100.

Stem cell mobilization, collection and selection

Donors received human granulocyte colony-stimulating factor (G-CSF) (Granocyte®, kindly provided by Rhône-Poulenc-Rorer, Brussels, Belgium) at a dose of 10-15 µg/kg from day -5 through day -1 before transplant. Collection of PBSC was carried out on days -1 and 0, using a continuous flow blood cell separator (CS3000+, Baxter-Fenwall Laboratories, Deerfield, IL, or Cobe Spectra, Lakewood, CO, USA). The volume of blood processed was 12-16 liters. The PBSC from the first day of harvest were stored overnight in the patient's own plasma. Immediately after the second harvest, PBSC from the first and the second days of harvest were pooled. CD34$^+$ cell selection was carried out using the Isolex 300i® magnetic cell separator (Nexell International, Wemmel, Belgium), according to the manufacturer's recommendations.

Donor lymphocyte infusions

Around day 60 post-transplantation, donors underwent 12-16 liter leukophereses on 2 consecutive days to collect lymphocytes. The collection from the first day of harvest was stored overnight in the patient's own plasma and pooled with the second harvest before processing by CD8$^+$ selection using the Nexell Isolex 300i®. The CD8-negative fraction was recovered and divided into 3 aliquots containing 2×10^6, 1×10^7 and 5×10^7 CD3$^+$ cells/kg recipient for patients from #1 to #13. The 2nd and 3rd aliquots were cryopreserved in 10% DMSO in a controlled-rate freezer. After an interim analysis showed little acute or chronic GVHD associated with DLI, the schedule of DLI was changed to 2 aliquots of 1×10^7 and 5×10^7 CD3$^+$ cells/kg recipient for patients 14 to 21. Aliquot 1 was injected fresh immediately after the CD8 depletion procedure (around day 60). Around day 100 (and 140 for patients 1 to 13), aliquot 2 (and 3) were thawed and infused into the patient. CD8-depleted DLI were not to be infused in case of an antecedent grade III or IV acute GVHD, or an antecedent extensive chronic GVHD, or active GVHD at the time of the scheduled infusion.

Laboratory analyses

Aliquots of the pooled PBSC as well as the CD34$^+$ selected fraction were incubated with phycoerythrin-conjugated anti-CD34 monoclonal antibody (HPCA2; Becton-Dickinson, Palo-Alto, CA, USA) for 20 minutes at 20°C, washed and fixed. A total of 1×10^5 cells was analyzed using a FACS-scan analyzer (Becton-Dickinson). The percentage of CD34$^+$ cells was defined with dot plot analysis using the whole nucleated cell population. The percentage of positive cells in the isotype control was subtracted from the CD34$^+$ percentage to give the final percentage of CD34$^+$ cells. Data acquisition was performed with the Cellquest software (Becton-Dickinson). Donor lymphocytes (before and after CD8 depletion) were similarly examined using double labeling with FITC- and PE- conjugated antibodies after treatment with a lysing solution.

Complete blood counts were determined using a Technicon H2 cell counter (Bayer Diagnostics, Diegem, Belgium). Percentages of reticulocytes were obtained by an automated cytofluorometric method using the thiazole orange analog DEQTC.[43]

Table 2. Composition of PBSC grafts and engraftment kinetics.

	PBSC collections		CD34 selected graft		Engraftment: time (days) to				
	CD34+	CD3+	CD34+	CD3+	500 PMN	20,000 Plts	100,000 Plts	1% ret.	RBC LT
	(×10⁶/kg)								
1	19.40	ND	3.41	ND	14	13	45	15	21
2	12.33	433	2.07	0.73	20	124	500	30	33
3	10.54	276	1.43	0.30	12	16	36	14	22
4	13.10	127	3.11	0.85	19	37	NA	25	NA
5	15.75	413	2.05	0.92	10	11	28	14	9
6	7.90	181	2.03	0.20	16	NA	NA	21	NA
7	13.69	358	6.14	0.03	10	12	33	14	35
8	14.31	458	7.74	0.28	13	17	22	19	11
9	7.06	242	2.05	0.11	15	24	45	21	10
10	8.68	375	4.06	0.10	11	19	NA	21	NA
11	6.49	280	3.87	0.13	11	21	148	130	173
12	8.76	309	5.67	0.26	11	13	18	18	13
13	5.59	302	3.45	0.18	9	15	37	20	NA
14	7.24	315	4.42	0.18	12	13	29	15	35
15	14.98	515	8.01	0.21	9	10	31	15	16
16	11.42	412	6.72	0.05	10	23	NA	14	NA
17	21.02	634	9.40	0.03	10	12	NA	NA	NA
18	6.57	228	5.65	0.58	11	16	46	18	14
19	13.29	656	5.86	0.01	11	20	NA	ND	31
20	6.68	624	2.04	0.24	10	10	14	13	17
21	5.93	435	2.73	0.18	9	23	NA	17	21
22	9.83	648	5.78	0.35	10	11	17	13	35
23	5.73	550	2.79	0.20	9	12	14	16	8
24	10.47	279	7.92	0.48	9	10	17	12	6
Median	10.15	375	3.97	0.20	11	15	31	17	19

ND = not done; NA = not achieved; PBSC = peripheral blood stem cells; PMN = neutrophils, Plt = platelets, Retic = reticulocytes, LT = last transfusion, RBC = red blood cells.

Table 3. Composition of lymphocyte infusions.

	CD8 cell content before CD8-depletion (×10⁶/kg R)	Cell content after CD8-depletion (×10⁶/kg R)			CD3+ cells infused (×10⁶/kg R)		
		CD8+ cells	CD4+ cells	CD56+ cells	DLI #1 (d60)	DLI #2 (d100)	DLI #3 (d140)
1	161	22.5	107	ND	2	10	50
2	93	1.1	63	40	2	10	50
3	71	0.3	42	12	2	10	41
4	–	–	–	–	–	–	–
5	99	0.3	46	11	2	10	32
6	–	–	–	–	–	–	–
7	169	1.5	ND	ND	2	141*	–
8	40	0.2	60	9	2	10	26
9	16	0.1	ND	ND	2	10	11
10	30	0.4	ND	ND	41*	–	–
11	118	2.8	73	39	2	10	50
12	179	20	89	37	2	–	–
13	110	1.7	133	34	2	10	50
14	83	0.6	119	42	10	50	–
15	95	6	109	65	10	50	–
16	–	–	–	–	–	–	–
17	–	–	–	–	–	–	
18	76	0.94	110	ND	10	50	–
19	–	–	–	–	–	–	–
20	98	0.7	46	13	10	40	–
21	41	1.1	102	31	10	50	–
22	126	3.7	ND	ND	10	50	–
23	117	4.6	84	24	10	50	–
24	33	0.6	37	16	10	31	–
Median	95	1.1	84	31			

*R: recipient; ND: not done; –: lymphocyte collection and/or infusion not performed (see text); *after relapse.*

Statistical analyses

Student's t-tests were used to compare cell subsets before and after CD8 depletion. The probability of GVHD, relapse, and survival as well as the speed of engraftment were studied by life-table analyses and Wilcoxon rank tests were used for comparisons between groups. Statistical analyses were carried out with Graphpad Prism (Graphpad Software, San Diego, CA, USA).

Results

Collection of PBSC, CD34 selection and engraftment kinetics

PBSC were collected by leukophereses on two consecutive days, except in one 70-year old donor (patient #6) who had to undergo 4 consecutive leukophereses (and two CD34 selection procedures) because of poor yields. Most donors experienced bone pain and/or cephalalgia that were easily controlled with paracetamol, but no other complication was noted. A median of 10.15 (5.59 to 21.02) $\times 10^6$ $CD34^+$ cells/kg and 375 (127 to 656)$\times 10^6$ $CD3^+$ cells/kg were collected (Table 2). After CD34 selection, a median of 3.97 (range 1.43 to 9.40) $\times 10^6$ $CD34^+$ cells/kg and 0.20 (range 0.01 to 0.92) $\times 10^6$ $CD3^+$ cells/kg recipient were infused (Table 2). This represents a 3.3 log reduction for $CD3^+$ cells. Neutrophil engraftment (> 0.5×10^9/L) occurred on day 11 (range 9 to 20) (Table 2). Partial (platelets > 20×10^9/L without transfusion) and complete platelet engraftment occurred at a median of 15 (range 10 to 124) and 31 (range 14 to 500) days, respectively. The median time to 1% reticulocytes was 17 days (range 12 to 130) and time to last RBC transfusions 19 days (range 6 to >365). Neutrophil engraftment tended to be faster in patients receiving > 4×10^6 $CD34^+$ cells/kg (11 vs 12 days, $p = 0.07$) but platelet and RBC engraftments were not affected. Neutrophil (10 vs 16 days, $p<0.001$) and platelet (12 vs 29 days, $p=0.009$) engraftments were faster in patients not receiving a short course of methotrexate.

Figure 1. Clinical outcome of the patients.

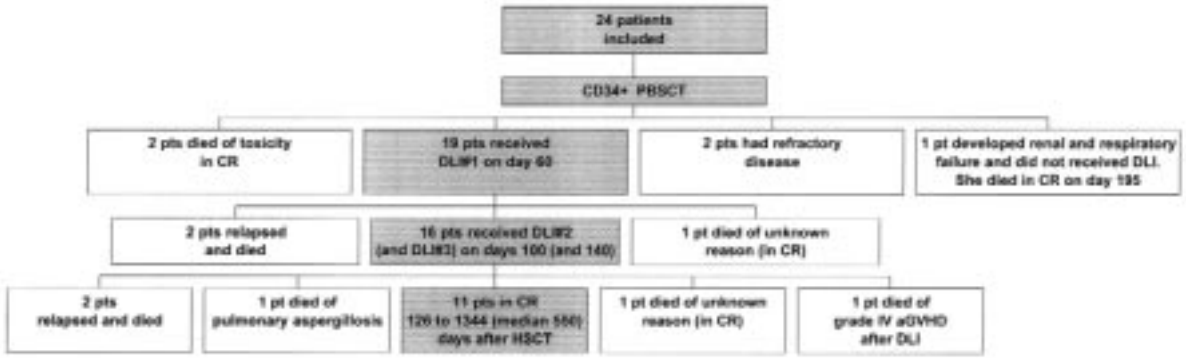

Table 4. Clinical data.

	GVHD							CMV			Follow-up	
	Prophylaxis		Acute GVHD		Chronic GVHD			Status	Reactivation	Other	Disease	Survival
	Drugs	Duration (days)	Grade	Treatment (steroids)	Grade	Onset	Treatment	Don/Rec	(PCR+)	complications	Status	Status
1	CyA + Mtx	213	0	No	No	–	No	+/–	No	No	CR	Alive (d 1344)
2	CyA + Mtx	381	II	Yes	No	–	No	–/–	No	No	CR	Alive (d 1015)
3	CyA	385	II	Yes	No	–	No	–/–	No	Pancreatitis	Relapse (d 315)	Dead (d 950)
4	CyA + Mtx	death	0	No	No	–	No	+/+	Yes (d 39)	MOF	CR	Dead (d 97)
5	CyA	378	I	No	No	–	No	+/+	No	No	CR	Alive (d 952)
6	CyA + Mtx	death	II	Yes	No	–	No	+/+	Yes (d 25)	MOF	CR	Dead (d 81)
7	CyA	97	I	No	No	–	No	–/–	No	No	Relapse (d 96)	Dead (d 313)
8	CyA + Mtx	>559	II	Yes	Limited	d 310	CyA	+/+	Yes (d 55)	Diabetes	CR	Alive (d 766)
9	CyA + Mtx	>459	I	Yes	Limited	d 255	CyA	–/–	No	Encephalopathy	CR	Alive (d 686)
10	CyA	60	0	No	No	–	No	+/+	Yes (d 26)	No	Relapse (d 60)	Dead (d 99)
11	CyA	273	I	No	No	–	No	+/+	Yes (d 30)	Sepsis (d 85)	CR	Alive (d 651)
12	CyA	death	0	No	No	–	No	–/–	No	No	CR	Dead (d 70)
13	CyA	death	0	No	No	–	No	+/+	Yes (d 40)	Pleural effusion	CR	Dead (d 351)
14	CyA	185	I	No	Extensive	d 150	CyA+PDN, UV	–/–	No	No	CR	Alive (d 449)
15	CyA	150	0	No	No	–	No	–/+	Yes (d 25)	No	Relapse (d 135)	Dead (d 309)
16	CyA	death	0	No	No	–	No	–/–	No	VOD	Refractory	Dead (d 44)
17	CyA	10	I	No	No	–	No	–/+	No	No	Refractory	Dead (d 38)
18	CyA	death	0	No	Limited	d 230	CyA+PDN	–/+	Yes (d 32)	No	CR	Dead (d 288)
19	CyA	40	I	No	No	–	No	+/+	Yes (d 18)	Respiratory failure	CR	Dead (d 195)
20	CyA	315	I	No	No	–	No	+/–	No	No	CR	Alive (d 315)
21	CyA	death	IV	Yes	No	–	No	–/–	No	No	CR	Dead (d 150)
22	CyA	> 168	II	Yes	No	–	No	+/+	Yes (d 45)	Hemorrhagic cystitis	CR	Alive (d 168)
23	CyA	> 126	I	No	No	–	No	–/–	No	Encephalopathy	CR	Alive (d 126)
24	CyA	> 140	0	No	No	–	No	–/+	Yes (d 52)	No	CR	Alive (d 140)

GVHD = graft–versus–host disease; CMV = cytomegalovirus; Don = donor; Rec = recipient; CyA = cyclosporine A; Mtx = short course of methotrexate; PDN = methylprednisolone; CR = complete remission; MOF = multi–organ failure; VOD = veno–occlusive disease of the liver; UVx = photopheresis.

Collection of donor lymphocytes, CD8 depletion and CD8-depleted DLI

Donor lymphocytes were collected on day 60 from all but 5 donors whose recipients died before or experienced serious complications (Table 3). As expected, CD8 depletion reduced the $CD8^+$ cell count but only by about 1.5 log (435×10^3 to 14×10^3 $CD8^+$ cells per 1×10^6 $CD3^+$ cells, $p<0.001$), leaving around 100% $CD4^+$ cells among T-lymphocytes. After CD8 depletion, the number of $CD3^+$ cells was sufficient for DLI 1 and 2 in 100% of the cases and DLI 3 in two thirds of them. On average, 90% (range 40 to 100%) of the scheduled dose of CD8-depleted lymphocytes could be procured. Infusion of 2, 10 and 50×10^6 $CD3^+$ cells/kg corresponded to doses of 0.02, 0.1 and 0.5×10^6 $CD8^+$ cells/kg, respectively.

Clinical data (Figure 1 and Table 4)

Five patients died before receiving DLI. Patients #6 (second transplant) and #4 developed many early serious complications and died on day 81 of influenza pneumonia and on day 99 of a polymicrobial infection, respectively. Patient #19 developed severe renal and respiratory failure and died of infection at day 195. Patient #16 died of CLL and veno-occlusive disease of the liver on day 44 and patient #17 died of leukemia on day 38. The other 19 patients were in CR on day 60 and received the first DLI. Two ALL patients (patients #7 and 10) relapsed between days 60 and 100 and received the pooled 2nd and 3rd aliquots. Patient #7 achieved a CR with chemotherapy and DLI but relapsed later and died on day 313. Patient #10 died of leukemia on day 100. In addition, patient #12 died suddenly at home of unknown reason while enjoying continuous CR and presenting no complication other than depression. The other 16 received the scheduled 2nd (and the 3rd for patients #1, 2, 3, 5, 8, 9, 10, 13) DLI on days 100 (and 140). Fourteen of the 16 patients remain in CR 126 to 1344 (median 400) days post-transplantation. Patient #15 developed a central nervous system relapse on day 135, was reinduced into remission with chemotherapy and radiotherapy but relapsed again and died on day 309. Patient #3 experienced a biopsy-proven massive relapse in the thymus on day 315 that completely regressed after cyclosporine discontinuation, but later relapsed again and she died on day 950. Finally, 3 patients died in CR after day 100: patient #13 died of pulmonary aspergillosis on day 351, patient #18 died suddenly at home of unknown reason on day 288 and patient #21 of acute grade IV GVHD on day 150. The remaining 11 patients were alive and disease-free 126 to 1344 (median 550) days after transplantation.

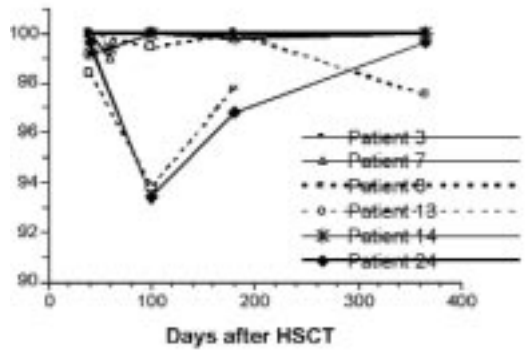

Figure 2. Evolution of chimerism in patients with a donor of the opposite sex.

Bone marrow chimerism

Evaluation of bone marrow chimerism was performed in 6/7 patients with a donor of the opposite sex (n=7) by FISH with X and Y probes (Figure 2). All of them were more than 98% chimeric on day 40. Two patients had mixed chimerism on day 100 after transplantation but developed full donor chimerism on day 180 following pre-emptive CD8-depleted DLI.

Acute and chronic GVHD (Figure 3)

Acute GVHD occurred in 15 patients. It was of grade I in 9 patients, of grade II in 5 patients (3 with 1 HLA mismatch) and of grade IV in 1 patient (HLA mismatch). Thus, grade II-IV acute GVHD occurred more frequently in HLA-mismatched (4/8 or 50%) than HLA-matched (2/16 or 13%) transplants ($p<0.05$). Before DLI, the 60-day actuarial incidence of grade II-IV GVHD was 17% (4 patients) but 2 additional patients developed grade II and IV GVHD after DLI to produce a 150-day (after DLI) incidence of 28%. For HLA-identical sibling transplants, the 60- and 150-day actuarial incidences of grade II-IV acute GVHD were 0 and 13%, respectively (Figure 3A). Chronic GVHD occurred in 4 patients while cyclosporine was being tapered off or after it had been discontinued. It was limited to the liver in 3 patients and extensive in 1 patient. Three of them (2 limited and 1 extensive) were mismatched transplants and one (limited) was a matched sibling transplant. The actuarial 2-year incidences of chronic GVHD and extensive chronic GVHD were 37 and 13%, respectively. For HLA-identical sibling recipients, the figures were 12 and 0%, respectively (Figure 3B). Finally, the incidences of acute and chronic GVHD were quite similar in the 2 schedules of DLI.

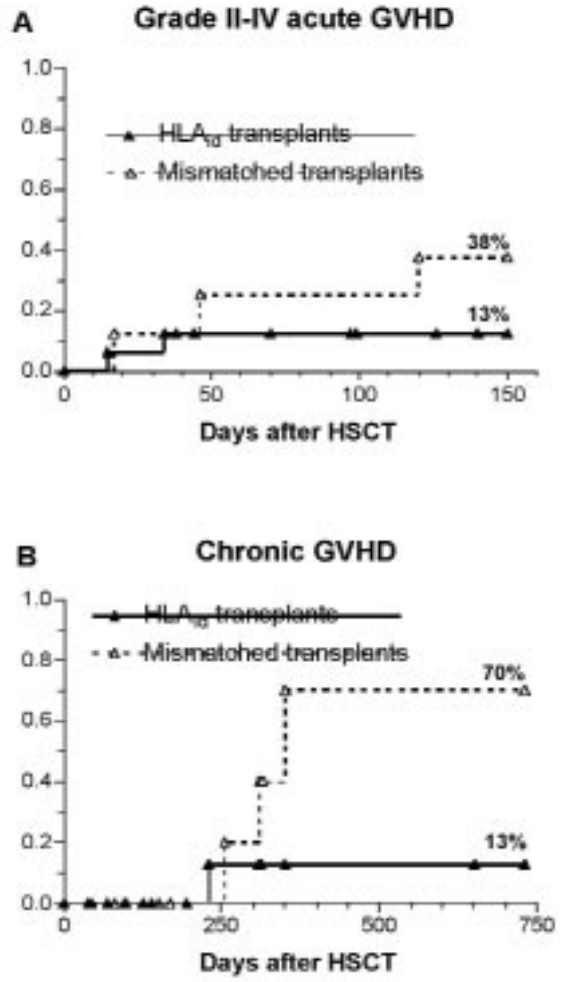

Figure 3. A. Cumulative incidence of grade II-IV acute GVHD in HLA-identical (N=16) versus mismatched (N=8) transplants. B. Cumulative incidence of chronic GVHD in HLA-identical (N=16) versus mismatched (N=8) transplants.

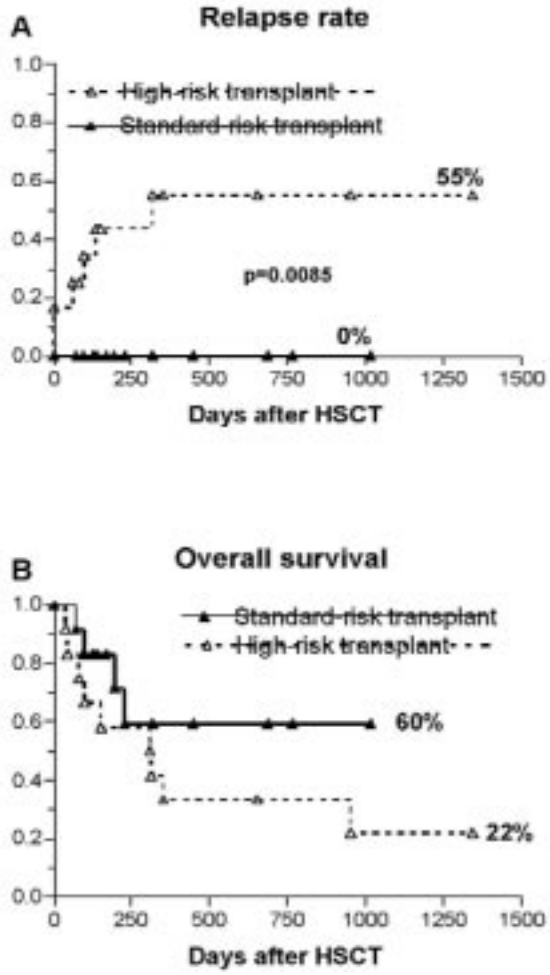

Figure 4. A. Cumulative incidence of relapse in standard-risk (n=12) versus high-risk (n=12) patients. B. Survival rates in standard-risk (n=12) versus high-risk (n=12) patients.

CMV reactivation

Eleven of the 24 patients experienced CMV reactivation (PCR positivity) before day 60 that was successfully reversed by ganciclovir treatment and none of them presented a clinical CMV infection.

Relapse and survival (Figure 4)

There was no relapse in the 12 standard risk patients but 6 of the high-risk patients relapsed (p<0.005) (Figure 4A). The 2-year probability of relapse was 0% for standard-risk patients and 55% for high-risk patients (p=0.0085). Analysis of minimal residual disease by reverse transcriptase-PCR in patient #8 demonstrated the persistence of BCR-ABL fusion transcripts in bone marrow cells on day 100 after transplantation that disappeared on day 180 (after DLI). Patients #11 and 22 had no evidence of residual disease by RT-PCR in any bone marrow analysis after the transplant. The actuarial 2-year probability of survival was 45%, 60% in standard-risk versus 22% in high-risk patients (Figure 4B).

Discussion

In agreement with previous studies,[17,19,21] CD34-selection resulted in our study in a 3.3 log elimination of T-cells while preserving hematologic reconstitution. Engraftment of neutrophils and platelets was prompt and significantly faster in patients who did not receive methotrexate. However, within the range studied, the number of

$CD34^+$ cells did not influence the speed of engraftment significantly. Prompt engraftment occurred with $CD34^+$ cell doses as low as 1.46×10^6/kg. Therefore, $CD34^-$selection preserved the engraftment capability of allogeneic PBSC.

As previously suggested by others,[17,22] our results evidenced that CD34 selection reduces the risk of acute GVHD. The actuarial 60-day (before DLI) probability of grade II-IV acute GVHD was 17% in our study. This rate compares very favorably with results from studies of HLA-identical siblings receiving unmanipulated PBSC or BM.[4,8] For example in the most recent report of the Seattle group, the 100-day incidence of grade II-IV acute GVHD was 64% in the PBSC group and 57% in the BM group.[8]

Recent evidence implicates a preparative regimen-related *cytokine storm* in the pathogenesis of acute GVHD following hematopoietic stem cell transplantation.[44] Therefore, delaying the infusion of donor lymphocytes until after this storm has subsided could result in a lower risk of acute GVHD. We chose to give DLI using CyA prophylaxis because of a previous report by Barrett *et al*.[45] showing that the risk of grade II-IV acute GVHD was significant (32%) and the GVL effect preserved (relapse rate in CML patients considerably less than after T-cell-depleted BMT without T-cell add-back) after T-cell-depleted BMT followed by infusion of substantial (5×10^7/kg) T-cell doses under cyclosporine prophylaxis. Previous studies have demonstrated that it is possible to maintain a GVL effect without GVHD by CD8-depletion of DLI given for leukemic relapse after BMT.[29,34,35,46] Therefore, we developed an original strategy of pre-emptive infusions of CD8-depleted donor lymphocytes given in incremental doses after CD34-selected PBSC transplantation. We recognize the heterogeneity of our patients, but they were treated in an uniform way and this does not interfere with our evaluation of GVHD. Nineteen of 24 patients received pre-emptive CD8-depleted DLI and DLI were never withheld because of GVHD. These DLI were very well tolerated in patients #1-13 (only 4 patients developed grade II acute GVHD) leading us to omit the short course of methotrexate (MTX) even in mismatched or older patients and then to infuse higher doses of CD8-depleted donor lymphocytes earlier in patients.[14-23] These doses again did not induce any severe acute GVHD except in patient #21 who developed grade IV acute GVHD and died of infection and GVHD. Although not all patients could receive the full third dose because of the significant loss of cells in the depletion procedure, on average 90% of the scheduled dose could be provided. Although low doses of $CD8^+$ cells were infused because the degree of $CD8^+$ cell depletion was moderate (only 1.5 log) and although we injected more $CD4^+$ cells because the total dose of T-cells was maintained at 5×10^7/kg, the procedure was efficient at preventing severe GVHD. For HLA-identical sibling transplants, the incidences of grade II-IV acute GVHD, chronic GVHD and extensive chronic GVHD were 13%, 12% and 0%, respectively. These incidences are lower than previously reported in HLA-identical BMT with methotrexate and CyA as GVHD prophylaxis[47] and even more so in HLA-identical PBSC transplants.[8] Therefore, our results show that it is possible to circumvent GVHD by delaying the addition of donor lymphocytes after CD34-selected transplantation.

Moreover, our results also compared favorably with those of previous studies of pre-emptive DLI after T-cell-depleted BMT or PBSC transplantation. A few other studies have investigated the feasibility of adding T-cells back, also to heterogeneous groups of patients, a few weeks to a few months after T-cell-depleted (TCD) transplantation. Barrett gave either 2×10^6/kg on day 30 and 5×10^7/kg on day 45 or 1×10^7/kg on day 30 to HLA-identical siblings after TCD BMT.[45] The probability of grade II-IV acute GVHD was 32% with schedule 1 and 100% with schedule 2 and the incidence of chronic GVHD was 46%. Naparstek gave weekly incremental doses of up to 10^7/kg before day 28 or 3 incremental infusions up to 10^7/kg between day 28 and 84 to HLA-identical siblings after TCD BMT.[38] DLI were only given to 56% of the patients in group 1 and their incidence of acute GVHD was 42% but there was little chronic GVHD. In group 2, the crude incidences of acute and chronic GVHD were 53% and 40%, respectively. Lee administered up to 3 doses ranging from 10^5 to 10^6/kg given between day 0 and day 100 after related or unrelated transplantation.[48] In related transplants, the incidences of acute grade II-IV GVHD and chronic GVHD were 52% and 56%, respectively. Martino treated 10 patients after HLA-matched related CD34-selected PBSC transplantation but only 4 received a single infusion of 10^7/kg donor lymphocytes and all developed chronic GVHD.[39] Recently, Alyea reported on 24 patients with multiple myeloma who received $1\text{-}3\times10^7$ CD8-depleted lymphocytes 6-9 months after a CD6 T-cell-depleted BMT.[49] The authors evidenced a GVL effect mediated by CD8-depleted DLI. However, only 14/24 (56%) patients received pre-emptive DLI and DLI induced grade II-IV acute or extensive chronic GVHD in 7 of them (50%). Thus, compared to our study, most previous reports used lower doses of T-

cells and many patients did not receive the scheduled DLI, and their incidence of acute and chronic GVHD was higher. Therefore, CD8-depletion appears to allow infusions of higher doses of donor lymphocytes while minimizing the risk of severe acute or chronic GVHD.

The mechanism and specificity of the GVL reaction remain uncertain.[29,50-53] Although the contribution of CD8+ T-cells cannot be dismissed, results of CD8-depleted DLI in CML or multiple myeloma patients suggest that CD4+ T-cells may be the primary mediators of GVL,[29,34,35,49,54,55] at least for these malignancies. Moreover, for malignancies less sensitive to CD4+ cells, CD4+ T-cells may recruit tumor-reactive CD8+ T-cells in the host and favor their amplification.[26,29,35] Therefore, pre-emptive infusion of large doses of CD4+ (and NK) cells in our study should be expected to preserve the GVL effect. The relapse rate was much lower than that previously described after T-cell-depleted transplantation.[18] We observed no relapse in the standard-risk patients and only 55% relapses in the high-risk patients. Interestingly, whereas previous studies have shown that the persistence of BCR-ABL transcripts after TCD BMT strongly predicted for relapse,[56] recurrence of CML could be prevented by DLI in one such patient. The two AML patients in whom leukemia persisted or recurred after transplant were very high-risk refractory patients, as was the patient with refractory CLL who also did not respond to the conditioning regimen. It remains to be seen whether earlier infusions of higher doses of lymphocytes could have any impact on such refractory diseases. We conclude that allogeneic CD34-selected PBSC transplantation followed by pre-emptive CD8-depleted DLI appears to be a promising strategy to separate the GVL effect from GVHD. CD34-selection of the graft and CD8-depletion of DLI resulted in substantial benefits for the patients in terms of acute and chronic GVHD despite the large proportion of HLA mismatches. The use of pre-emptive DLI appeared to maintain the GVL effect in a high-risk population but this remains to be demonstrated in more homogeneous groups of patients. Large randomized studies are needed to determine whether this strategy could improve overall survival as well as the quality of life of the patients and to investigate the infusion of specific cytotoxic T-lymphocytes instead of CD8-depleted DLI.

Contributions and Acknowledgments

YB designed the study and wrote the paper. FB analyzed the data and wrote the paper. JS and EB took care of the donors and collected PBSC and lymphocytes. NS-L performed the laboratory analyses on PBSC and lymphocyte products. FB, J-PH, GF and YB took care of the patients.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

What is already known on this topic

Transplantation of CD34+ selected cells from peripheral blood of allogeneic donors associates with a lower risk of acute and chronic graft-versus-host disease than unmanipulated transplants. Relapse risk may be increased in this setting, as a consequence of T-cell depletion, which leads to a decreased graft-versus-leukemia effect.

What this study adds

CD8-depleted lymphocyte infusions starting on day 60 may be safely administered without triggering acute or chronic graft-versus-host disease. The relapse rate after this approach was similar to that observed in a control group of non-T-cell depleted transplants.

Manuscript processing

This manuscript was peer reviewed by two external referees and by Dr. Jordi Sierra, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Dr. Sierra and the Editors. Manuscript received July 24, 2001; accepted October 6, 2001.

Potential implications for clinical practice

This report demonstrates the feasibility of allogeneic CD34+ selected stem cell transplantation followed by the infusion of engineered cell-depleted fractions.

Jordi Sierra Gil, Deputy Editor